The Effects of Oestradiol on Nucleoside Transport in Rat Uterus

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1. By using the non-metabolized cytidine analogue, cytosine arabinoside, it was possible to examine the mechanism of nucleoside transport in the immature rat uterus in the absence of intracellular utilization of the permeant. It was demonstrated that the uptake of cytosine arabinoside is not accumulative and that it can be competitively inhibited by the addition of a second nucleoside, uridine. Introduction of a concentration gradient of uridine from the medium towards the intracellular water promotes the counterflow of cytosine arabinoside out of the cells against its concentration gradient. These properties indicate that a facilitated-diffusion system is involved in nucleoside transport in the uterus. Further counterflow studies have shown that the transport system has a broad specificity for purine and pyrimidine nucleosides and that it is distinct from the processes that mediate the uptake of sugars, amino acids and purine and pyrimidine bases. 2. Oestradiol injection has no effect on the initial rate of cytosine arabinoside uptake *in vitro*. The increased amount of the analogue taken up per uterus is simply due to the expansion of the uterine volume that accompanies oestrogen action. 3. It is concluded that the striking increase in uridine uptake, observed *in vivo* in uteri from oestrogen-treated rats, does not result from an increase in the initial rate of nucleoside transport into the intracellular space of the tissue.

Means & Hamilton (1966) have shown that administration of oestradiol to ovariectomized rats stimulates both the amount of nucleoside taken up by the uterus and the rate of its incorporation into RNA. These responses were too closely related in time to determine whether increased RNA synthesis was a cause or a consequence of increased uptake. Billing, Barbiroli & Smellie (1969) treated rats with actinomycin-D to measure nucleoside transport in the absence of RNA synthesis and found that oestradiol promoted a smaller, but still significant, increase in uptake even when incorporation into RNA was decreased by 80%. Assuming that the hormone has no effect on the utilization of nucleoside for processes other than nucleic acid synthesis, they concluded that stimulation of nucleoside transport is an early and independent response of the uterus to oestrogen action.

Subsequently, it has been shown that oestradiol changes not only the rate of uterine RNA synthesis, but also the size of the uterine pools of acid-soluble nucleotides and the rates of nucleotide synthesis from precursor nucleosides and bases (Oliver & Kellie, 1970; J. M. Oliver, unpublished work). These results suggested that the effect of oestradiol on nucleoside transport should be re-examined under conditions that differentiate between the sequential

processes of uptake, nucleotide synthesis and incorporation of nucleoside triphosphates into RNA.

In the present work, a permeant that is not metabolized by the immature rat uterus was used to determine the mechanism of nucleoside transport and the effect of oestradiol on this specific uptake process.

EXPERIMENTAL

Oestradiol, leucine and purine and pyrimidine bases were purchased from the Sigma (London) Chemical Co. Ltd., London S.W.10, U.K.; cytosine arabinoside was from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Polyethyleneimine-cellulose for column chromatography was obtained from E. Merck A.-G., Darmstadt, Germany; Baker-flex cellulose polyethyleneimine sheets for t.l.c. were from the J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.; MN Polygram Cel 300 UV₂₅₄ sheets were from Macherey, Nagel und Co., Düren, Germany. NCS solubilizer was supplied from the Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A., and Fischer's medium was from the Grand Island Biological Co., Grand Island, N.Y., U.S.A. [³H]Cytosine arabinoside was purchased from Schwarz BioResearch Inc., Orangeburg, N.J., U.S.A., and [³H]uridine and [¹⁴C]sucrose were from The Radiochemical Centre, Amersham, Bucks., U.K.

In all experiments uteri were taken from immature

female rats (40–50g) of the Wistar strain. The animals were killed by cervical dislocation, the abdomen was opened and the uterus removed on to a filter paper moistened with 0.9% NaCl. The organ was dissected free of connective tissue and collected in 0.9% NaCl at room temperature. Unless otherwise noted, incubations were carried out at 37°C with shaking and gassing with O₂ + CO₂ (95:5) in Fischer's medium (Fischer & Sartorelli, 1964), in which uteri maintain a linear rate of RNA synthesis for extended periods (J. M. Oliver, unpublished work).

In uptake studies, groups of three uteri (40–90mg of tissue) were incubated in stoppered test tubes in 1ml of medium containing [³H]cytosine arabinoside (1mM; 0.5μCi/ml) and [¹⁴C]sucrose (1mM; 0.4μCi/ml). At the end of the incubation period the uteri were removed, rinsed briefly in ice-cold 0.9% NaCl and blotted dry on filter paper. They were transferred to preweighed counting vials, their wet weights were recorded and the tissue was dissolved by sonication in the presence of 1ml of NCS solubilizer. Toluene scintillation fluid (15ml) was added and ¹⁴C and ³H radioactivity was measured by the channels-ratio method by using 3 channels of a Packard Tri-Carb model 3375 liquid-scintillation spectrometer. Portions of the media were also dissolved and counted for radioactivity.

The method of Roskoski & Steiner (1967a) was used to determine the uptake of cytosine arabinoside into the intracellular water of the uterus. The 'permeant space', defined as the volume of tissue water required to maintain the permeant in the tissue at the concentration of the incubation medium, was calculated for both cytosine arabinoside and sucrose from the formula:

$$\text{permeant space } (\mu\text{l/g}) = \frac{\text{c.p.m. of permeant/g of tissue}}{\text{c.p.m. of permeant}/\mu\text{l of medium}}$$

Since sucrose is excluded from the intracellular space then: intracellular cytosine arabinoside space (μl/g) = cytosine arabinoside space (μl/g) – sucrose space (μl/g).

RESULTS

Effect of oestradiol on nucleoside uptake in vivo. Fig. 1 confirms the observations of Means & Hamilton (1966) and of Billing *et al.* (1969) that oestradiol promotes a striking increase in the rat uterus. This could result from a more rapid delivery of uridine to the tissue, from an increased rate of nucleotide transport into the uterine cells, or from an increased rate of intracellular metabolism of the permeant. Fig. 1 also shows that a very much smaller increase, which simply parallels the increase in wet weight, is measured when cytosine arabinoside is injected in place of uridine.

Metabolism of cytosine arabinoside. Cytosine arabinoside, an inhibitor of cell growth in both normal and neoplastic tissues of mouse and man, has been widely used in the chemotherapy of experimental tumours and against human leukaemia. Phosphorylation by deoxycytidine kinase

Fig. 1. Effect of oestradiol on nucleoside uptake *in vivo*. Immature female rats (40–50g) were injected intraperitoneally with oestradiol (5μg). After various time-intervals they were divided into two groups of six animals and injected with 3μCi each of [³H]cytosine arabinoside (1.1Ci/mmol) or [³H]uridine (1.0Ci/mmol). They were killed 30min later and their uteri were dissected free of connective tissue, weighed, dissolved in NCS solubilizer and counted for radioactivity. Results, averaged from duplicate groups of three pooled uteri, are expressed as percentages above control values. ▲, Cytosine arabinoside uptake (control value, 1005 c.p.m./uterus); ■, uridine uptake (control value, 1378 c.p.m./uterus); ●, wet wt. (control value, 17.7 mg/uterus).

is a prerequisite for growth inhibition and the drug is inactivated by deamination to uracil arabinoside, a non-metabolizable analogue of uridine (Sartorelli & Creasy, 1969).

Available evidence suggests that cytosine arabinoside may not be metabolized in the rat. Evans, Musser, Mengel, Forsblad & Hunter (1961) showed that it was unable to inhibit the growth of three different types of rat tumours or to produce any toxic reactions in the host rats; Camenier & Smith (1965) observed that no deamination of cytosine arabinoside occurs in rat tissues, and Reel & Gorski (1968) found no evidence of any incorporation into RNA during incubation *in vitro* of rat ovaries with the drug. The lower increment in its uptake *in vivo* observed in Fig. 1 also suggests that cytosine arabinoside may not be removed from its concentration gradient across the cell membranes of the rat uterus by incorporation into nucleotides and nucleic acids.

This possibility was investigated *in vitro* by in-

cubation of duplicate groups of five uteri in 3 ml of Fischer's medium containing [^3H]cytosine arabinoside (1 mM; 1.5 μCi). After 1 h the tissue was removed, rinsed briefly in ice-cold 0.9% sodium chloride, frozen in liquid N_2 and the acid-soluble fraction was extracted with ice-cold 4% (v/v) perchloric acid as described by Oliver & Kellie (1970). Portions of both the media and extracts were chromatographed on plastic-backed plates of polyethyleneimine-cellulose and the developed sheets were cut into 1 cm strips and counted for radioactivity in toluene scintillator. All of the added counts accompanied the nucleoside and base 'carriers' to the solvent front and no radioactivity was detected at the origin with the nucleotides. Further portions of media and extracts were applied to columns of polyethyleneimine-cellulose and the fraction containing nucleosides and bases was separated from that containing nucleotides as described by Oliver & Kellie (1970). Elution of the columns with water to collect bases and nucleosides recovered 98% of the added radioactivity and no further radioactivity was recovered after elution of the nucleotides with 1 M-triethylammonium bicarbonate. It was concluded that no phosphorylation of cytosine arabinoside occurs during 1 h of incubation *in vitro*.

Samples of the media and extracts were also chromatographed on plates of MN 300-cellulose in butan-1-ol-water (43:7, v/v). All of the radioactivity on the developed sheets coincided with the u.v.-absorption due to non-radioactive cytosine arabinoside 'carrier', indicating that no deamination of the base moiety or cleavage of the arabinose linkage had occurred.

Transport of nucleosides in vitro. Fig. 2 shows a time-course of the uptake of cytosine arabinoside *in vitro* measured over 1 h. The water content of the immature rat uterus, measured by drying five replicate groups of four uteri to constant weight at 110°C, was 82% (820 $\mu\text{l/g}$) and the total cytosine arabinoside space (upper curve) corresponded to 100% of the total cell water after 15 min of incubation. No further uptake of nucleoside occurred, showing that the uterus does not concentrate cytosine arabinoside relative to the medium. The middle curve shows the rate at which sucrose, which is excluded from the intracellular water, appears in the extracellular space. Equilibration of permeant between the medium and the interstitial water was complete within 10 min of incubation; after this time, sucrose occupied approx. 54% of the total water space. The lower curve, by difference, shows that the uptake of cytosine arabinoside into the intracellular water occurs linearly for approx. 10 min to reach a maximum value of 375 $\mu\text{l/g}$ at equilibrium.

Fig. 3 shows that addition of 10 mM-uridine to the medium considerably decreases the initial rate of

Fig. 2. Uptake of cytosine arabinoside in the rat uterus *in vitro*. The permeant spaces were measured as described in the Experimental section. Each point represents the mean of at least two determinations. ■, Sucrose space; ▲, cytosine arabinoside space; ●, intracellular cytosine arabinoside space.

Fig. 3. Effect of uridine on the intracellular distribution of cytosine arabinoside. The intracellular cytosine arabinoside space was measured in duplicate as described in the Experimental section. ■, No addition to medium; ●, 10 mM-uridine initially present in medium; ▲, 10 mM-uridine added after 30 min of incubation.

uptake of cytosine arabinoside into the intracellular space. This is evidence that the two nucleosides may compete for a common transport system. Addition of 10 mM-uridine to an incubation mixture at equilibrium with 1 mM-cytosine arabinoside resulted in a rapid efflux of cytosine arabinoside from the tissue into the medium against its concentration gradient. This demonstration of counterflow, defined as a shift in the distribution of one permeant as a result of a concentration difference across the cell membrane of a second permeant that competes for the same transport site, is further

Table 1. *Effect of extracellular permeants on intracellular cytosine arabinoside space*

Duplicate groups of three uteri were equilibrated by incubation for 30 min at 37°C in (a) 1 ml of Fischer's medium or (b) 1 ml of Krebs-Ringer phosphate medium (Dawson, Elliott, Elliott & Jones, 1959) containing [³H]cytosine arabinoside (1 mM; 0.5 µCi) and [¹⁴C]sucrose (1 mM; 0.5 µCi). A second permeant (0.1 ml) was then added to a final concentration of 10 mM in the medium and incubation was continued for a further 10 min before removal of the tissue and measurement of the intracellular cytosine arabinoside space.

Addition to medium	Intracellular cytosine arabinoside space (µl/g)
(a) None	374.4
Cytosine arabinoside	115.2
Cytidine	105.2
Uridine	156.0
Inosine	204.6
Adenosine	168.2
Cytosine	405.6
Uracil	380.5
Hypoxanthine	385.8
Adenine	378.7
(b) None	377.6
Uridine	157.2
Leucine	395.4
Glucose	404.8

Table 2. *Effect of oestradiol on the distribution of permeants in the immature rat uterus*

Water content was calculated from the difference between the wet and dry weights of five replicate groups of four uteri from immature rats injected 4 h previously with oestradiol [0.1 ml of a 50 µg/ml solution in ethanol-0.9% NaCl (1:4, v/v)] or with ethanol-0.9% NaCl alone. Replicate groups of three uteri were incubated for 30 min at 37°C in Fischer's medium containing [³H]cytosine arabinoside (1 mM; 0.5 µCi) and [¹⁴C]sucrose (1 mM; 0.4 µCi) to determine the effect of 4 h pretreatment with oestradiol on the uterine distribution of these permeants.

	Control	Pretreated with oestradiol (4h)
Wet wt. of uterus (mg)	21.1	29.7
Dry wt. of uterus (mg)	3.8	4.2
Water content (µl/g)	820	860
Cytosine arabinoside space (µl/g)	820	860
Sucrose space (µl/g)	440	470

evidence that these nucleosides share a common carrier-mediated transport system.

Table 1 summarizes the results of a series of similar counterflow experiments. All of the purine and pyrimidine nucleosides tested were able to promote an efflux of cytosine arabinoside against

Fig. 4. Effect of oestradiol on cytosine arabinoside uptake *in vitro*. The initial rate of cytosine arabinoside uptake into the intracellular space was measured as described in the Experimental section by using duplicate groups of three uteri from control rats (●) and from animals pretreated for 4 h with 5 µg of oestradiol (■) for each determination.

its concentration gradient but the addition to the medium of purine or pyrimidine bases, leucine or glucose did not alter the intracellular cytosine arabinoside space. This is evidence that the immature rat uterus contains a carrier system that has a broad substrate specificity for nucleosides and that is independent of the transport of bases, sugars and amino acids.

Effect of oestradiol on nucleoside uptake in vitro. Table 2 summarizes the effect of 4 h pretreatment of immature rats with oestradiol on the uterine distribution of water, cytosine arabinoside and sucrose. The hormone increases the water content of the tissue and the cytosine arabinoside content expands to occupy all of the available water space. The increased sucrose space indicates that oestrogen promotes the absorption of water into both the extracellular and intracellular spaces of the tissue. From these data it is apparent that oestradiol has no effect on the distribution of the nucleoside analogue throughout the available intracellular and extracellular space of the uterus when incubation is continued for a sufficient time to reach equilibrium.

The initial rate of uptake of cytosine arabinoside into the intracellular water of uteri from control animals and from animals pretreated for 4 h with oestradiol is plotted in Fig. 4. This shows that the rate at which the nucleoside occupies the intracellular space is not affected by hormone injection. Identical influx curves were obtained with uteri from animals exposed to the hormone for 2 h and 6 h.

DISCUSSION

The movement of molecules across cell membranes can occur either by passive diffusion or by carrier-mediated processes. In passive diffusion, where permeants simply move in and out of cells along their individual concentration gradients, neither saturation of the transport process nor competition between permeants is observed. In mediated transport, a component of the membrane facilitates permeation and uptake obeys Michaelis-Menten kinetics. These systems can be saturated at high substrate concentrations, competition occurs between permeants for uptake, and counterflow, which is a shift in the distribution of one substrate produced by a concentration gradient of a second substrate, can be demonstrated. Facilitated diffusion is a type of mediated transport in which the driving force is simply the concentration gradient of permeant across the cell membrane. Active transport systems are mediated processes that show the characteristic properties of saturability, competition and counterflow and, in addition, have the ability to accumulate substrates in an energy-dependent manner.

By using a permeant that is not subject to intracellular metabolism it has been established that a specific, carrier-mediated process is responsible for the uptake of nucleosides in the immature rat uterus. No accumulation of nucleoside against a concentration gradient occurs, indicating that transport occurs by facilitated diffusion. This process is independent of the facilitated diffusion of sugars (Roskoski & Steiner, 1967a), and of the active transport of amino acids (Roskoski & Steiner, 1967b; Riggs, Pan & Feng, 1968) in the uterus. It is also independent of the mechanism involved in purine and pyrimidine base uptake.

Although nucleoside transport has not yet been examined in any other complex mammalian tissue Oliver & Paterson (1970) have established that uptake into erythrocytes occurs by a process of facilitated diffusion. Indirect evidence also exists for a nucleoside-specific carrier system in several types of mammalian cells in tissue culture (Skehel, Hay, Burke & Cartwright, 1967; Hausen & Stein, 1968; Kessel & Shurin, 1968; Scholtissek, 1968; Steck, Nakata & Bader, 1969), in sea urchin eggs (Piatigorsky & Whiteley, 1965) and in cells of the Ehrlich ascites carcinoma (Jacquez, 1962). Active transport systems for nucleoside uptake have been described in several different micro-organisms (Pourquie, 1970; Schilz & Terry, 1970; Peterson, Boniface & Koch, 1967).

It was found that oestradiol has no effect on the initial rate of nucleoside uptake *in vitro* into the immature rat uterus. At equilibrium more radioactivity from cytosine arabinoside was recovered

in the uteri from oestrogen-treated rats, but this was due to expansion of the size and water content of the uterus and not to an increased rate of transport. Since the systems mediating sugar and amino acid uptake in uterus are both stimulated by oestradiol (Roskoski & Steiner, 1967a,b; Riggs *et al.* 1968), this is further evidence for the independence of the nucleoside transport process.

Billing *et al.* (1969) observed that oestradiol increases the amount of an injected dose of radio-active nucleoside which is recovered in the rat uterus *in vivo* and concluded that the hormone stimulates nucleoside transport in the tissue. In the present investigation more uridine was recovered in uteri from hormone-treated rats in similar experiments *in vivo*, but the difference in the rate of uptake between control and oestrogen-treated tissue was abolished when uridine was replaced *in vitro* by cytosine arabinoside. These results suggest that the increase measured *in vivo* is due either to a more rapid delivery of nucleoside to the uterus or to a more rapid removal of nucleoside for the synthesis of uterine nucleotides and nucleic acids. It is not due to an increase in the initial rate of nucleoside transport or to a change in the equilibrium distribution of nucleoside in the oestrogen-treated uterus.

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