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# Transport of Inositol into the Distal Cauda Epididymidis of the Rat

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**The transport of [$^3$H]myo-inositol into the lumen of the rat distal cauda epididymidis was studied by luminal perfusion *in vitro*. Entry was time and tubule length-dependent and saturable transport could be demonstrated with $V_{max}$ of 237 pmol/(30 min. cm) and $K_t$ of 1 mM. Secretion of unlabeled inositol into the epididymal lumen was maintained for 5 hours at a concentration 6 times greater than that of the bathing solution. The turnover time of the epithelial pool of inositol was 4.5 hours, from which the intracellular concentration was estimated to be 26.6 mM. Transport was not reduced by metabolic inhibitors, and it was demonstrated that exchange diffusion across the basolateral membranes could drive the uptake in this region.**

**Key words: inositol, transport, epididymis.**

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The high concentrations of inositol that bathe mature spermatozoa in the epididymis of the rat (Hinton et al, 1980) arise from epididymal secretions (Setchell and Hinton, 1981). Part of this inositol originates from the bloodstream (Hinton and Howards, 1982; Cooper, 1982; Sujarit et al, 1985), and both *in vivo* (Hinton and Howards, 1982) and *in vitro* (Cooper et al, 1985) studies have suggested that the uptake of inositol into the epididymis is saturable. This investigation was designed to characterize the transport of inositol in the distal cauda epididymidis perfused *in vitro*.

## Materials and Methods

### *Perfusions*

Adult Sprague-Dawley rats were used (mean ± SEM weight 322 ± 8 g). The luminal perfusion technique described by Cooper et al (1985) was employed but modified by use of a Compact Harvard Infusion pump and alteration of the composition of the bathing (peritubular) fluid to include 60 μM L-carnitine (a generous gift from Sigma Tau, Industrie Farmaceutiche, Riunite SPA, 0014 Rome), rather than the racemic mixture, and fraction V bovine serum albumin (Sigma Chemie, München) rather than the unfractionated preparation. 2-[$^3$H]myoinositol (S.A. 16.3 Ci/mmol) and [$^{14}$C]carboxyinulin (S.A. 7.9 Ci/mmol) from Amersham International were added to peritubular fluid to monitor transport and account for leakage, respectively. The mean (± SEM) temperature of the perfusions was 33.0 ± 0.05 C.

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In memory of the late D. E. Brooks.

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### Calculations of Transport Rates

The concentration of each isotope in the bath and in sequential 30-minute samples of outflowing perfusate was measured by liquid scintillation spectrophotometry as described earlier (Cooper et al, 1986). The concentration in the sample was expressed as a percentage of that in the bath and the percentage obtained for [$^{14}$C] (considered leakage or nonspecific entry) was subtracted from that of [$^{3}$H]. The net transport of inositol to the lumen from the bath per 30 minutes was calculated from the corrected entry of isotope, the specific activity of inositol in the bath, and the perfusion rate.

### Saturation and Competition Studies

In saturation experiments, the concentration of [$^{3}$H]-inositol was kept constant as described above while the concentration of unlabeled inositol in peritubular fluid was increased from 12.5 μM to 10 mM. Isomers of inositol (Sigma Chemie, Calbiochem or generous gifts from Dr. J. Angyal, University of New South Wales, Australia; Dr. L. Anderson, University of Wisconsin, U.S.A.; and Dr. C. E. Ballou, University of California) were included at 1 mM in the presence of tracer and 12.5 or 50 μM of myoinositol.

### Active Transport Studies

Two experimental designs were used to elucidate the nature of the transport. In stop-flow experiments, continuous perfusion was halted for 4 hours during immersion of the tubule in peritubular fluid with either intraluminal fluid in the lumen, or intraluminal fluid to which [$^{3}$H]-myoinositol and myoinositol at the same concentration as in the bath had been added, together with [$^{14}$C]inulin. At the end of this time, the total contents of the tubule and catheters were flushed out with unlabeled medium into preweighed vials, and the total isotope content was determined. The concentration of radioactivity in the tubule lumen during the period of stopped flow was determined by dividing the total isotope content by an estimate of the volume of the tubule, derived from the tubule content of tracer.

In continuous perfusion experiments, the volume marker [$^{14}$C]inulin was added to luminal fluid and the specific activity of [$^{3}$H]myoinositol was equal to that of the bath. To block glycolysis of respiration, the peritubular fluid was modified by removing glucose and substituting 5 mM deoxyglucose or by removing pyruvate or lactate and substituting 5 μM oligomycin. After correcting for water absorption, the activity in the lumen at each time point was expressed as a percentage of that in the bath.

### Exchange-Diffusion of Inositol

Tubules from the distal cauda epididymidis of three 400-g rats were unraveled in ice-cold peritubular fluid and divided into proximal and distal regions by 5/0-ligatures that acted to contain the luminal contents. Each pair of distal tubules and each pair of proximal tubules from each animal were incubated separately in 1.9 ml peritubular fluid containing [$^{3}$H]inositol (1.1 μCi/ml) and [$^{14}$C]inulin (0.2 μCi/ml) at 33 C for 3 hours with shaking at 70 strokes per minute. At the end of incubation, each pair of tubules was rinsed 2× in peritubular fluid before one was transferred to 1 ml of isotope-free peritubular fluid and the other to 1 ml of isotope-free peritubular fluid containing 50 mM myoinositol. Incubation at 33 C, with shaking as above, continued for 60 minutes, during which time duplicate 10-μl aliquots were removed at intervals for assessment of radioactivity. After incubation, the tissues were blotted dry and weighed. The resulting efflux of tracer into the medium was taken as an indirect reflection of loss from the tissue. Wash-out of interstitial tracer, not removed during the rinses, was accounted for by the appearance of [$^{14}$C]inulin in the medium: the corresponding amount of tritium was substracted from the total amount of this isotope in the medium. Finally, the resulting concentration of inositol from intratubular sources was corrected for the weight of the tubule.

### Analysis of Radiometabolites

Aliquots of pooled bath media and pooled perfusates, together with myoinositol and glucuronic acid standards, were applied to silica plates and processed as in Cooper (1982) to analyze the radioactivity of pooled samples of luminal perfusate and the corresponding bath fluids. Radiometabolites from epididymal tubules incubated in [$^{3}$H]inositol from the exchange-diffusion experiments were also analyzed. Frozen tubules were thawed, homogenized in 7 ml of 0.5 M HCl in an Ultratorrax blender, and then sonicated twice for 15 seconds. The mixture was partitioned against 7 ml of $CHCl_3$:MeOH (1:2, v/v) vortexed, and centrifuged at $500_{gmax}$ for 5 minutes. The upper phase was removed and the interphase was washed twice (Berridge, 1983).

The combined upper phases were frozen at −80 C and freeze-dried. Five hundred μl of water were added, duplicate 10-μl aliquots were taken for liquid scintillation counting, and samples of the remainder were separated by the TLC system described in Cooper, 1982. Carbohydrates were localized with a periodocuprate stain (Dawson et al, 1986) The lower phases were pooled, dried under air at 37 C, and the residue was dissolved in 100 μl MeOH. After assessing the radioactivity, samples were applied to activated, oxalated (Jolles et al, 1981), 250-μm silica plates and developed for 15 cm in the solvent system of Gumber and Lowenstein (1986) against standards of phosphatidylinositol, phosphatidylinositol-4-monophosphate and phosphatidylinositol-1,4-bisphosphate visualized with iodine vapor.

Thin-layer plates were scanned for β-emission (Berthold Linear Analyser LB2832) before and after covering with aluminum foil to locate the $^{14}$C peaks. The counts remaining after background substraction were expressed as a percentage of the total counts in the lane.

### Total Secretion of Inositol

A luminescence assay (Gudermann and Cooper, 1986) was used to quantify the concentration of inositol in sequential samples obtained from the perfused epididymis.

### Statistics

Wilcoxon's Stratified test was used to check the significance of results from exchange diffusion and the Kruskal-Wallis test for active transport.

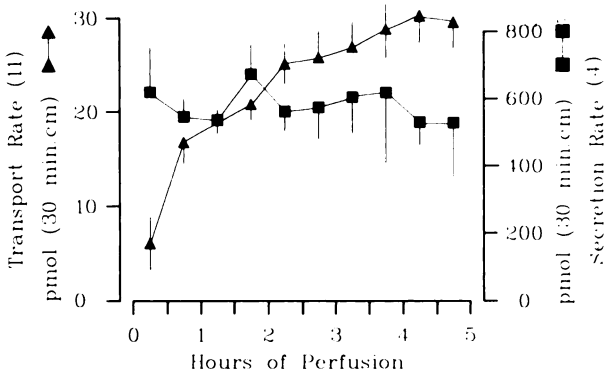


**Fig. 1.** Secretion and transport of myo-inositol into the lumen of the distal cauda epididymidis perfused *in vitro*. The transport of inositol from the organ bath (left hand ordinate [▲]) and the total secretion of inositol (right hand ordinate [■], during 5-hour perfusion (abscissa) during immersion in a solution containing 50 μM myoinositol. Values are the mean ± SEM from the number of experiments in parentheses.

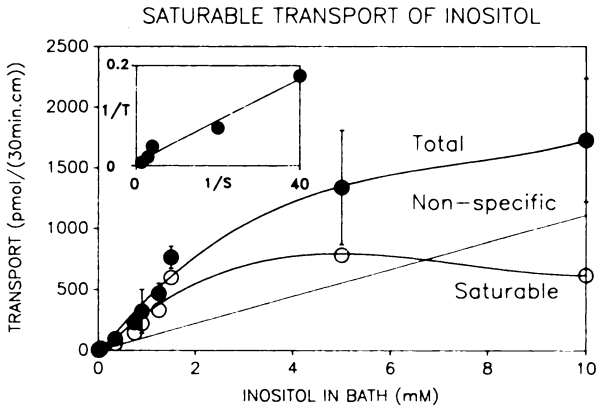


**Fig. 2.** The transport of [³H]myoinositol from the bath to the lumen of the distal cauda epididymidis perfused *in vitro* (ordinate) as a function of the inositol concentration in the bath (abscissa). Values are the mean ± SEM of five values taken 180 minutes after the beginning of the perfusion; where no error bars are seen they lie within the symbol. Inset: Double reciprocal plot (1/bath concentration of inositol as ordinate; 1/mean saturable transport rate as abscissa).

## Results

### *Movement of Myoinositol to the Epididymal Lumen*

Radioactivity entered the lumen of the rat epididymis within the first 30 minutes during perfusion *in vitro*. At a bath concentration of 50 μM, myoinositol radioactivity rose steadily to reach a plateau (approaching 35% of bath levels) by 4 to 5 hours (Fig. 1). At this concentration, there was a correlation between the length of the perfused tubule and the extent of transport of inositol to the lumen, whether the latter was obtained from the mean plateau level reached (last three or four values) or a mean of the rising values during 30 to 300 minutes of perfusion ($r$ = 0.499, N = 17, y = 15.09 x + 152.2).

### *Carrier-mediated Transport*

Increasing the amount of inositol in the bath increased linearly (slope 313 pmol/(30 min. cm)) the amount of inositol transported to the lumen up to 1.5 mM. At concentrations above this, the rate of transport was less, so that saturable transport could be determined when the nonspecific component (111 pmol/(30 min. cm per mM)) was substracted (Fig. 2). Double reciprocal (Lineweaver Burke) plots of the data (Fig. 2, inset) indicated $V_{max}$ to be 237 pmol/(30 min. cm) and the $K_t$ 1.01 mM. At the concentrations tested, there was no marked inhibition of inositol transport in the presence of isomers (data not shown).

### *Nature of Transport*

During 4-hour stop-flow experiments, the concentration of [³H]inositol in the lumen did not exceed that in the bath when resorption of water was taken into account, whether the fluid had been initially isotope-free or had initially contained [³H]inositol at the same specific activity as the bath.

During continuous perfusion of isotope-containing fluids through the tubules, luminal radioactivity exceeded the initial bath concentration of 94.8 ± 8.8% (Fig. 3). This transfer was independent of the nature of the fluid in the lumen and was not prevented by metabolic inhibitors in the medium (Fig. 3).

### *Exchange Diffusion*

The efflux of tissue [³H]inositol into the peritubular fluid during the 60-minute wash-out period rose slowly in both distal and proximal regions. In the presence of 50 mM of unlabeled inositol, however, efflux was greater and exceeded the control after 15 minutes (more in distal segment, Fig. 4). For both regions this reached statistical significance at the 1% level.

### *Analysis of Radiometabolites*

Two major peaks of radioactivity were found in the bath and in pooled perfusates. That remaining at the origin was [¹⁴C]inulin and the other migrated with [³H]inositol.

Most (96%) extracted tissue metabolites were water-soluble. They were clearly separable into three major peaks, one remaining at the origin that represented 15% of total activity, and two abutting peaks migrating just before ($R_f$ 0.32) or with ($R_f$ 0.4)

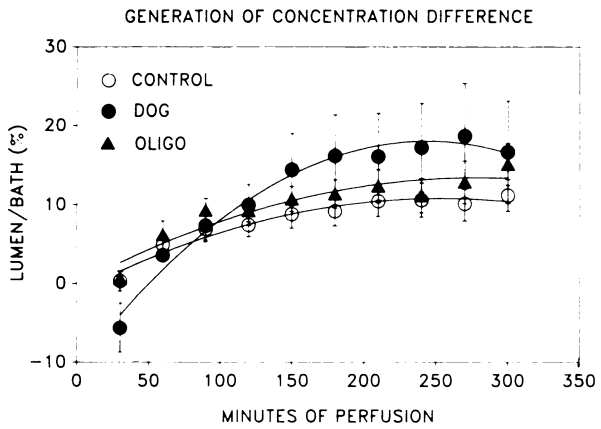


**Fig. 3.** Transport of [$^3$H]myoinositol to the lumen of the distal cauda epididymidis perfused *in vitro* when bath and lumen contained initially the same concentration of myoinositol, after correcting for concentration due to water absorption. Normal bath conditions (O), bath containing 5 μM oligomycin and no lactate or pyruvate (oligo) (▲) or 5 mM 2-deoxyglucose and no glucose (DOG) (●). The luminal activity is expressed as a percentage of that in the bath less 100% (ordinate) against time (abscissa).

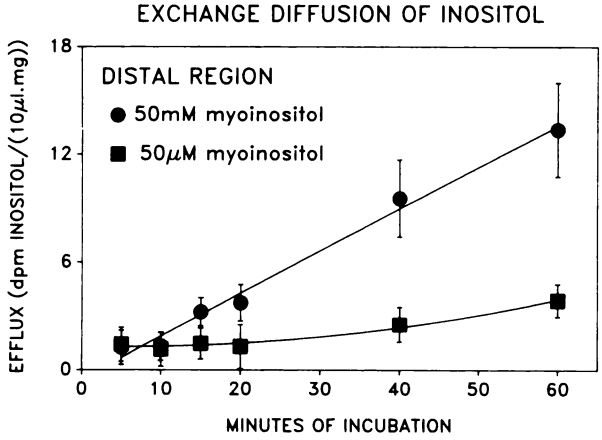


**Fig. 4.** Movement of labeled [$^3$H]inositol from tubules of the distal half of the distal cauda epididymidis into medium. Distal cauda tubules were preincubated in tracer for 3 hours and then transferred to media containing either 50 μm (■) or 50 mM (●) myoinositol for 60 minutes. The total efflux of inositol at the times indicated (abscissa) is corrected for bound and interstitial tracer and the weight of the tubule (ordinate).

inositol, which accounted for 22% and 12% of the activity, respectively. The nature of the metabolites was not established.

### *Secretion of Unlabeled Inositol*

The concentration of inositol in luminal fluids was high even within the first 30-minute sample, although the perfusing medium contained no inositol. In seven experiments, a mean (± SEM) concentration of 368.2 ± 19 μM was obtained. In four others a constant mean secretion rate of 9.34 ± 0.96 nmol/30 minutes or 570.5 ± 60.6 pmol/(30 min. cm) was maintained for 5 hours. The resulting secretion contrasts with the transport of inositol from the bath in Fig. 1. From the secretion rate, the turnover time (4.5 h), and the literature values of the epithelial height in this region (Reid and Cleland, 1957), an average intracellular concentration of inositol in the epithelium was calculated to be 26.6 mM.

## Discussion

The present work extends earlier observations concerning the inhibitory effect of 10 mM unlabeled inositol on the transport of labeled inositol into the lumen of the distal cauda epididymidis (Cooper et al, 1985) to provide information on the maximal velocity and transport constant. Both constants were greater than the equivalent values for carnitine established in the same preparation, in which plasma concentrations regulate luminal secretion (Cooper et al, 1986). The high $K_t$ for inositol (c.1 mM) denies plasma concentrations (c.50 μM) any such role and emphasizes the importance of secretion from synthesized pools.

The 5-hour perfusion period permitted a steady state to be established between extratubular tracer and endogenous inositol secretion, which in turn enabled calculation of intracellular inositol concentrations of close to 30 mM. This supports the inference of a large epithelial pool (Cooper, 1982; Cooper et al, 1985). Since this value is close to the concentration of inositol found in epididymal fluid in the cauda (Hinton et al, 1980; Cooper, 1982), no active transport would be necessary to bring inositol into the lumen from the cell if the apical membrane exhibited some permeability towards the hexitol. This mechanism has been suggested by Spector (1978) for inositol transported into cerebrospinal fluid and postulated for the epididymis by Hinton and Howards (1982).

It follows that transport of inositol from the bath into the epithelial cell should take place against a concentration difference. The failure to block transport with metabolic inhibitors was surprising. However, active transport in this region of the duct was not demonstrated in a micropuncture study when luminal concentrations failed to exceed blood levels during 3 hours of infusion (Hinton and Howards, 1982). This finding contrasts with the situation in the caput epididymidis and suggests that the organ util-

izes two different mechanisms of inositol transport in different regions.

One explanation could be that the movement into the cell is mediated by exchange diffusion, by which membrane carriers that are responsible for the efflux of inositol from the cell return to the cytoplasm carrying extracellular tracer into the cell. Support for this mechanism came from the greater efflux of [$^3$H]inositol from preloaded tissue in the presence of extra-tubular inositol. Exchange diffusion of inositol also occurs in plasma membranes from the kidney (Takenawa and Tsumita, 1974; Hammerman et al, 1980). The apparent uphill movement of tracer to the cell would be fueled by continued synthesis of myo-inositol by the epididymis. Although the extent of this activity remains debatable (Cooper, 1986) and the effect of metabolic blockers has not been studied, they would not be expected to reduce such exchange until the intracellular pool was substantially depleted.

Although it is usual in transport studies to employ nonmetabolizable tracers, these are not available for inositol. Thus, interpretation of the data could be confused by the metabolism of inositol by epididymal tissue. Although only free cyclitol was found to be transported to the sperm-free lumen, as previously found *in vitro* (Cooper et al, 1985), water-soluble metabolites were extractable from whole tissue in the present study, in addition to lipid-soluble metabolites previously reported (Voglmayr and Amann, 1973; Voglmayr, 1974).

Finally, a comment can be made concerning the nature of nonspecific transport in the epididymis. Since the epididymal epithelium has been considered "leaky" owing to its electrical properties (Cheung et al, 1978), it is possible that, in common with other such epithelia (Whittembury et al, 1980, 1985), transport by a paracellular route occurs. Even inulin may be dragged by solvent through tight junctions, which would be exaggerated in the present study, and *in situ*, by osmotic differences across the epithelium. Another route that has been described recently *in vitro* is that of fluid-phase transcytosis (Cooper et al, 1987).

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