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*Topical Review*

# Role of Organic Osmolytes in Adaptation of Renal Cells to High Osmolality

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## Introduction

A striking feature of the urinary concentrating process is that it entails a variable elevation of urea and NaCl in renal medullary blood and interstitial fluid. The concentrations of these substances regularly reach levels that would be lethal in the general circulation, yet the renal medullary cells evidently survive and function. They adapt by accumulating certain organic osmolytes (Schimassek, Kohl & Bucher, 1959; Balaban & Knepper, 1983; Bagnasco et al., 1986). This mechanism is evolutionarily ancient (Yancey et al., 1982). Within mammals it is largely confined to the kidney medulla, which is the only tissue in which the cells are exposed to such an extreme stress.

Accumulation of "compatible" organic osmolytes is the response to water stress of all organisms, including bacteria, plants, and animals, whose cells adapt to high-salt environments (Yancey et al., 1982). The compatible organic osmolytes comprise three classes of compounds: (i) sugar alcohols or polyols, (ii) neutral free amino acids and related solutes, and (iii) methylamines plus urea. In renal medullas four particular organic osmolytes predominate (Bagnasco et al., 1986), namely sorbitol and inositol which are polyols, glycerophosphorylcholine (GPC), which is a methylamine, and betaine, which is both a methylamine and an amino acid derivative.

**Key Words** osmoregulation · sorbibol · betaine · inositol · glycerophosphorylcholine · aldose reductase

## Levels of Organic Osmolytes in Renal Medullary Cells Correlate with Extracellular NaCl and Urea Concentrations

When normal animals are dehydrated and their urine is very concentrated, there is a gradient of NaCl and urea in the renal medulla (Atherton, Green & Thomas, 1970). Both solutes increase towards the tip of the papilla, as illustrated in Fig. 1. Under these conditions there are gradients of sorbitol (Wirthensohn, Beck, & Guder, 1987; Schmolke, Beck & Guder, 1989; Wirthensohn et al., 1989; Yancey & Burg, 1989), GPC (Wirthensohn et al., 1987; Yancey & Burg, 1989), and betaine (Yancey & Burg, 1989) that parallel those of sodium and urea. Inositol is also higher in the medulla than in the cortex, but in contrast to the other organic osmolytes, inositol is as high or higher in the outer compared to the inner medulla (Cohen, Hruska & Daughaday, 1982; Bagnasco et al., 1986; Yancey, 1988; Schmolke et al., 1989; Wirthensohn et al., 1989; Yancey & Burg, 1989).

Following experimental perturbations that induce diuresis, the organic osmolytes generally decrease (e.g., Fig. 2). However, there are exceptions. For example, inner medullary betaine and sorbitol rise (Heilig, Stromski & Gullans, 1989), as does NaCl (Wald, Scherzer & Popovtzer, 1989), during the diuresis caused by hypertonic saline administration. Also, the pattern of change depends on how the diuresis is produced (Cohen et al., 1982; Bagnasco et al., 1986; Gullans et al., 1988; Wolff, Eng & Balaban, 1988; Yancey, 1988; Blumenfeld et al., 1989; Schmolke et al., 1989; Wirthensohn et al., 1989; Wolff et al., 1989). Despite these differences, two generalizations seem to hold: (i) The sum of the intracellular compatible organic osmolytes correlates highly with medul-

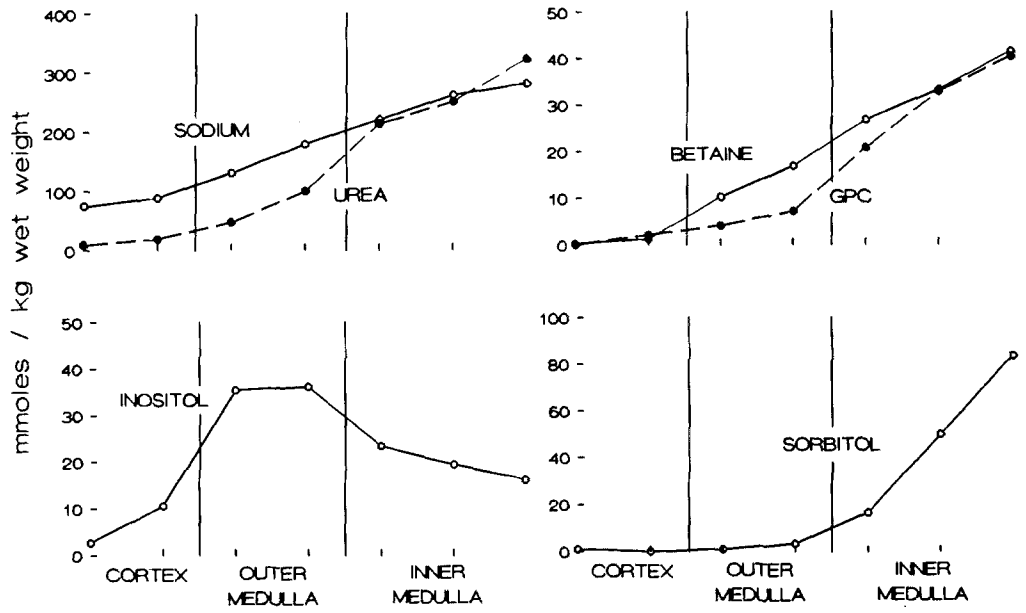


**Fig. 1.** Distribution of sodium, urea, and compatible organic osmolytes in kidneys of antidiuretic rabbits. Data are from Yancey and Burg (1989); the figure is reproduced from Garcia-Perez and Burg (1990) with permission of the publisher

lary extracellular NaCl and (ii) GPC correlates highly with medullary urea (Yancey, 1988; Yancey & Burg, 1989). Insight into the basis for these relations and their functional consequences has come both from studies of tissue cultures and from comparison to the adaptive strategies of other organisms.

## Renal Cells in Tissue Culture Osmoregulate by Accumulating the Same Organic Osmolytes as do Renal Medullary Cells in Vivo

PAP-HT25 is a line of rabbit renal medullary epithelial cells that can survive and grow in hypertonic medium (Uchida et al., 1987). These cells were observed to accumulate enough organic osmolyte (mainly sorbitol) in high NaCl medium so that cell volume and intracellular Na and K remain essentially normal (Bagnasco et al., 1987). Thus, sorbitol, by balancing the osmotic pressure of the high external NaCl, allowed the cells to keep both a normal intracellular ionic composition and volume.

Because sorbitol was the only organic osmolyte initially observed to be accumulated to an appreciable extent by PAP-HT25 cells, several additional renal cell lines were screened to find models for studying the other osmolytes (Nakanishi, Balaban & Burg, 1988). MDCK (dog kidney) cells were selected because they grow well in hypertonic medium and accumulate betaine, inositol, and GPC under those conditions, even though they do not accumulate sorbitol (Fig. 3). Studies of the MDCK cells revealed the conditions required for accumulation of inositol (Nakanishi, Turner & Burg, 1989*a*), betaine (Nakanishi, Turner & Burg, 1990), and GPC (Nakanishi & Burg, 1989*a*). Based on those results, betaine and urea (as well as NaCl to produce hypertonicity) were added to the medium bathing PAP-HT25 cells, and under those conditions the PAP-HT25 cells accumulated substantial amounts of betaine, inositol, and GPC, in addition to sorbitol (Fig. 3) (Moriyama, Garcia-Perez & Burg, 1989*a*). The MDCK and PAP-HT25 cell lines provide models to study the mechanisms involved in organic osmolyte accumulation and to analyze the effects of the osmolytes.

## Hypertonicity Induces Sorbitol Accumulation by Slowly Increasing the Activity of Aldose Reductase, an Enzyme that Catalyzes Synthesis of Sorbitol

Sorbitol is synthesized from glucose in a reaction catalyzed by aldose reductase. When PAP-HT25 cells grown at a normal osmolality are switched to high NaCl medium, there is a six hour lag, following which aldose reductase activity increases gradually for two to three days and sorbitol accumulation follows (Fig. 4) (Uchida et al., 1989).

The rise in aldose reductase activity is due to an increase in the amount of the enzyme protein (Bedford et al., 1987), and the protein rises because its synthesis rate is increased (Moriyama, Garcia-Perez & Burg, 1989*b*). Aldose reductase synthesis

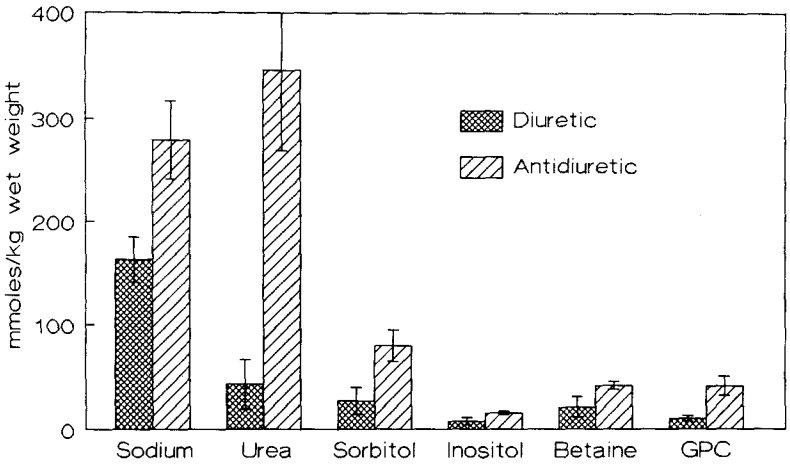


**Fig. 2.** Comparison of the amounts of osmolytes in the tips of the renal papillas in diuretic *vs.* antidiuretic rabbits. Data are from Yancey and Burg (1989); the figure is reproduced from Garcia-Perez and Burg (1990) with permission of the publisher

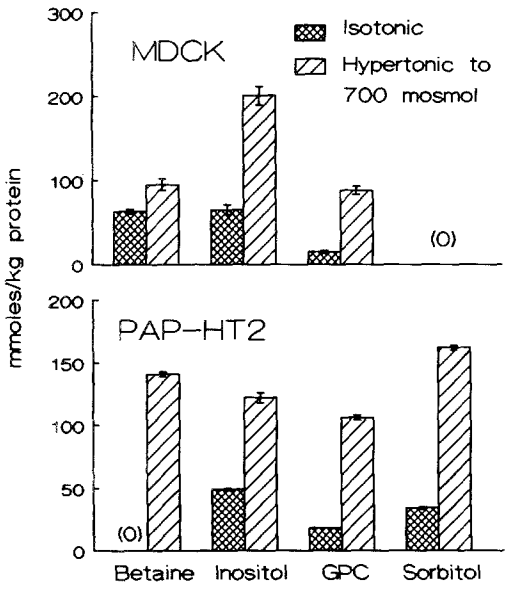


**Fig. 3.** Effect of medium osmolality on organic osmolytes in renal cells in tissue cultures. The medium was either isotonic (≈300 mosmol/kg) or hypertonic (≈700 mosmol/kg with added NaCl and urea). Data for MDCK are from Nakanishi et al. (1990) PAP-HT25 was from Moriyama et al. (1990); the figure is reproduced from Garcia-Perez and Burg (1990) with permission of the publisher

and degradation were measured by $^{35}$S-methionine pulse-chase, followed by immunoprecipitation. Synthesis rate (Fig. 5, left panel) does not change for three hours after the osmotic pressure is elevated. By 24 hours it peaks at approximately 15 times the baseline value, then it decreases to a steady level approximately six times greater in hypertonic medium than in medium of normal osmolality. The decrease in synthesis rate after 24 hours coincides with the accumulation of sorbitol (Fig. 4). As also shown in Fig. 5 (right panel), if the rise in sorbitol is prevented by using the aldose reductase inhibitor, Tolrestat, the rate of synthesis continues to increase beyond 24 hours. Conversely, when the experiment is designed so that the cells accumulate sorbitol from an exogenous source, not requiring intracellular synthesis, translation of aldose reductase declines (Moriyama et al., 1989*b*). Thus, intracellular sorbitol somehow exerts a negative feedback on its own continued production by ultimately affecting aldose reductase protein synthesis rate. The degradation rate of aldose reductase protein is slow (half-time approximately 6 days) and does not differ between normal and hypertonic medium. Therefore, the rise of aldose reductase protein in hypertonic medium is entirely due to increased synthesis.

In order to investigate how hypertonicity increases aldose reductase protein synthesis, aldose reductase cDNA was cloned from PAP-HT25 cells, and the cloned cDNA was used as a probe to measure mRNA abundance (Garcia-Perez et al., 1989). When the medium is made hypertonic by adding NaCl, aldose reductase mRNA increases. The time course and magnitude of the increase closely match the increase in aldose reductase protein synthesis rate (Fig. 5, left panel). Therefore, the increased translation of enzyme protein is due to a rise in its mRNA. Using nuclear run-on assays, we recently determined that the increase in aldose reductase mRNA abundance follows a rise in its transcription initiation rate (Smardo, Burg & Garcia-Perez, 1990). It is not yet clear whether changes in aldose reductase mRNA stability also play a role.

When osmolality is decreased from a high to normal level, both aldose reductase activity and protein fall slowly (half-time of 6 days) to their baseline

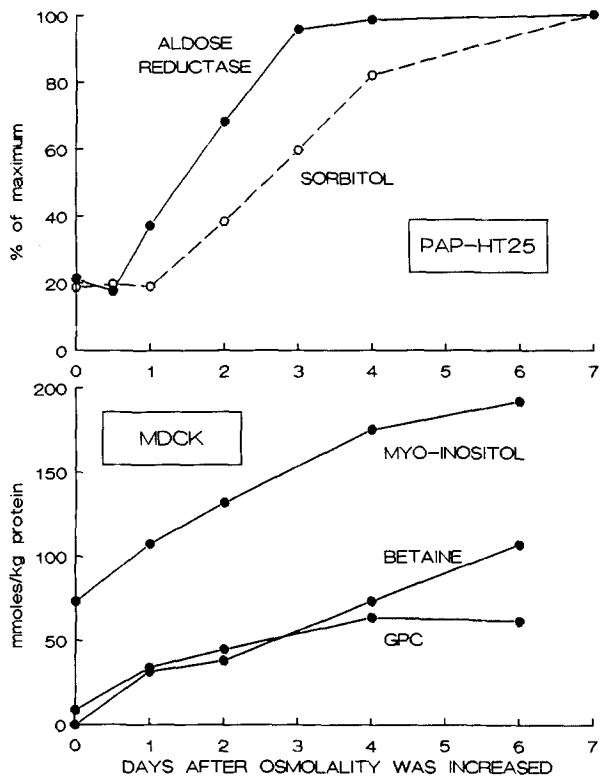


**Fig. 4.** Slow time course of the rise in aldose reductase activity and sorbitol in PAP-HT25 cells (top panel) and of GPC, inositol and betaine in MDCK cells (bottom panel). The top panel is reproduced from Uchida et al. (1989) and the bottom panel from Nakanishi et al. (1990) with permission of the publishers

level (Bagnasco et al., 1988). In contrast, the abundance of aldose reductase mRNA decreases rapidly, returning close to the baseline value within one day (Garcia-Perez et al., 1989).

## Decreased Extracellular Tonicity Induces Rapid Efflux of Sorbitol

The level of sorbitol in PAP-HT25 cells decreases very rapidly by efflux from the cells when medium NaCl is lowered and the cells swell (Bagnasco et al., 1988). PAP-HT25 cells grown in medium made hyperosmotic by adding NaCl have a very low permeability to sorbitol. However, when the medium osmolality is decreased abruptly from 600 to 300 mosmol/kg by lowering the NaCl concentration, sorbitol efflux immediately increases more than 150-fold. Approximately half of the cell sorbitol appears in the medium within 15 min. By this time the sorbitol efflux rate constant has decreased again to only twice the original value in the hyperosmotic medium. The cell sorbitol continues to fall after 15 min, but more slowly. It is less than 25% of the initial value after 24 hours and is close to baseline after 3 days. Rat renal papillary cells in suspension also lose sorbitol rapidly to the medium when NaCl (but not urea) concentration is decreased (Wirthensohn et al., 1989; Grunewald & Kinne, 1989).

The rapid efflux of sorbitol from PAP-HT25 cells results from increased sorbitol permeability (Siebens & Spring, 1989). While sorbitol is exiting the cells at a fast rate, there is also a large increase in the influx measured with $^{14}$C-sorbitol. When PAP-HT25 cells grown in 500 mosmol/kg medium are switched to a 300 mosmol/kg solution, $^{14}$C-sorbitol uptake increases 71-fold. The initial increase in permeability occurs within 30 seconds, and, if the osmolality is then restored to the original value again, sorbitol permeability returns to its original low level within 30 seconds. The permeability pathway is selective. Permeability to some other polyols is also enhanced, although to a smaller extent (sorbitol > mannitol > inositol). In contrast, there is little change in permeability to sucrose, raffinose, or L-glucose. The pathway does not saturate with concentrations of sorbitol up to 315 mM, nor is it competitively inhibited by high concentrations of other polyols. Therefore, the $K_m$ must be very high. A large number of putative inhibitors fail to prevent the increase in sorbitol permeability. The most effective is quinidine. 1 mM quinidine inhibits the increased sorbitol permeability by 82%. The sorbitol permeability increase is also blocked by reducing the temperature to 0°C. Presumably, the sorbitol efflux is mediated by a carrier or a selective channel, but the nature of this novel transporter and its mediation remain to be elucidated.

Whatever its basis, the rapid efflux of sorbitol ensures that the cells do not remain swollen when the osmolality decreases. In this sense, the response is analogous to the rapid efflux of KCl from many different cell types during the volume regulatory decrease (Eveloff & Warnock, 1987) and the efflux of taurine from Erlich cells that follows a fall in osmolality (Hoffmann, 1985).

Sorbitol can be catabolized to fructose in a reaction catalyzed by sorbitol dehydrogenase (Hers, 1960). This enzyme is present in the kidney, but it is much lower in the medulla than in the cortex (Heinz, Schlegel & Krause, 1975; Clampitt & Hart, 1978; Chauncey, Leite & Goldstein, 1988). Its activity also is low in PAP-HT25 cells and is not measurably affected by medium osmolality. Also, no fructose is detectable in the cells under any of the conditions studied, consistent with the absence of significant sorbitol degradation. Therefore, there is no evidence for significant sorbitol degradation in

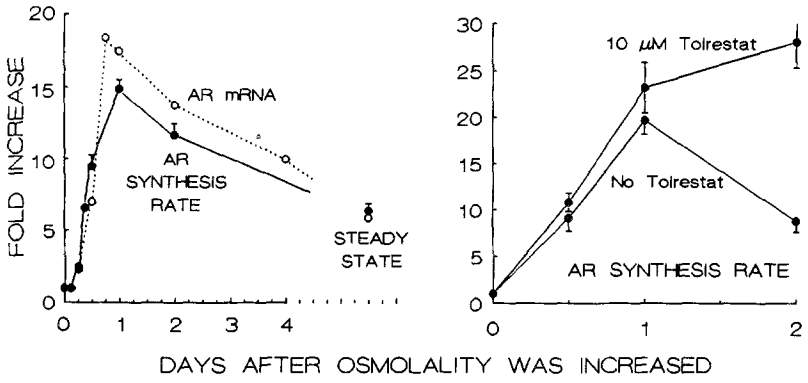


**Fig. 5.** Effect on aldose reductase synthesis rate and mRNA abundance of increasing the tonicity of the medium bathing PAP-HT25 cells. Left panel: time course of the increase in aldose reductase synthesis rate and mRNA abundance (data from Moriyama, Garcia-Perez & Burg, (1989*c*) and Garcia-Perez et al. (1989). Right panel: effect of the aldose reductase inhibitor, Tolrestat, on the synthesis rate (reproduced from Moriyama et al., 1989*c*) with permission of the publisher

the cell cultures. On the other hand, sorbitol dehydrogenase activity increases in rabbit inner medullary collecting ducts after a day of diuresis, suggesting that sorbitol catabolism may play a role in lowering inner medullary sorbitol under those conditions (Sands & Schrader, 1990).

## High Intracellular Ionic Strength Induces Aldose Reductase

The signal for induction of aldose reductase activity and subsequent sorbitol accumulation has been examined by comparing the effect of raising medium osmolality with different solutes (Fig. 6 and Uchida et al., 1989). As already indicated, high extracellular NaCl increases aldose reductase activity. Raffinose, a trisaccharide that does not enter cells readily, is as effective as NaCl, indicating that neither extracellular Na nor Cl is necessary. In contrast, raising the osmolality with either urea or glycerol, which penetrate these cells readily, fails to increase aldose reductase activity. Therefore, increased osmolality *per se* is not a sufficient signal. When osmolality is increased by adding raffinose, cell water decreases, and cell sodium and potassium concentrations increase. The changes persist for at least 24 hours until sorbitol content begins to rise. Addition of urea, on the other hand, does not cause any significant change in cell water or electrolyte concentration, just as it fails to induce increased aldose reductase activity. Based on these correlations, the trigger for induction of aldose reductase appears to be prolonged extracellular hypertonicity.

The mechanism by which prolonged extracellular hypertonicity increases renal medullary cell aldose reductase activity might well involve either the reduction in cell volume or the increase in intracellular Na or K, both of which precede the rise in enzyme activity. To distinguish between these possibilities, ouabain was added. Ouabain generally decreases cell potassium and increases cell sodium and volume. Medium osmolality was increased by adding NaCl either without ouabain or with different concentrations of the drug. Aldose reductase activity did not correlate significantly with cell volume or sodium, making it unlikely that either is directly involved in inducing enzyme activity. Aldose reduc-

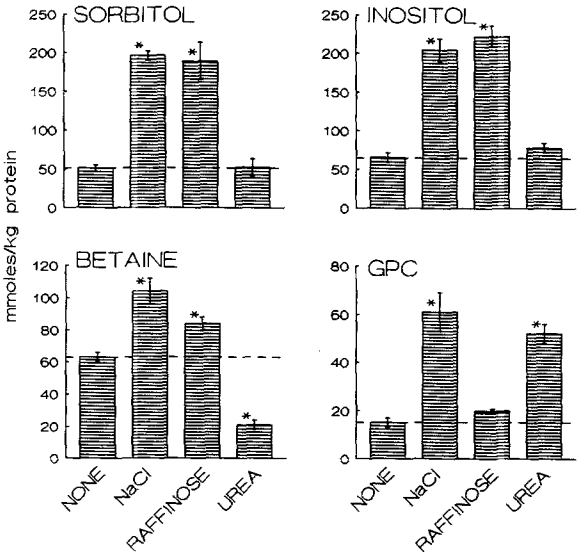


**Fig. 6.** Effect of medium NaCl, raffinose, and urea on accumulation of the different organic osmolytes by renal cells in tissue culture. Data for sorbitol are from PAP-HT25 cells (Uchida et al., 1989); data for inositol, betaine, and GPC are from MDCK cells (Nakanishi et al., 1990)

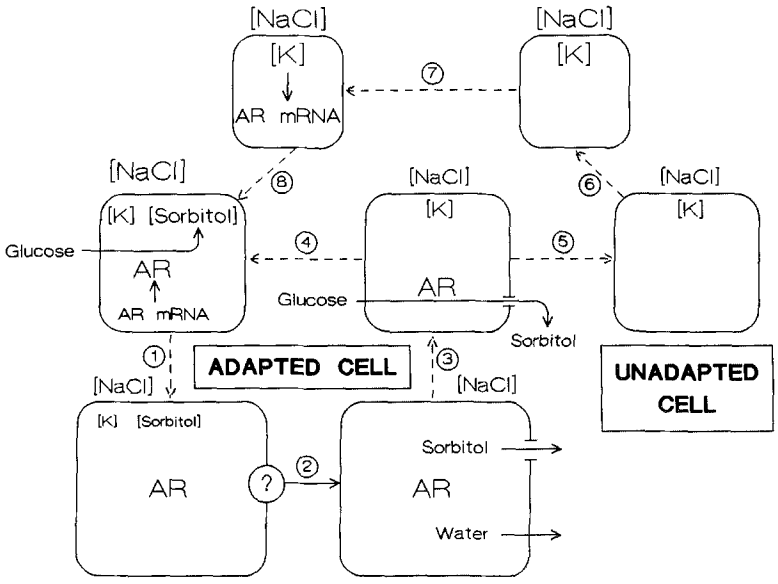


**Fig. 7.** Osmoregulation of sorbitol in renal medullary cells. In the state of chronic antidiuresis, the cells are adapted, meaning that they contain high levels of aldose reductase. During a transient diuresis, sorbitol concentration falls rapidly by efflux (steps *1* to *3*). Then, when antidiuresis is reestablished, sorbitol is accumulated again by synthesis, catalyzed by the aldose reductase, which has persisted during the diuresis (step *4*). If the diuresis is prolonged, however, as in diabetes insipidus, aldose reductase falls and the cells become unadapted (step *5*). Then, for sorbitol to reaccumulate, antidiuresis must induce synthesis of new aldose reductase (steps *6* to *8*), a process requiring more than one day. *See* the text for additional details (reproduced from Garcia-Perez and Burg (1990) with permission of the publisher

tase activity did correlate, however, with cell potassium concentration ($r = 0.72$, $P < 0.05$) and even better with the sum of cell potassium plus sodium concentration ($r = 0.99$, $P < 0.001$) (Uchida et al., 1989). Thus, the signal that induces increased aldose reductase activity apparently is a rise in the total concentration of sodium plus potassium salts (or ionic strength) in the cells.

## Cellular Adaptation to Hypertonicity Affects Sorbitol Accumulation

Based on a model originally proposed for yeast (Brown & Edgley, 1980), the different phases of cellular adaptation involved in osmoregulation by mammalian renal cells have been identified (Burg, 1988). The different states of adaptation are illustrated for sorbitol in Fig. 7. The model is meant to apply both to cells in vivo and in tissue culture.

Once aldose reductase has been induced in tissue culture, it is very stable. Its half-life is approximately one week regardless of the osmolality. This is the adapted state. Although adapted cells do not quickly alter their aldose reductase activity, they can vary sorbitol rapidly by changing permeability to it (Fig. 7, steps *1* to *4*). Renal medullary cells apparently can become similarly adapted in vivo. The urine osmolality and medullary extracellular NaCl concentration remain high in animals that are chronically antidiuretic because they are not ingesting much fluid. Under those conditions aldose reductase activity is high and does not decrease within hours (Cowley et al., 1990) to days (Sands & Schrader, 1990) after a diuresis starts.

Adapted cells can rapidly control their sorbitol content by varying their permeability. Extrapolating to renal medullary cells in vivo from the results in cell culture, the following sequence can be visualized. When a diuresis ensues and the renal medullary extracellular NaCl concentration falls, the cells swell (Fig. 7, step *1*), and sorbitol permeability increases (step *2*). The resultant efflux of sorbitol and water restores cell volume, but without any change in aldose reductase (step *3*). Although its production continues, sorbitol does not accumulate in the cells because its permeability remains high enough so that the sorbitol leaves the cells as fast as it is formed. If, within a few days, the diuresis ends and extracellular NaCl increases, the permeability falls and the sorbitol that is being synthesized is retained in the cells, increasing sorbitol over a few hours to compensate for the higher NaCl (step *4*). Sorbitol is able to accumulate again rapidly because the machinery for synthesizing it has been maintained during the transient diuretic state.

On the other hand, when the diuretic state is prolonged and extracellular NaCl remains low, aldose reductase activity falls slowly over several days, as the enzyme degrades, and the cells eventually become unadapted (step *5*). This occurs in pathological states like diabetes insipidus. If the condition causing the diuresis is corrected, the renal medullary extracellular NaCl increases, as the kidney makes more concentrated urine (step *6*). This triggers the transcription of aldose reductase mRNA and translation of enzyme protein (step *7*), returning the cells to the adapted state within several days (step *8*). This sequence of events occurs in the Brattleboro strain of rats, that have congenital diabetes

insipidus because of lack of antidiuretic hormone (ADH). When they are given ADH, their renal medullary $Na^+$, aldose reductase mRNA, aldose reductase activity, and sorbitol (which are initially low) increase considerably within a few days (Cowley et al., 1990). The process is slow, however. Sorbitol rises gradually during the first 3 days (Schmolke et al., 1989) and is even higher at 12 days than at 3 (Blumenfeld et al., 1989).

The model in Figure 7 refers specifically to sorbitol. However, the concept of adapted and unadapted states could apply to all four renal organic osmolytes. Although the mechanisms for accumulating GPC, betaine, and inositol differ from that for sorbitol, all are induced and decay relatively slowly. Yet, cellular levels of all four renal medullary organic osmolytes decrease rapidly by efflux, when extracellular tonicity falls.

### Hypertonicity and/or High Urea Induce Accumulation of GPC Synthesized from Choline

When Ullrich (Ullrich, 1959) originally discovered the large amount of GPC in dog renal medulla and recognized the osmotic consequences of its intracellular location, he suggested that the GPC is synthesized from phosphatidylcholine (PC) and that degradation of the GPC controls its abundance. Synthesis of GPC from PC is catalyzed by phospholipases and lysophospholipases (Dawson, 1955), and GPC is degraded in kidneys to choline and $\alpha$-glycerolphosphate in a reaction catalyzed by GPC diesterase (Baldwin & Cornatzer, 1968). Ullrich reasoned that GPC diesterase activity controls the level of GPC, based on two observations; (i) GPC diesterase activity is higher in the renal cortex than in the medulla, which could explain the higher level of GPC in the medulla, and (ii) addition of NaCl and urea to homogenates of cortical tissue inhibits GPC diesterase activity. Thus, the high levels of urea and NaCl in the renal medulla during antidiuresis could be inhibiting GPC diesterase, and thus elevating GPC. On the other hand, recent measurements failed to show differences in renal medullary GPC diesterase between diuretic and antidiuretic rats (Kanfer & McCartney, 1989), which does not support this theory.

GPC is higher in MDCK cells when they are grown for a long time in medium made hyperosmotic by adding NaCl or NaCl plus urea (Nakanishi et al., 1988). When MDCK cells grown at normal osmolality are switched to the hyperosmotic media, GPC rises gradually over a week or more (Fig. 4) (Nakanishi & Burg, 1989*a*). This increase in GPC was studied in detail to determine the mechanism involved.

Tissue culture media routinely contain choline, and it was present in the experiments described above. When cells grown in choline-containing medium of normal osmolality are switched to hyperosmotic medium containing no choline, GPC rises during the first two days, but then it falls back to the baseline level (Nakanishi, Turner & Burg, 1989*b*). In contrast, if choline is present in the hyperosmotic medium the level of GPC continues to rise. Thus, GPC apparently is synthesized from choline taken up from the medium, and, even when there is no exogenous choline, some metabolic intermediate already formed in the cells (presumably PC) can provide for a transient increase in GPC. Choline is taken up into MDCK cells by sodium-independent transport, but the choline transport is not affected by medium osmolality. Therefore, transport of choline is not rate limiting for osmoregulation of GPC accumulation.

Synthesis of GPC from PC also does not seem to be the controlling step. High urea and NaCl decrease the rate of synthesis of GPC from choline by MDCK cells in hyperosmotic medium (measured by $^{14}C$ incorporation), rather than increasing it, as would be expected from increased phospholipase activity (Zablocki et al., 1990). That points again to degradation of GPC as the controlling step. Consistent with this theory, GPC diesterase activity is decreased by 25% in MDCK cells adapted to high NaCl or high NaCl plus urea (Nakanishi & Burg, 1989*a*). However, this measured decrease in GPC diesterase seems small to explain the large increase in GPC, and we feel that further experiments are needed to reach a firm conclusion.

When cells adapted to high NaCl are returned to a normal osmolality, the GPC diesterase activity returns to normal within 12 hours, and there is efflux of choline from these cells (Nakanishi & Burg, 1989*a*). In contrast, there is no immediate increase in GPC diesterase when cells adapted to a mixture of high NaCl plus urea are returned to a normal osmolality, and these cells lose GPC to the medium. The decrease in GPC observed in MDCK cells after lowering the osmolality is relatively small (only 10 to 15% during the first three hours) compared to the much more rapid fall in sorbitol in PAP-HT25 cells discussed earlier. Presumably, the loss of GPC occurs because of an increase in permeability, as is the case for sorbitol, but the permeability has not been directly measured.

Like sorbitol, GPC increases when NaCl is added to the medium. However, GPC accumulation is also triggered by elevating urea alone (Fig. 7 and Nakanishi & Burg, 1989*a*; Nakanishi et al., 1989*b*; Moriyama, Garcia-Perez & Burg, 1990), whereas sorbitol accumulation is not. Elevated urea concentration does not increase the intracellular ionic

strength. Thus, high intracellular ionic strength is not necessary for GPC accumulation, even though it induces sorbitol accumulation (see above), and may also induce the GPC accumulation that occurs when extracellular NaCl is elevated. The fact that urea causes GPC to rise is particularly meaningful when we consider that GPC is a methylamine and that methylamines are "counteracting osmolytes" that can oppose the harmful effects of high urea (*see below*).

## Betaine is Accumulated by Osmoregulated Transport

Betaine is higher in MDCK cells grown in medium made hyperosmotic by the addition of NaCl than in medium of normal osmolality (Nakanishi et al., 1988). Any betaine in MDCK cells is taken up from the medium. In the original experiments the formulation of the medium did not include betaine (Nakanishi et al., 1988, 1990) because betaine is not needed for cell growth. However, fetal calf serum had been added to the medium, and it contains approximately 180 $\mu$M betaine (Nakanishi et al., 1990). Therefore, with the routine addition of 10% serum, the medium contains approximately 18 $\mu$M betaine. Then, the intracellular betaine concentration is 6 mM in MDCK cells grown in medium of normal osmolality (315 mosmol/kg) and 66 mM in medium made hypertonic to 615 mosmol by adding NaCl (Nakanishi et al., 1988). In the hypertonic medium the cell to medium betaine ratio exceeds 1000, implying a high affinity transport mechanism.

MDCK cells contain no betaine, when grown in betaine-free, defined medium, regardless of the osmolality (Nakanishi et al., 1990). However, when betaine is present, and the medium is made hypertonic by adding NaCl, cell betaine rises slowly over a week or more (Fig. 4) (Nakanishi et al., 1990). Hyperosmolality, achieved by adding raffinose, also induces betaine accumulation, but added glycerol and urea do not. Thus, the same solutes that induce sorbitol accumulation also induce accumulation of betaine. In this respect, the osmotic regulation of betaine closely resembles that of sorbitol. As will be seen, there are other similarities, as well. In the original studies of PAP-HT25 cells, no betaine accumulation was noted (Bagnasco et al., 1987). However, PAP-HT25 cells require so little serum (2%), that there is little betaine in the medium. When betaine is added to the medium, PAP-HT25 cells accumulate it, and to a much greater extent under hypertonic than under isotonic conditions (Moriyama et al., 1989*a*), similar to the result with MDCK.

MDCK cells take up betaine from the medium by active, sodium-dependent transport (Nakanishi et al., 1990). There are high affinity ($K_m$ = 0.12 mM) and low affinity ($K_m$ = 5.6 mM) betaine transport sites. Chronic hypertonicity does not affect the affinities, but does increase both $V_{max}$s by approximately the same amount. The $V_{max}$s are approximately 10 times higher in cells grown in medium made hypertonic to 615 mosmol/kg by adding NaCl. Only a small part of the increases in $V_{max}$ is due to the greater $Na^+$ gradient into the cells. When the transport measurements are all made with the same medium NaCl (hypertonicity compensated by mannitol), the $V_{max}$s are still elevated more than sevenfold, compared to the isotonic control. Thus, hypertonicity increases the intrinsic betaine transport capacity of the cells. The number of transporters (or less likely, their turnover rates) apparently increases.

When cells are acutely exposed to hypertonic medium, sodium-dependent betaine influx rises. It is maximal after one day, then falls as betaine and the other osmolytes accumulate in the cells. If there is no betaine in the medium (and cells), the transport capacity still increases after one day of hypertonicity, but then it does not fall significantly after that time (Nakanishi et al., 1990).

After the medium is made hypertonic, both betaine transport rate and aldose reductase synthesis rate peak at one day, then decrease again. A possible explanation is that elevated intracellular ionic strength stimulates both increases. Then, as betaine and/or sorbitol accumulate and the ionic strength falls back towards normal, this common stimulus is reduced.

There is little direct evidence as to whether there is active betaine transport that is osmoregulated in renal medullary cells in vivo, as there is in tissue cultures. In support of this possibility, slices from rat inner medulla concentrate $^{14}$C-betaine to a slice-to-medium ratio of 2.3 (Lohr & Acara, 1990). If betaine transport by renal medullary cells is an important factor in vivo, the betaine presumably originates elsewhere and is carried to the renal medulla by the circulation. The blood levels of betaine, measured by $^1$H NMR, were 80 and 120 $\mu$M in two normal humans (Grasdalen et al., 1987). Betaine is produced by oxidation of choline in liver (Mann & Quastel, 1937; Mann, Woodward & Quastel, 1938; Wilken, McMacken & Rodriquez, 1970) and kidney (Haubrich, Wang & Wedeking, 1975) mitochondria and is present in the diet. Within the kidney it is produced by proximal tubule cells (Wirthensohn & Guder, 1982). In addition, betaine can be synthesized directly from choline in renal medullary cells, which contain choline dehydrogenase activity (Grossman & Hebert, 1989; Lohr & Acara, 1990). However,

there is little change in the renal medullary choline dehydrogenase activity when rats accumulate large amounts of betaine in their renal medullas following salt loading (Grossman & Hebert, 1989).

Renal choline metabolism in vivo has been investigated by NMR (Eng, Berkowitz & Balaban, 1990). $^2$H-choline, which is infused into rabbits, is rapidly cleared from the blood and converted to $^2$H-betaine which appears in liver and in the renal cortex and inner medulla. Furosemide administration rapidly eliminates the betaine from the renal medulla, but not from the cortex or liver.

## Inositol also is Accumulated by Osmoregulated Transport

Many aspects of inositol accumulation by kidney cells in tissue culture resemble those of betaine. One difference, however, is that inositol is more important as a nutrient. It is routinely included in tissue culture medium to support cell growth. If the inositol is removed from a defined medium bathing MDCK cells, the transport capacity for inositol into the cells increases considerably (Nakanishi et al., 1989*a*). Betaine transport also is slightly greater when it is chronically absent from the medium (Nakanishi et al., 1990), but the difference is much less than for inositol.

Chronic exposure of renal tissue cultures to high NaCl increases the amount of inositol in the cells (Nakanishi et al., 1988). In a defined medium of normal osmolality (320 mosmol) and with 120 $\mu$M inositol, MDCK cells contain approximately 90 mmol/kg protein of inositol (Nakanishi et al., 1989*a*). When they are switched to medium made hyperosmotic to 700 mosmol with NaCl plus urea (but still containing inositol), cell inositol rises over a week or more to approximately three times the initial level (Fig. 4) (Nakanishi et al., 1989*a*). However, if they are switched to hyperosmotic medium containing no inositol, additional inositol does not accumulate in the cells. Increasing the osmolality with NaCl, raffinose, sorbitol, or dextrose (to 500 mosmol) has essentially the same effect as increasing it with NaCl plus urea (to 700 mosmol) (Nakanishi et al., 1989*a*, 1990). However, increasing the osmolality (to 500 mosmol) with urea alone (Nakanishi et al., 1989, 1990) or with glycerol (Nakanishi et al., 1990) does not induce inositol accumulation. The results are essentially the same with PAP-HT25 cells (Moriyama et al., 1989*a*, 1990).

MDCK cells take up inositol by active, sodium-dependent transport, that is inhibited by phlorizin (Nakanishi et al., 1989*a*). There are at least two transport sites which differ in their affinities. Under isotonic conditions, the $K_m$ of the higher affinity inositol transport site is $\approx$39 $\mu$M. When the cells are grown chronically in medium made hyperosmotic to 915 mosmol by addition of NaCl plus urea, $V_{max}$ increases almost threefold without a significant change in $K_m$. The increased sodium gradient provided by the higher medium NaCl accounts for only part of the rise in $V_{max}$. If the medium NaCl is equilized during the transport measurement (hypertonicity maintained by mannitol), the $V_{max}$ still rises almost twofold. Considering that hypertonicity increases the $V_{max}$ for inositol transport, but not the $K_m$, the number of transporters (or less likely, their turnover rates) probably is increased. In the remainder of the studies summarized here, medium NaCl was equalized during the transport measurements, so that differences in inositol transport are attributable to changes in intrinsic transport capacity and not to altered sodium gradients.

When MDCK cells are acutely exposed to medium made hyperosmotic to 700 mosmol with NaCl plus urea, sodium-dependent inositol uptake increases, reaches a peak by one day, then decreases as inositol accumulates in the cells (Nakanishi et al., 1989*a*). Thus, there apparently is a feedback by which increased cell inositol reduces its own transport. If the medium contains no inositol, so that inositol does not accumulate following the increase in osmolality, the transport capacity continues to rise after the first day. Also, if the osmolality is increased with raffinose, the transport rate increases to higher levels than when the osmolality is increased with NaCl. Presumably, the greater sodium gradient, when medium NaCl is elevated, allows the cells to accumulate sufficient inositol with less increase in intrinsic transport capacity than when hypertonicity is achieved with raffinose.

Hypertonicity increases the $V_{max}$ for inositol transport without any change in $K_m$. This could result either from increased uptake per transporter or from an increase in the number of functioning transporters. Although it is not yet clear which mechanism is involved, experiments involving expression of mRNA in *Xenopus* oocytes suggest that the latter is the case (Kwon et al., 1990*a*,*b*). These experiments were initiated in order to clone the cDNA encoding the inositol transporters. mRNA purified from MDCK cells acutely exposed to hypertonic medium is size fractionated using sucrose gradient centrifugation. The individual fractions are then microinjected into the oocytes, and transport activity is determined by measuring the uptake of $^3$H-inositol into the oocytes. A fraction of the mRNA with a size range of 2–6 kilobases is enriched for expression of inositol transport. Furthermore, mRNA only from cells exposed to hypertonicity in-

duces $^3$H-inositol uptake by the oocytes, while mRNA from isotonic cells does not. Probably, the mRNA codes for the inositol transporter, and more functional transporters are translated when the MDCK cells are placed in hypertonic medium.

There is little direct evidence that active inositol transport accounts for its osmoregulation in renal medullary cells in vivo, as in tissue culture. In support of this possibility, slices from rat inner medulla concentrate $^{14}$C-inositol to a slice-to-medium ratio of 27 (Goldstein, 1989). If transport is responsible for the osmoregulation in vivo, then the inositol presumably originates elsewhere and is carried to the renal medulla by the circulation. The normal blood level of inositol is 25 to 60 $\mu$M in different species (Clements & Reynertson, 1977; Cohen et al., 1982), which is approximately the $K_m$ of the high affinity transport site in MDCK cells. On the other hand, inositol is also synthesized *de novo* from glucose by numerous organs, including the kidney (Eisenberg, 1967), and is present in the diet (Holub, 1986). Inositol catabolism occurs only in the kidney, where the rate is 200-fold greater in slices from cortex and outer medulla than in papillary slices (Howard & Anderson, 1967). Conceivably, the osmoregulatory accumulation of inositol by renal medullas in vivo could be by synthesis, not by transport, as is found in cell culture, but there is, as yet, no direct evidence concerning this.

## When Tonicity Decreases, Inositol and Betaine Rapidly Leak from Renal Cells, and the Active Influx of these Compounds Gradually Decreases

When MDCK (Nakanishi & Burg, 1989*b*) or PAP-HT25 (T. Moriyama, A. Garcia-Perez, and M. B. Burg, *unpublished observations*) cells are switched from hypertonic to isotonic medium, there is a rapid, transient efflux of both inositol and betaine. 15 to 20% of cell inositol and 30 to 50% of betaine exit within 10 to 15 minutes. The inositol efflux apparently is due to increased permeability since, under those conditions, $^3$H-inositol flux into PAP-HT25 cells also increases transiently (Siebens & Spring, 1989).

Active influx of inositol and betaine falls gradually, which contributes to the eventual loss of these compounds from the cells (Nakanishi & Burg, 1989*b*). Sodium-dependent inositol influx decreases approximately 30% within 3 hours and back close to baseline by 12 hours. Sodium-dependent betaine influx falls approximately 25% by 3 hours, 50% by one day, and back to baseline by the end of the second day (Nakanishi & Burg, 1989*b*).

Thus, decreased tonicity reduces cell inositol and betaine both by a transient, rapid efflux from the cells and a slower decrease in their active transport into the cells.

## Organic Osmolytes Serve as Compatible Solutes; Methylamines are also Counteracting Solutes

Excessive concentrations of ions generally "perturb" the structure and function of biological macromolecules. For example, high NaCl or KCl alters the kinetics of enzymes in cell homogenates. In contrast, high concentrations of certain organic osmolytes do not have such effects, which is the theoretical reason why cells adapt to hypertonic environments by accumulating these electrically neutral, compatible organic osmolytes. In this fashion the cells are able to maintain their volume, while still keeping their intracellular ion concentration at the level that is optimal for protein and nucleic acid function (Yancey et al., 1982). In support of this hypothesis, the accumulation of compatible organic osmolytes has been shown to help sustain the growth of bacteria in hyperosmotic medium.

In addition to this role, methylamines, such as the betaine and GPC that are present in the kidney medulla, can offset deleterious effects of the urea. High concentrations of urea are present in kidney medullas as part of the renal concentrating mechanism. Urea is a potent destabilizer of protein structure and generally an inhibitor of function. Methylamines are effective stabilizers of protein structure, and they counteract the effects of urea. They are most effective as counteracting solutes when combined with urea at a 1 : 2 ratio (methylamine : urea) (Yancey et al., 1982).

Recent studies have provided direct evidence for a similar critical role of compatible organic osmolytes in renal cells (Yancey, Burg & Bagnasco, 1990). The test used was cloning efficiency, which quantifies survival and growth of cells in tissue culture. PAP-HT25 cells were tested in hypertonic medium under conditions (no medium betaine or urea) in which sorbitol is their main organic osmolyte. An aldose reductase inhibitor was added to prevent them from accumulating sorbitol. The inhibitor had no effect on cloning efficiency while the cells were in isotonic medium, but greatly reduced it in hypertonic medium. Thus, accumulation of sorbitol is important for the survival and growth of these cells in hypertonic medium, consistent with its role as a compatible osmolyte.

Betaine is a methylamine that can counteract the destabilizing effects of urea, as demonstrated in isolated preparations of enzymes and other macromolecules (Yancey et al., 1982). More recently, be-

taine has been found to have a similar effect on renal cells in culture. As already described, both urea and betaine are readily taken up by MDCK cells from their medium. Cloning efficiency of MDCK cells declines when the osmolality is increased above 400 mosmol by adding urea (Yancey & Burg, 1990). However, when betaine is also added, increasing the osmolality even further, cloning efficiency is restored towards normal levels. The results are similar with PAP-HT25 and CHO cells. Thus, betaine counteracts the harmful effects of urea on living renal cells, consistent with a role as a counteracting osmolyte.

Given this effect of betaine, it seems paradoxical that MDCK (Nakanishi et al., 1990) and PAP-HT25 (Moriyama et al., 1990) cells accumulate less, rather than more, betaine in response to high urea concentrations (Fig. 7). Although there is no direct evidence that GPC counteracts the destabilizing effects of urea, this probably is the case, judging from its chemical structure (Somero, 1986). As already mentioned, elevation of urea induces accumulation of GPC, but not of the other organic osmolytes. Thus, renal cells prefer GPC to betaine as their counteracting osmolyte in tissue culture. The same seems also to be the case in vivo, judging from the observation that the renal medullary level of GPC highly correlates with that of urea (Yancey & Burg, 1989; Yancey, 1988).

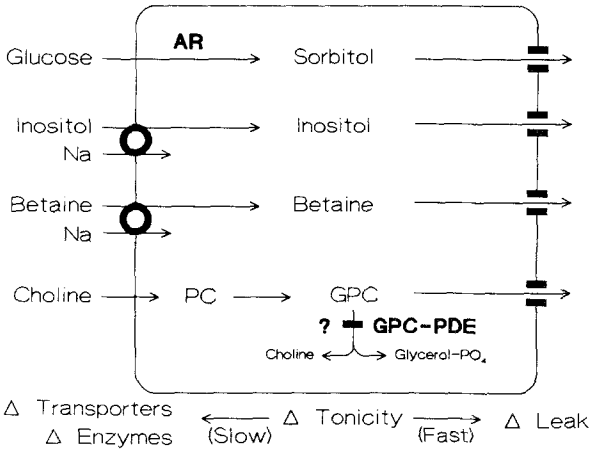


**Fig. 8.** Mechanisms of organic osmolyte accumulation and loss in renal medullary cells. When the extracellular osmolality is altered, transporters and enzymes change (Δ) slowly (at left), but permeability changes rapidly (at right). (Reproduced from Garcia-Perez and Burg (1990) with permission of the publisher

## The Occurrence of Four Different Renal Medullary Organic Osmolytes Provides the Safety Factor of Redundancy of their Protective Effects

The two roles postulated for renal medullary organic osmolytes, i.e. as compatible and also counteracting substances, could in principle be satisfied by only two solutes, one polyol and one methylamine. However, there are four major renal medullary organic osmolytes. While it is not entirely clear why there are so many, one advantage could be that the redundancy provides a safety factor. Any one of the organic osmolytes can vary because of factors other than tonicity and urea concentration. Extraneous factors may influence availability of particular substrates, there may be competing reactions, and the relevant enzymes and transporters might be inhibited by drugs or toxins. In these cases, when the availability of one of the organic osmolytes is restricted, it is advantageous if another can substitute for it.

An example is that in PAP-HT25 cells betaine and sorbitol are often reciprocally related (Moriyama et al., 1989*a*). Cell sorbitol and betaine vary inversely when cell betaine content is altered by changing its concentration in the medium or when cell sorbitol content is varied by inhibiting aldose reductase. There is a similar relation in vivo. When antidiuretic rats are given an inhibitor of aldose reductase, sorbitol falls in their inner medullas, but there is a compensating rise in betaine (P. H. Yancey, *personal communication*). The rise in betaine may protect the renal medullary cells from what would otherwise be a harmful effect of the aldose reductase inhibitors. Recall that the aldose reductase inhibitor Tolrestat greatly decreases cloning efficiency of PAP-HT25 cells in hypertonic medium, in the absence of betaine (Yancey et al., 1990). Under those conditions, addition of betaine to the medium (and cells) protects them from the harmful effect of Tolrestat (T. Moriyama, A. Garcia-Perez, and M. B. Burg, *unpublished observation*). This may be the reason why aldose reductase inhibitors do not have any apparent renal toxicity in vivo.

## Summary and Conclusions

Kidney cells accumulate organic osmolytes in order to protect themselves from the high concentrations of NaCl and urea in the blood and interstitial fluid of the renal medulla. The renal medullary organic osmolytes are sorbitol, inositol, betaine and GPC. The concentrations of these solutes in renal medullary cells are osmoregulated in resposne to extracellular NaCl and urea concentration, as summarized in Fig. 8 (the putative controlled steps are highlighted). Sorbitol accumulates by synthesis from glucose, catalyzed by aldose reductase. Hypertonicity increases

the transcription of the gene that encodes this enzyme. GPC is synthesized from choline, and the amount retained apparently may be conrolled by the activity of GPC diesterase, an enzyme that catabolizes GPC. Inositol and betaine are taken up from the medium by sodium-dependent transport, and this transport is increased by hypertonicity. Control of these processes is slow (hours to days), but a decrease in tonicity causes a transient, rapid efflux of the solutes, which prevents the cells from becoming overly distended. Similar strategies are used by all types of cells, including bacteria and those in plants and animals, that can adapt to hyperosmotic stress.

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