Aust. J. Biol. Sci., 1975, **28**, 109–14

Osmotic Effects of Sorbitol Accumulation in Monkey Kidney Epithelial Cell Cultures

J. C. Hutton,[A] *P. J. Schofield,*[A] *J. F. Williams*[A] *and F. C. Hollows*[B]

[A] School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033.
[B] Department of Ophthalmology, Prince of Wales Hospital, Randwick, N.S.W. 2031.

Abstract

It has been observed that primary monolayer cell cultures derived from monkey kidney cortex behave in a similar manner to the mammalian lens in that they accumulate high concentrations of sorbitol when they are incubated in medium containing a high glucose concentration (33 mM). An investigation was undertaken to determine whether the accumulation of sorbitol by these cultures results in cellular damage by an osmotic mechanism similar to that which has been proposed to occur in the lens.

Phase-contrast microscopy and histochemical investigations revealed that no changes in the cell size, cell growth rate, or cell staining properties occurred as a result of exposure of the cell cultures to a glucose concentration of 33 mM for up to 4 days. The cell protein and myo-inositol concentrations and fatty acid composition were also unaffected, as was the incorporation of radioactivity from [U-^{14}C]leucine into cell protein.

The rate of ^{86}Rb influx of the cell cultures was decreased and the rate of ^{86}Rb efflux was increased by incubation in medium containing a glucose concentration of 33 mM.

From these observations it was concluded that the accumulation of sorbitol by monkey kidney epithelial cell cultures did not exert a pathabolic influence upon their growth and metabolism, and that these cells, unlike the lens, have the capacity to compensate adequately for changes in transmembrane osmotic gradients induced by sorbitol accumulation.

Introduction

In the presence of a high glucose concentration, as in diabetes, or a high galactose concentration, as in experimentally induced galactosaemia, there is an accumulation of the corresponding polyols sorbitol and dulcitol in various tissues, including lens (Van Heyningen 1959; Kinoshita 1965). Under these conditions cataract formation occurs and the pathogenesis of such cataract formation has been linked primarily to an abnormal functioning of the polyol pathway. The accumulation of the polyol results in effects such as osmotic swelling of the lens cells, defects in amino acid concentrating mechanisms, and changes in cation pump mechanisms (Kinoshita 1965). It has been suggested that these disturbances culminate in the irreversible impairment of cell function and eventually result in cellular disruption.

However, sorbitol accumulation in the diabetic state is not restricted to the lens; increased concentrations have been found in other tissues such as peripheral nerve where the accumulation has been implicated in the pathogenesis of diabetic peripheral neuropathy (Stewart *et al.* 1967) and in the kidney (Gabbay and O'Sullivan 1968). Thus a crucial point is whether sorbitol accumulation is involved in the development of diabetic nephropathy and, if so, by what mechanism. From the studies on diabetic cataractogenesis and peripheral neuropathy, it appears that the mechanism by which polyol accumulation exerts its primary effect is by causing osmotic stress to the

particular cell as a result of the high intracellular concentration of polyol. The accumulation of sorbitol in the kidney could cause an osmotic insult to this organ which may ultimately result in irreversible damage to the renal cells.

We have previously observed that monkey kidney epithelial (MKE) cells accumulate polyols when incubated in media containing a high concentration of glucose or galactose (33 and 28 mM respectively); the intracellular concentrations of the respective polyols (Hutton *et al.* 1974) are similar to those observed in the lens and peripheral nerve in the diabetic or galactosaemic states. The present paper relates the investigations into the effects of such accumulations on the morphology, the transmembrane transport of ^{86}Rb, the rate of protein synthesis, and the fatty acid composition in MKE cells. The objective of these investigations was to determine whether MKE cells, like lens cells, sustained osmotic damage as a consequence of their accumulation of sorbitol. Contingent upon the results of this investigation was the question of whether a general model for the osmotic effect of polyol accumulation could be formulated and if a particular cell of the kidney did suffer osmotic damage, could this damage be related to the development of nephropathy *in vivo*.

Materials and Methods

Primary monolayer cultures of epithelial cells derived from trypsinized monkey kidney cortex were grown by the method of Hutton *et al.* (1974). The cell suspensions and medium 199 were obtained from Commonwealth Serum Laboratories, Parkville, Vic. Growth of cell cultures and possible changes in cell morphology were monitored by phase-contrast microscopy. In some instances cells were grown on glass coverslips in the culture vessels and subsequently stained with haematoxylin and PAS by the conventional methods. Free carbohydrate concentrations of cell suspensions subjected to ultrasonic vibrations were determined by the method of Hutton *et al.* (1974), and protein by the method of Lowry *et al.* (1951).

^{86}*Rb Influx Studies*

MKE cell cultures were preincubated for 36 h at 37°C in medium 199 containing either 5·5 or 33 mM glucose. ^{86}RbCl ($8 \cdot 8 \times 10^5$ c.p.m./ml medium; Australian Atomic Energy Commission, Lucas Heights, N.S.W.) was then added, and the incubations continued. At various time intervals, the incubations were terminated, and the radioactivity in samples of suspensions of cells which were subjected to ultrasonic vibrations determined by scintillation counting.

In control experiments disodium 3,3′-tetramethylene glutarate (TMG) was added (5 μmol/ml medium) and the intracellular radioactivity determined after a 4 h incubation in the presence of ^{86}RbCl.

^{86}*Rb Efflux Studies*

Cultures were preincubated as above, and the ^{86}RbCl added and the incubation continued for a further 4 h. The medium was then removed, the cells rapidly washed four times with fresh medium, and fresh medium without ^{86}RbCl then added. At various intervals after the change of medium, the radioactivity of 0·1-ml samples of the medium was determined. At the completion of the incubation, the radioactivity of a suspension of the cells subjected to ultrasonic vibrations was determined.

Incorporation of Radioactivity from [U-^{14}C]Leucine into Protein

MKE cell cultures were preincubated for 28 h at 37°C in medium 199 which contained either 5·5 or 33 mM glucose. L-[U-^{14}C]leucine (Radiochemical Centre, Amersham, U.K.) was then added to each incubation mixture (0·93 μCi/ml medium) and the cultures incubated for a further 4 h. At the completion of the incubation, the cells were subjected to ultrasonic vibrations, bovine serum albumin fraction V added as carrier protein, and the total protein precipitated with 10% (w/v) trichloroacetic acid. After centrifugation, the radioactivity of the supernatant was determined by scintillation counting. The precipitated protein was successively extracted with L-leucine in trichloroacetic acid, ethanol, ether–ethanol–chloroform (2:1:1 v/v), ether, and acetone and then dried *in vacuo*. The dried

pellet was dissolved in 88% (w/v) formic acid, and the radioactivity determined. Counting efficiencies were determined by the use of [1-^{14}C]hexadecane as an internal standard.

Fatty Acid Composition of a Total Lipid Extract

MKE cell cultures were grown for 4 days in medium 199 containing 5·5 mM glucose, and then for 4 days in medium 199 containing either 5·5 or 33 mM glucose. After subjection to ultrasonic vibration, the cell suspension was lyophilized, resuspended in water, and the total lipids extracted by the method of Folch *et al.* (1957). The lipid extract was methylated with BF_3, and the fatty acid methyl esters extracted, dried, redissolved in light petroleum and purified by passage through a Florisol column. The fatty acid profile of the esters was determined by gas chromatography on a Packard 7300 gas chromatograph with a 2 m by 4 mm column of Gas Chrom. P coated with 15% (w/v) diethyleneglycol succinate (DEGS). Heptadecanoic acid was added at the first step of the extraction procedure as an internal standard.

Results

The cells of the primary cell cultures were typically epitheloid in morphology, ranging in diameter from 7 to 14 μm. Within most cultures, isolated large cells (17–21 μm diameter) and small populations of small (5–7 μm diameter) fibroplastic cells were observed. These atypical cells collectively constituted less than 0·5% of the total cell number.

Exposure of cultures of MKE cells for periods of up to 4 days to high glucose (33 mM) medium did not affect their size or appearance under phase-contrast microscopy, or the incidence of mitotic events. There were no qualitative microscopic differences in the rate of growth of cell cultures over a 4-day period of exposure (4th–8th day post-inoculation) to media containing glucose at a concentration of either 5·5 or 33 mM. The protein content of 8-day-old cultures (9 mg protein per culture vessel) was not affected by the prior exposure of the cells to elevated glucose concentrations for 4, 6, 24, 36 or 96 h.

Cell cultures incubated for 4 h in medium containing 33 mM glucose accumulated high concentrations of sorbitol (210 μmol/g protein), and also fructose (10 μmol/g protein) and glucose (25 μmol/g protein). The concentrations of these intermediates in MKE cell cultures which were incubated for 96 h in high glucose medium did not vary significantly from their concentrations after only a 4 h incubation. Neither fructose nor sorbitol were detected in the incubation media after either a 4- or 96-h incubation period. The lower limit of sensitivity is approximately 20 nmol, and each culture, of approximately 10 mg wet weight, contained approximately 300 nmoles of intracellular sorbitol. The sorbitol concentration of the MKE cells was routinely determined on four pooled samples, i.e. equivalent to approximately 1200 nmol sorbitol. Had equilibrium been established between the MKE cells and the medium with respect to sorbitol concentrations, the sorbitol concentration of the medium would thus be approximately 30 nmol/ml, and hence detectable in the medium itself or readily detectable in medium following concentrating procedures. The concentration of myo-inositol in MKE cell cultures (8·4 μmol/g protein) was not affected by a high glucose incubation of either 4 or 96 h duration.

For cultures incubated in both low or high glucose media, the initial rate of ^{86}Rb influx (Fig. 1*a*) was approximately 20% lower for cultures incubated in high glucose medium than that observed in cultures incubated in low glucose medium.

The ratio of the intracellular to extracellular radioactivities attained at equilibrium for cultures incubated in high glucose medium was 75 ± 6% (n = 6) of the value

determined for cultures incubated in low glucose medium. In the presence of 5 mM TMG, the corresponding percentage was 127 ± 20% (n = 4). TMG prevents polyol synthesis by inhibiting aldose reductase (Kinoshita *et al.* 1968).

For MKE cultures incubated in both low or high glucose media, the initial rate of efflux of ^{86}Rb (expressed as the percentage of the initial intracellular radioactivity) which appeared in the media per minute (Fig. 1*b*) was higher for cultures incubated in high glucose media than in cultures incubated in low glucose media.

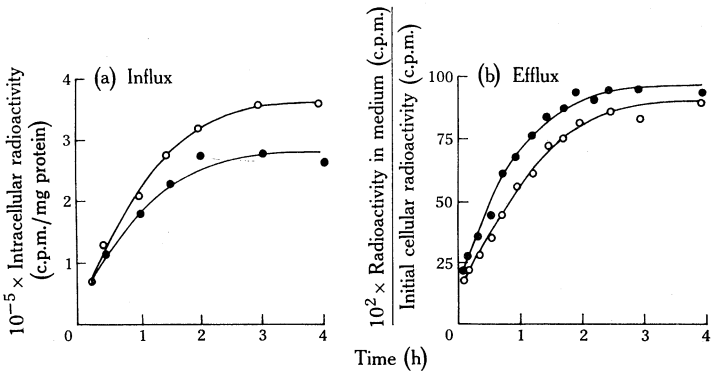

Fig. 1. Effect of incubation in high glucose concentrations on ^{86}Rb flux in monkey kidney epithelium cell cultures. Primary monolayer cultures of epithelium cells (7 days old) were preincubated for 36 h in medium 199 which contained either 5·5 mM (○) or 33 mM (●) glucose. For influx studies (*a*), ^{86}RbCl (8·8 × 10^5 c.p.m./ml medium) was then added to the medium and the incubations were terminated at various time intervals up to 4 h. The accumulation of radioactivity in the cells over these intervals was determined by liquid scintillation counting. For efflux studies (*b*), cultures were incubated for 4 h in the presence of ^{86}RbCl (8·8 × 10^5 c.p.m./ml medium) and then in fresh media without radioisotope. The appearance of radioisotope in the fresh medium was determined by liquid scintillation counting. Each plotted value is the mean of determinations from four cultures.

When monolayer cultures of MKE cells (7 days old) were incubated for 28 h at 37°C in medium 199 which contained either 5·5 mM (low) or 33 mM (high) glucose, and 9·3 μCi of L-(U-^{14}C)leucine was added to the media for the final 4 h of incubation, the incorporation of radioactivity, as determined by liquid scintillation counting, into a protein and a trichloroacetic acid-soluble fraction was not significantly affected by incubation in the medium containing the higher glucose concentration. This is shown in the following tabulation in which the radioactivity is expressed as μCi/g protein and each value is the mean ± S.E.M. of five determinations:

Incubation medium	Protein	Acid-soluble fraction
High glucose	32·8 ± 1·2	10·5 ± 0·5
Low glucose	31·8 ± 0·7	13·6 ± 1·3

Also when MKE cell cultures were grown for 4 days in medium 199 which contained 5·5 mM glucose and then in medium 199 which contained either 5·5 mM (low) or 33 mM (high) glucose for a further 4 days, and a total lipid extract of the cells was then prepared, and its fatty acid composition was determined by gas–liquid chromatography, the fatty acid composition of the cultures contained a remarkably low

percentage of linoleic acid and of other polyunsaturated fatty acids. This is illustrated in the following tabulation in which each value is the mean of two determinations:

Growth medium	Major fatty acid components (g/100 g total fatty acid)					
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
High glucose	4·3	26·5	10·6	13·8	43·2	1·4
Low glucose	5·2	27·0	12·4	13·4	39·1	2·6

This finding was apparently related to the absence of suitable metabolic precursors of these essential fatty acids in the media. The fatty acid profile was not affected significantly by changes in the concentration of glucose in the growth media.

Discussion

The failure to find significant concentrations of acyclic hexitol in the media surrounding MKE cells after their incubation in high glucose media suggested that the plasma membranes of these cells, like those of other cells *in vivo* (Wick and Drury 1951), are impermeable to acyclic polyols. It follows that the accumulation of sorbitol by cultures of MKE cells resulted in a hyperosmolar state within the cell. Assuming the cells contained 15% (w/w) protein and 80% (w/w) water, the magnitude of the osmotic gradient due to sorbitol accumulation under a high glucose incubation was approximately 50 m-osmolar. This intracellular concentration of polyol, and hence concentration gradient, is of the same order as that found for acyclic polyol accumulation in the lens in the diabetic or galactosaemic state (Quan-Ma and Wells 1965; Kuck 1966). Furthermore, since the osmotic gradient approaches the concentration gradient of K^+ in the normal cell, it may be expected that the cells under these conditions would sustain osmotic damage. However, since the cell morphology, growth rate, amino acid incorporation into protein, and fatty acid profile were not significantly affected it would appear that the cells are not appreciably affected by the intracellular sorbitol accumulation.

The observed decrease in the rate of ^{86}Rb influx and the increase in the rate of ^{86}Rb efflux from MKE cell cultures incubated in a high concentration of glucose suggested that an alteration in the intracellular cation concentrations had occurrred, and that the cells exhibited an increased leakage of the ^{86}Rb ion. The finding that TMG restored the equilibrium ratio of the intracellular to extracellular radioactivity further suggested that the effect on ^{86}Rb transport was secondary to sorbitol accumulation by these cells. This finding is similar to that demonstrated by Chylack and Kinoshita (1969) for lens exposed to a high glucose concentration *in vitro*; in this instance TMG both prevented sorbitol synthesis and preserved the normal cation balance of the lens throughout the 8-day incubation. The MKE cells show similar changes in ^{86}Rb transport to those shown by the lens, but these alterations only occur after other disturbances had begun to appear in the lens during the cataractogenic process. However, the MKE cells do not exhibit such osmotically induced changes and in this respect it may be relevant that the swelling and the loss of myo-inositol (Broekhuyse 1968), which has been shown to occur in lens as a consequence of the intracellular accumulation of high concentrations of acyclic hexitol *in vitro*, was not presently observed in the MKE cells exposed to high glucose concentrations.

MKE cells, when injected intracerebrally into rats, assembled in groups giving rise to tubular structures suggesting that the MKE cell cultures originate from renal tubular epithelium and maintain at least some functional properties of the parental

cells (Sigel *et al.* 1958). The present findings that MKE cell cultures, unlike lens, did not suffer pathabolic osmotic disturbances from the intracellular accumulation of acyclic hexitols may be related to the specialized physiological function of the renal tubules in the maintenance of cation and anion balance *in vivo*. It follows from the above observations that a general model for the osmotic effects of polyol accumulation in mammalian cells cannot be presently established. In addition, these findings, together with the reported localization of sorbitol in the medulla (Gabbay and O'Sullivan 1968), suggest that sorbitol accumulation does not have a direct causal effect in the development of diabetic glomerulosclerosis, particularly since isolated glomeruli from diabetic rats contain no sorbitol and isolated glomeruli incubated *in vitro* in medium containing 33 mM glucose show no sorbitol accumulation (Hutton *et al.*, unpublished data). However, the possibility that sorbitol accumulation may have some unknown, indirect effect on renal function cannot be definitively eliminated solely on the basis of these data.

Acknowledgments

F.C.H., P.J.S., and J.F.W. gratefully acknowledge the support of the National Health and Medical Research Council of Australia. J.C.H. gratefully acknowledges the support of a Commonwealth Post-graduate Award.

References

Broekhuyse, R. M. (1968). Changes in myo-inositol permeability in the lens due to cataractous conditions. *Biochim. Biophys. Acta* **163**, 269–72.

Chylack, L. T., and Kinoshita, J. H. (1969). A biochemical evaluation of a cataract induced in a high glucose medium. *Invest. Ophthalmol.* **8**, 401–12.

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509.

Gabbay, K. H., and O'Sullivan, J. B. (1968). The sorbitol pathway in diabetes and galactosaemia: enzyme and substrate localisation and changes in kidney. *Diabetes* **17**, 300.

Hutton, J. C., Williams, J. F., Schofield, P. J., and Hollows, F. C. (1974). Polyol metabolism in monkey kidney epithelial cell cultures. Sorbitol synthesis. *Eur. J. Biochem.* **49**, 347–53.

Kinoshita, J. H. (1965). Cataracts in galactosemia. *Invest. Ophthalmol.* **4**, 786–99.

Kinoshita, J. H., Dvornik, D., Kraml, C., and Gabbay, K. H. (1968). The effect of an aldose reductase inhibitor on the galactose-exposed rabbit lens. *Biochim. Biophys. Acta* **158**, 472–5.

Kuck, J. F. R. (1966). Sorbitol pathway metabolites in the diabetic rabbit lens. *Invest. Ophthalmol.* **5**, 65–73.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–75.

Quan-Ma, R., and Wells, W. W. (1965). The distribution of galactitol in tissues of rats fed galactose. *Biochem. Biophys. Res. Commun.* **20**, 486–90.

Sigel, M. M., Scotti, T. M., Wryk, M. A., and Dorsey, M. (1958). Tubule formation in rat brains by monkey kidney cells previously grown *in vitro*. *Nature (Lond.)* **182**, 1034–5.

Stewart, M. A., Sherman, W. R., Kurien, M. M., Moonsammy, G. T., and Wisgerhof, M. (1967). Polyol accumulations in nervous tissue of rats with experimental diabetes and galactosemia. *J. Neurochem.* **14**, 1057–66.

Van Heyningen, R. (1959). Formation of polyols by the lens of the rat with "sugar" cataract. *Nature (Lond.)* **184**, 194–5.

Wick, A. N., and Drury, D. R. (1951). Action of insulin on the permeability of cells to sorbitol. *Am. J. Physiol.* **166**, 412–23.

Manuscript received 26 July 1974