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DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE ENHANCEMENT OF α-METHYL-D-GLUCOSIDE ACCUMULATION BY KIDNEY CORTEX SLICES

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SUMMARY

Cyclic adenosine 3′,5′-monophosphate and N^6-2′-*O*-dibutyryl cyclic adenosine 3′,5′-monophosphate increase the accumulation of α-methyl-D-glucoside by cortical slices from rat, rabbit, dog and human kidney. The characteristics of the effect have been studied in rat tissue. At least 90 min of exposure of the tissue to cyclic nucleotide prior to onset of glucoside accumulation is required as well as presence of the cyclic nucleotide during the accumulation phase. Inhibition of protein synthesis does not abolish the effect of N^6-2′-*O*-dibutyryl cyclic adenosine 3′,5′-monophosphate. The cyclic nucleotide causes an increase in the initial entry rate of α-methyl-D-glucoside into cells and an increase in the intracellular steady state concentration. The cyclic nucleotide does not affect the apparent K_m of the glucoside entry process but increases the maximum velocity of accumulation.

INTRODUCTION

Numerous observations have been made of cyclic adenosine monophosphate (cyclic AMP) effects on membrane transport of water[1,2] and ions[1,3] as well as sugars[4,5] and amino acids[6] in various model systems. With regard to non-ionic solutes, dibutyryl cyclic AMP has been shown to inhibit glucose transport in adipose cells[4]. On the other hand, Schimmel and Goodman[5] have reported a cyclic nucleotide related increase in the non-concentrative uptake of D-xylose by adipose tissue. Weiss *et al.*[6] have shown that cyclic AMP and dibutyryl cyclic AMP increase the concentrative uptake of amino acids by renal cortical slices. We have studied the effect of cyclic nucleotides on the accumulation of α-methyl-D-glucoside, a model sugar[7,8], by kidney cortical cells from rat, rabbit, dog and human. Our results indicate that dibutyryl cyclic AMP enhances the accumulation of this sugar in kidney cortex of all the species examined. The nature of this effect has been examined in rat renal cortex slices, and our observations form the basis of this report.

METHODS

Tissues

Kidneys were obtained from non-fasting male Sprague-Dawley rats weighing 150–200 g killed by stunning and decapitation, adult male white rabbits killed by cervical fracture, and mongrel dogs killed by exsanguination. One human kidney specimen was obtained from a patient undergoing nephrectomy for a renal cell carcinoma (courtesy of Dr Harry Schoenberg, Hospital of the University of Pennsylvania, Philadelphia, Pa. (U.S.A.)). Slices of cortex, approx. 0.4 mm thick, weighing approx. 20 mg, were prepared with a Stadie–Riggs microtome.

Determination of sugar accumulation

The technique for measuring sugar accumulation by kidney cortex slices was similar to that used previously for studies of amino acid accumulation[10] as well as sugar uptake in both rat[7,11] and human kidney[8,12]. In the present experiments, however, an incubation prior to addition of the α-methyl-D-glucoside or other sugars was employed in which the kidney cortex slices were placed in 30-ml stoppered plastic bottles containing either 2 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, or buffer containing various nucleotides (0.2 mM dibutyryl cyclic AMP, and 1 mM cyclic AMP, cyclic GMP, AMP or ATP). The bottles were gassed for 30 s with O_2–CO_2 (95:5, v/v), sealed and incubated at 37 °C in a Dubnoff shaker for various periods of time, usually 2 h. In certain experiments cycloheximide (1 mM) was added to both the treated and untreated tissues.

In studies employing rat tissue three cortical slices, one from each of three rats were incubated together in each bottle so that paired control and treated tissues were compared. In each individual experiment at least triplicate flasks were prepared for each variable examined. When using rabbit, dog and human kidney, single slices were incubated in each bottle and triplicate or quadruplicate determinations were made for each datum.

Following the incubation period described above, the bottles were opened and α-methyl-D-glucoside (unlabeled and ^{14}C labeled) was added to achieve a concentration of 2 mM and 0.1 μCi/ml. The bottle was regassed and incubated further for a designated period of time, usually 60 min. In experiments to determine whether continued presence of cyclic nucleotide was required in order to observe the effect, the first incubation was terminated by removing the slices, rapidly rinsing them in 0.9% saline and transferring them to new buffers containing the sugar substrate.

Tissue concentration of α-methyl-D-glucoside was determined as described previously[7,8]. After the second incubation phase, the tissues were removed, rinsed quickly in 0.9% saline, blotted, weighed and placed in tubes containing 2 ml of water which were heated in a boiling water bath for 6 min. 0.2-ml aliquots of the tissue extract and incubation medium were assayed for radioactivity by liquid scintillation techniques[7,8,10].

The accumulation of sugar is expressed as the distribution ratio, the ratio of cpm/ml intracellular fluid to cpm/ml medium using inulin space of slices to determine that portion of tissue radioactivity due to extracellular substrate. The difference between total tissue water and inulin space was used to assess the magnitude of the intracellular fluid[13,14]. The inulin space and total tissue water in rat is 25% and 80%,

respectively, of wet tissue weight[13]. These values for dog are 39% and 79%; rabbit, 30% and 75.5%, respectively. In human kidney cortex inulin space varies for each specimen. In the specimen used here the inulin space was 30% wet weight while the tissue water was 80% wet weight. These were unchanged when nucleotides were added to the buffer.

The distribution ratio indicates a concentration gradient of α-methyl-D-glucoside since on silica gel thin-layer chromatography of tissue extracts of rat and human cortex, only radioactive glucoside is present[7,12] and no phosphorylated sugars are precipitated on treatment of extracts with $Ba(OH)_2$ and $ZnSO_4$. The glucoside gives rise to only negligible amounts of $^{14}CO_2$ and therefore, may serve as a non-metabolizable model substrate[7,12]. The distribution ratio may be converted to concentration of sugar in the intracellular fluid by multiplying this number by the substrate concentration. This has been done to derive the velocity in concentration dependence studies. The velocity was corrected for a diffusion component[15].

MATERIALS

Chemicals

α-Methyl-D-[U-^{14}C]glucoside (52.2 Ci/mole), D-[1-^{14}C]-xylose (28.0 Ci/mole), and 3-*O*-methylglucose (2.7 Ci/mole) were obtained from Calatomic (Los Angeles, Calif.). D-[1-^{14}C]Galactose, (6 Ci/mole) was purchased from New England Nuclear (Boston, Mass.). The labeled α-methyl-D-glucoside was found to be pure by thin-layer chromatography[9]. Unlabeled α-methyl-D-glucoside was obtained from Pfanstiehl Co (Waukegan, Ill.) and found to be pure by gas–liquid chromatography[7]. D-Galactose and D-xylose were also purchased from Pfanstiehl, 3-*O*-methylglucose from Calbiochem (Los Angeles, Calif.), and 2-deoxy-D-glucose from Nutritional Biochemical (Cleveland, Ohio). All nucleotides were purchased from Calbiochem.

RESULTS

Effect of nucleotides on α-methyl-D-glucoside accumulation

The accumulation of the glucoside by kidney cortex slices of the rat, rabbit, dog, and human in the presence of various nucleotides is shown in Table I. With pretreatment of tissue with nucleotides for 120 min the renal cortex of all four species demonstrated enhanced intracellular concentration of α-methyl-D-glucoside in the presence of dibutyryl cyclic AMP and cyclic AMP. The distribution ratio in the rat tissue, as a result of incubation with 0.2 mM dibutyryl cyclic AMP, increased from 2.52 ± 0.12 to 3.35 ± 0.11, in rabbit from 2.95 ± 0.11 to 9.14 ± 0.94, in dog tissue from 5.72 ± 0.23 to 7.03 ± 0.40, and in human from 7.53 ± 0.71 to 12.05 ± 1.15. The rabbit tissue responded most profoundly to dibutyryl cyclic AMP while the dog responded the least. Cyclic AMP at five times the concentration of the dibutyryl derivative elicited similar effects but the response was not as great in rabbit tissue. Cyclic GMP also produced enhanced glucoside accumulation although the effect was not significant in dog tissue. AMP and ATP, on the other hand, were without effect on the distribution ratio.

The time necessary for pretreatment of rat tissue slices with 0.2 mM dibutyryl

TABLE I

EFFECT OF NUCLEOTIDES ON α-METHYL-D-GLUCOSIDE ACCUMULATION BY KIDNEY CORTEX SLICES OF VARIOUS SPECIES

Tissues were incubated with or without the nucleotide in 2 ml Krebs–Ringer bicarbonate buffer at 37 °C for 120 min. The flask was then opened and both radioactive and unlabeled glucoside was added to give a 2 mM concentration and 0.1 μCi/ml. The distribution ratios shown were determined after 60 min of further incubation. The number of determinations are shown in parentheses.

Nucleotide	*Distribution ratio (mean ± S.E.)*			
	Rat	*Rabbit*	*Dog*	*Human*
None	2.52 ± 0.12 (9)	2.95 ± 0.11 (7)	5.72 ± 0.23 (8)	7.53 ± 0.71 (4)
Dibutyryl cyclic AMP (0.2 mM)	3.35 ± 0.11 (10)*	9.14 ± 0.94 (7)*	7.03 ± 0.40 (8)***	12.05 ± 1.15 (4)**,
Cyclic AMP (1 mM)	3.22 ± 0.19 (7)**	6.99 ± 0.43 (4)*	7.13 ± 0.45 (7)***	11.32 ± 0.66 (4)**
Cyclic GMP (1 mM)	3.28 ± 0.13 (7)*	5.73 ± 0.26 (4)*	6.26 ± 0.26 (4)§	11.70 ± 1.02 (4)**,
AMP (1 mM)	2.72 ± 0.09 (5)§	2.95 ± 0.20 (3)§		7.02 ± 0.38 (3)§
ATP (1 mM)	2.38 ± 0.06 (5)§	2.74 ± 0.10 (3)§		9.10 ± 0.83 (4)§

* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.02$.
§ Not significant.

cyclic AMP in order to enhance glucoside accumulation is shown in Table II. The distribution ratio was determined after a 60-min incubation with labeled α-methyl-D-glucoside which followed various periods of exposure of tissue to the nucleotide. It appears that at least 90 min of incubation of slices with cyclic nucleotide is required before the later increase in cell content of α-methyl-D-glucoside is observed. Distribution ratio increased from 2.60 to 3.80. Table II shows also that incubation in the first medium containing cyclic nucleotide did not cause the cells to increase α-methyl-D-glucoside accumulation if the slices were transferred to buffer without nucleotide during the accumulation phase of the experiment. Once the effect of the cyclic nucleotide was established its continued presence in the incubation medium was required in order to observe the effect.

Incubation of rat kidney cortex in various concentrations of dibutyryl cyclic AMP from 0.05 to 5 mM caused enhanced accumulation of the glucoside in both rat and rabbit tissue. The maximum increase of accumulation was about 40% with rat tissue and was observed at 0.2 mM and 1 mM in two experiments. Because of the narrow range between control and dibutyryl cyclic AMP-treated tissue values in the rat, a reasonable curve of concentration dependence on the nucleotide was not obtained. In rabbit tissue where the maximum effect is a 3-fold increase in accumulation of glucoside, a progressive enhancement was observed from 0.05 mM to a maximum at 1 mM nucleotide. Cyclic AMP itself at 1 mM produced the same effect as 0.2 mM dibutyryl cyclic AMP in rat, dog, and human kidney tissue but was less effective in the rabbit (Table I).

TABLE II

EFFECT OF DURATION OF EXPOSURE TO DIBUTYRYL CYCLIC AMP AND ITS DELETION FROM INCUBATION MEDIUM ON GLUCOSIDE ACCUMULATION BY RAT KIDNEY CORTEX

Tissues were initially incubated for the times shown with or without 0.2 mM dibutyryl cyclic AMP. They were then transferred to fresh medium containing 2 mM α-methyl-D-glucoside and 0.1 μCi/ml. The dibutyryl cyclic AMP exposed tissues were transferred to medium with or without 0.2 mM dibutyryl cyclic AMP. The distribution ratio was determined after 60 min and is the average of triplicate flasks each containing three slices, one from each of three rats. The values are the average of triplicates.

Initial incubation time (min)	*Initial incubation medium*	*Glucoside incubation medium*	*Distribution ratio*
0		buffer	2.86 ± 0.11
		dibutyryl cyclic AMP	2.94 ± 0.13
30	buffer	buffer	2.29 ± 0.12
	dibutyryl cyclic AMP	buffer	2.27 ± 0.11
	dibutyryl cyclic AMP	dibutyryl cyclic AMP	3.03 ± 0.25
60	buffer	buffer	2.75 ± 0.12
	dibutyryl cyclic AMP	buffer	2.75 ± 0.17
	dibutyryl cyclic AMP	dibutyryl cyclic AMP	3.03 ± 0.10
90	buffer	buffer	2.60 ± 0.10
	dibutyryl cyclic AMP	buffer	2.81 ± 0.02
	dibutyryl cyclic AMP	dibutyryl cyclic AMP	3.80 ± 0.17*
120	buffer	buffer	2.59 ± 0.14
	dibutyryl cyclic AMP	buffer	2.87 ± 0.06
	dibutyryl cyclic AMP	dibutyryl cyclic AMP	3.93 ± 0.29*

* Significantly different from values for incubation in buffer during uptake with $P < 0.05$.

TABLE III

EFFECT OF DIBUTYRYL CYCLIC AMP ON MONOSACCHARIDE ACCUMULATION BY RAT KIDNEY CORTEX SLICES

Tissues were incubated for 120 min with or without 0.2 mM dibutyryl cyclic AMP. Monosaccharides were then added to the flask to give a concentration of 2 mM and 0.1 μCi/ml and the incubation continued for 60 min. The number of determinations are shown in parentheses.

Sugar	*Distribution ratio (mean ± S.E.)*	
	Control	*Dibutyryl cyclic AMP*
α-Methyl-D-glucoside	2.88 ± 0.11 (5)	3.87 ± 0.13 (5)*
D-Galactose	1.40 ± 0.05 (6)	1.44 ± 0.07 (6)**
3-*O*-Methyl-D-glucose	0.86 ± 0.02 (6)	0.85 ± 0.04 (6)**
2-Deoxy-D-glucose	2.38 ± 0.10 (5)	2.25 ± 0.12 (5)**
D-Xylose	0.90 ± 0.10 (6)	0.96 ± 0.09 (6)**

* $P < 0.001$.
** Not significant.

Effect of dibutyryl cyclic AMP on accumulation of monosaccharides by rat kidney cortex

The influence of dibutyryl cyclic AMP on the galactose, 3-*O*-methylglucose, 2-deoxyglucose and xylose accumulation by rat kidney is shown in Table III. The enhancement of α-methyl-D-glucoside uptake is evident with a distribution ratio of 2.88 ± 0.11 in controls and 3.87 ± 0.13 in nucleotide-treated tissue. The nucleotide does not enhance the accumulation of D-galactose, a sugar which shares the α-methyl-D-glucoside transport system[12] but is rapidly metabolized[16]. It did not increase the accumulation of poorly transported 3-*O*-methylglucose[17] or D-xylose, nor of 2-deoxyglucose which is transported by a system other than that shared by glucose, galactose and α-methyl-D-glucoside[18].

Influence of cycloheximide on the dibutyryl cyclic AMP effect

Weiss *et al.*[6] have shown that cycloheximide, a known inhibitor of protein synthesis, when incubated together with dibutyryl cyclic AMP eliminates the nucleotide-associated increase in amino acid accumulation. The result of a similar study with α-methyl-D-glucoside as substrate is shown in Table IV. Dibutyryl cyclic AMP caused the same increase in the distribution ratio whether cycloheximide was present or not.

TABLE IV

EFFECT OF CYCLOHEXIMIDE ON ENHANCED ACCUMULATION OF α-METHYL-D-GLUCOSIDE BY DIBUTYRYL CYCLIC AMP

Tissues were incubated for 120 min in either buffer alone, 1 mM cycloheximide, 0.2 mM dibutyryl cyclic AMP or cycloheximide *plus* dibutyryl cyclic AMP. α-Methyl-D-glucoside was added to the flask to give a concentration of 2 mM and 0.1 μCi/ml and the incubation continued for 60 min. The number of determinations is shown in parentheses.

Condition	*Distribution ratio (mean ± S.E.)*
Control	2.49 ± 0.10 (9)
Cycloheximide	2.65 ± 0.11 (7)**
Dibutyryl cyclic AMP	3.35 ± 0.18 (9)*
Cycloheximide + cyclic AMP	3.24 ± 0.13 (9)*

* $P < 0.001$.
** Not significant.

Kinetic parameters of α-methyl-D-glucoside accumulation

The time course of glucoside uptake after treatment of slices for 120 min is shown in Fig. 1. In rat and rabbit tissue incubated with dibutyryl cyclic AMP there is both an increase in the initial entry rate and the steady-state concentration gradient. The effect of dibutyryl cyclic AMP treatment on the rate of accumulation which is dependent on substrate concentration was also assessed in rat tissue. The data shown in Fig. 2 was obtained in three experiments in which glucoside accumulation was measured for 15 min after tissues had been pretreated with dibutyryl cyclic AMP for 120 min. The lines drawn were determined to be the best fit of the data using a Monroe 1775 computer. The equation for the control curve was $Y = 0.4330X + 0.0996$ while that obtained in presence of the nucleotide was $Y' = 0.2461X' + 0.0547$. The nucleotide

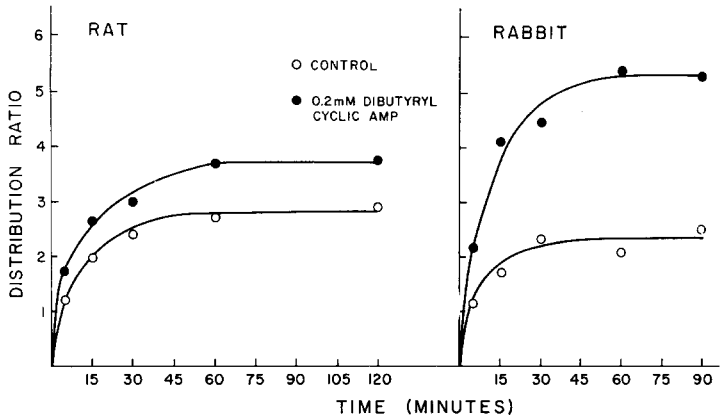

Fig. 1. The influence of dibutyryl cyclic AMP on the time course of the accumulation of α-methyl-D-glucoside by rat and rabbit renal cortical slices. Tissues were incubated for 120 min with or without 0.2 mM dibutyryl cyclic AMP. α-methyl-D-[U-^{14}C]glucoside was then added to a concentration of 2 mM and 0.1 μCi/ml and the incubation continued for the time indicated. Each point represents the mean of triplicate determinations.

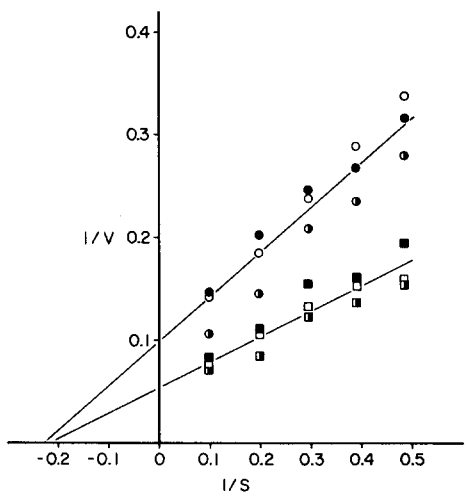

Fig. 2. Lineweaver–Burk plot of the velocity of α-methyl-D-glucoside accumulation as a function of substrate concentration. V is velocity in mM per liter per 15 min and S is substrate concentration in mM. Slices were incubated for 120 min with or without dibutyryl cyclic AMP, then transferred to new medium corresponding to that of the prior incubation but containing various concentrations of α-methyl-D-[U-^{14}C]glucoside, 0.1 μCi/ml. The incubation was continued for 15 min. Each point is the mean of triplicate determinations. The data represents three experiments with controls indicated by circles and dibutyryl cyclic AMP treated indicated by the squares. Corresponding points in each experiment are indicated by whether the symbols are open, solid or half-solid.

did not alter the apparent transport K_m for the glucoside (control 4.35:dibutyryl cyclic AMP 4.50 mM) but increased the maximum velocity of accumulation from 10.0 to 18.3 mmoles/l per 15 min. The data for each individual experiment was analyzed by computer curve fitting and the resulting K_m and V values were averaged and

examined by the t test for paired values. There was no significant difference in apparent K_m values but a significant difference in V values with a P value of 0.01.

DISCUSSION

Our studies show that the uptake of α-methyl-D-glucoside by renal cortical cells from several species is enhanced when they are exposed to cyclic nucleotides. The process has characteristics both similar to and different from the increase in amino acid accumulation by dibutyryl cyclic AMP-treated rat kidney cortex observed by Weiss *et al.*[6]. The main similarity is the requirement for preincubation of the tissue with cyclic nucleotide in order to clearly see the effect. The chief differences are that once initiated, the effect on sugar uptake requires continued presence of dibutyryl cyclic AMP and is not eliminated by cycloheximide. This disparity could possibly be related to the one major difference in experimental procedure. Weiss *et al.*[6] added 5 mM glucose to the incubation media. Because glucose is a competitive inhibitor of α-methyl-D-glucoside we could not add it to our incubation medium. There is no indication that glucose in the medium is necessary for optimal membrane transport function of rat renal cortex slices[19].

The reason for the preincubation requirement cannot be stated with certainty. A likely possibility is a slow penetration of the cyclic nucleotide into the cell. The continued need for the compound throughout the incubation to see effects on sugar uptake is consistent with maintenance of a critical intracellular level of cyclic AMP. The lack of interference by cycloheximide suggests that new protein formation is not involved in the process. Weiss *et al.*[6] on the basis of the pretreatment requirement for a cyclic AMP effect and its inhibition by cycloheximide suggest that the time lag represented the time required for increased synthesis of a component of the active transport process. Since activation of protein kinase and protein phosphorylation is a known effect of cyclic AMP in kidney, this must also be considered as a possible mechanism[20].

α-Methyl-D-glucoside was used as a model sugar representative of the glucose–galactose transport system. In the rat and human it is non-metabolized, its uptake is competitively inhibited by glucose and galactose, and it demonstrates counterflow phenomena with these sugars. Our lack of ability to show the cyclic nucleotide effect on ^{14}C accumulation with [1-^{14}C]galactose does not negate an effect on the glucose–galactose transport system since the metabolism of galactose by kidney to intracellular phosphorylated compounds and CO_2 is so rapid as to make accurate transport studies questionable[16]. Where there is limited ability to accumulate a sugar such as 3-*O*-methylglucose[17] or xylose, there was no enhancement of uptake. Likewise, no effect was seen on 2-deoxyglucose uptake, a process independent of the α-methyl-D-glucoside–glucose-galactose transport process[18]. The fact that cyclic GMP produces a similar effect as cyclic AMP would indicate that the enhancement may be a general effect of cyclic nucleotides.

The uptake experiments shown in Fig. 1 demonstrate an increase in initial rate of entry of the sugar. Concentration dependence of uptake clearly shows that there is no alteration of the apparent transport K_m, *i.e.* no change in the affinity of the saturable site(s) that may be involved in the transport process. The increase in maxi-

mum velocity could be explained by an increase in the number of saturable sites or an increased efficiency of those already present.

Although kinetic analysis of α-methyl-D-glucoside uptake in kidney may be satisfied by a simple two-compartment system with influx and efflux components[21], the actual localization of sugar entry and exit to luminal or anti-luminal cell borders has not been determined. By using an *in vitro* technique with slices, we cannot fully relate cellular accumulation to transepithelial transport in the intact kidney. Localization of cellular sugar entry has been assessed *in vivo* by Silverman *et al.*[22] in which glucose and galactose were found to enter dog cortex cells *via* both luminal and anti-luminal membranes. Deetjen and Boylan[23], however, in microperfusion studies of D-glucose in isolated rat nephrons could not demonstrate glucose movement from peritubular capillary into the tubule lumen.

One cannot equate the findings of enhanced sugar or amino acid uptake in slices to increased tubular reabsorption *in vivo*. In fact, the opposite may be the case for amino acids. Although parathyroid hormone stimulates cyclic AMP production and causes enhanced accumulation of amino acids by rat kidney slices[6], excessive parathyroid hormone is associated with decreased amino acid reabsorption and aminoaciduria[24]. Halver[25], however, has reported an elevated tubular reabsorptive capacity for glucose in patients with hyperparathyroidism, a finding over which there is some dispute[26]. It may well be that the *in vivo* increase in renal cortical cyclic AMP may be associated with divergent effects on amino acid and sugar reabsorption while *in vitro* increase in cyclic AMP may enhance accumulation of both types of compounds. This would depend on the basic differences of the amino acid and sugar transport processes and the underlying nature of cyclic AMP on both processes. Our findings suggest that cyclic AMP may play a role in regulation of renal cortal sugar reabsorptive mechanisms. The above-mentioned effects of parathyroid hormone on reabsorption may be only one facet. It is certainly possible that normal reabsorption of sugar depends on optimal levels of cyclic AMP interacting with membrane transport modalities.

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