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The effect of reducing ATP levels on reorientation of the secondary palate

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SUMMARY

The force for directing palate shelf reorientation appears to be associated with elements of the presumptive hard palate (Brinkley & Vickerman, 1979; Bulleit & Zimmerman, 1985). The palatal elements that mediate this process do not require palate cells to be metabolically active for expression of the force. This contention was demonstrated using an *in vitro* system that allows substantial reorientation of the hard palate to occur. ATP levels were reduced by treatment with metabolic inhibitors and the degree of reorientation was measured 1 h following pretreatment with inhibitors. Treatment of cultured embryonic heads under anoxic conditions with 2,4-dinitrophenol or KCN had no effect on the degree of reorientation occurring *in vitro*. These agents reduced ATP levels by 71 % and 62 %, respectively. Treatment of cultured heads with 2-deoxy-D-glucose under anoxia also had no effect on reorientation. This treatment reduced ATP levels in embryonic heads by 92–94 %. A similar reduction was observed if ATP levels were measured in palate tissue alone. The treatment of cultured heads with 2-deoxy-D-glucose and anoxia not only reduced levels of ATP but also reduced CTP, GTP and UTP. These results indicate that palate shelf reorientation is independent of cellular metabolic activity and supports the hypothesis that reorientation is dependent on a pre-existing infrastructure within the palate shelves.

INTRODUCTION

Morphogenesis of the mammalian secondary palate involves a number of events, including shelf growth, reorientation from a vertical to horizontal position and subsequent medial edge fusion. Reorientation is a rapid morphogenetic event that is believed to require a force intrinsic to the palate shelves (Walker & Fraser, 1956). This is supported by observations that reorientation can occur in cultured embryonic mouse heads in the absence of the tongue, brain and circulation (Brinkley, Basehoar & Avery, 1978). Furthermore, the intrinsic force appears to be primarily associated with the presumptive hard palate rather than soft palate, since when the hard and soft palate are separated the hard palate reorients while the soft palate fails to reorient (Brinkley & Vickerman, 1979; Bulleit & Zimmerman, 1985). The components of the presumptive hard palate that mediate this force may include an active contractile system within the mesenchyme, the

Key words: palate, reorientation, ATP, mouse.

accumulation of a hyaluronic acid (HA)-rich extracellular matrix, or elements of the epithelium surrounding the shelves (Larsson, 1960; Pourtois, 1972; Pratt, Goggins, Wilk & King, 1973; Babiarz, Allenspach & Zimmerman, 1975; Brinkley, 1980, 1984; Luke, 1984). Recently we have shown that the oral epithelium on the presumptive hard palate plays an integral role in directing this process (Bulleit & Zimmerman, 1985).

How these components function to direct reorientation is still not well understood. They may require metabolically active cells for the expression of the force necessary for reorientation. Alternatively, they may simply define a palatal infrastructure that is required for expression of reorientation and is independent of metabolic energy (Brinkley & Vickerman, 1979). This latter possibility is supported by observations that reorientation *in vitro* could occur under anoxic conditions in which ATP generated by oxidative phosphorylation would be inhibited (Wee, Babiarz, Zimmerman & Zimmerman, 1979). To substantiate these findings we have measured reorientation in cultured embryonic mouse heads following pretreatment with inhibitors of oxidative phosphorylation and glycolysis. The results indicate that palate reorientation is independent of tissue ATP levels since an almost complete reduction in ATP as measured in embryonic heads or palate shelves had no effect on shelf reorientation *in vitro*.

MATERIALS AND METHODS

Animals and culture system

A/J mice (Jackson Laboratories) were used in these studies. Matings were carried out by placing individual females with males overnight. The presence of a vaginal plug the following morning was taken as evidence for pregnancy and designated day zero. On day 14 of gestation animals were killed by cervical dislocation and gravid uteri were placed on ice. This is approximately 24 h prior to *in situ* shelf reorientation (Andrew, Bowen & Zimmerman, 1973). Embryos were removed and examined; those with cleft lip were eliminated. The remaining embryos were staged using a morphological rating system described by Walker & Crain (1960). Embryos with a rating of 5–7 were used for culture.

The culture system employed was the same as described by Bulleit & Zimmerman (1985). Heads were removed followed by excision of mandibles and tongues. A cut was also made down the midline of the cranium which allowed better access of medium to tissues. A transverse cut was made in the palate two thirds the distance from the anterior end to separate the presumptive hard and soft palate (Diewert, 1978) since the force for reorientation primarily resides in the hard palate. Heads were placed in 15×45 mm vials containing 3 ml Dulbecco's Modified Eagles Medium (DMEM, Gibco) and 10 % foetal bovine serum (FBS, M.A. Bioproducts) or in phosphate-buffered saline containing Ca^{2+} and Mg^{2+}. The vials were gassed with a mixture of 95 % O_2, 5 % CO_2 and sealed with rubber stoppers. The vials were placed in a rotating culture apparatus (approximately 30 rev min^{-1}) for up to 3 h.

Treatment with inhibitors of oxidative phosphorylation and glycolysis

In an effort to maximally inhibit palatal cellular metabolic activity and ATP production inhibitors were used at relatively high concentrations in the presence of anoxic conditions. Embryonic heads were placed in either Dulbecco's Modified Eagles medium (M.A. Bioproducts) with 10 % foetal bovine serum containing 5 mM-2,4-dinitrophenol (DNP) or

10 mM-potassium cyanide (KCN), or in phosphate-buffered saline with Ca^{2+} and Mg^{2+} containing 20 mM-2-deoxy-D-glucose (2-DG). The vials were then gassed with 100 % N_2 and sealed with rubber stoppers. The embryonic heads were incubated for 1 h under these conditions with the tongue remaining between the palate shelves to prevent reorientation. Following this 1 h pretreatment the tongues were removed and shelves separated into hard and soft palate. The heads were then placed back into the same media containing inhibitors and regassed with 100 % N_2. The cultures were incubated for an additional 1–2 h under these conditions.

Quantitation of reorientation in vitro

The amount of reorientation occurring during culture was measured using palate shelf index, PSI (Wee, Wolfson & Zimmerman, 1976). Following culture, heads were fixed in Bouin's solution for 24 h. Fixed heads were cut coronally using a razor blade. Razor-blade sections were made by cutting the palate shelves in the middle of each third of the palate, allowing for measurement of anterior hard palate, posterior hard palate, and soft palate PSI, presented previously as anterior, mid, and posterior PSI, respectively (Bulleit & Zimmerman, 1985). The transverse sections were placed on end and visualized using a dissecting microscope. Values of 1–5 were assigned to express the degree of palate shelf reorientation. A number of 1 was assigned for a completely vertical and a 5 for a completely horizontal palate. Numbers 2, 3 or 4 were designated intermediate elevation of one-quarter, one-half or three-quarters of the way to the horizontal position. Experimental results were expressed as mean PSI ± standard error for the anterior and posterior hard palate which undergo reorientation in this culture system.

Measurement of ATP levels

ATP levels were measured using high-performance liquid chromatography (HPLC) similar to that described by Ritter & Bruce (1979). In the majority of experiments embryonic heads were either removed from the embryo or taken directly from culture vials and immediately immersed in liquid nitrogen. In one experiment palate shelves were removed from embryonic heads as rapidly as possible and immediately frozen. Some ATP may have been hydrolysed during the 1–5 min dissection procedure. However the levels measured were similar to those analysed in embryonic heads.

The frozen tissue was then ground in a solid-CO_2-cooled mortar and pestle containing frozen 0·5 M-perchloric acid. This material was thawed at 4 °C, transferred to centrifuge tubes and centrifuged at 8000 rev min^{-1} for 10 min. The supernatant was removed and the pellet washed twice with 0·5 M-$HClO_4$. The combined supernatants were neutralized with 4 M-KOH, and the pH adjusted to 7·5. 0·5 M-NH_4HCO_3 (pH 7·5) was used to buffer the solution. The pellet was used to determine total protein content by the method of Lowry, Rosebrough, Farr & Randall (1951). Three embryonic heads were combined for each sample. When ATP was measured in isolated palate tissue, eight shelves were combined for each sample. ATP concentrations were expressed as mean $\mu g\ mg^{-1}$ protein ± standard error of three samples. Statistical analysis of the difference between mean ATP concentrations of treatment groups and controls was performed using a two-tailed Student's *t* test.

The ATP assay was performed on a model 204 liquid chromatograph (Waters Assoc.) equipped with a Partisil 10 SAX anion exchange column (Whatman) followed by a model 440 u.v. detector (Waters Assoc.) operated at 254 nm. The liquid chromatograph was equipped with a Data Module model 730 (Waters Assoc.). Elution was carried out using a non-linear gradient from a 0·01 M-$NH_4H_2PO_4$ (pH 3·2) starting buffer to a 0·6 M-$NH_4H_2PO_4$ (pH 4·4) limiting buffer at a flow rate of 1·0 ml min^{-1} for 80 min.

Light microscopy

For light microscopy, heads were fixed in Karnofsky's fixative, as modified by Singley & Solursh (1980). Isotonic sodium phosphate buffer (0·2 M) was used instead of sodium cacodylate buffer. Heads were fixed overnight followed by dehydration in a graded series of ethanol

(50–100 %) with a final passage through 100 % propylene oxide. The heads were then embedded in Poly/Bed 812 (Polyscience, Inc.). Sections were cut at 1–2 μm, stained in Richardson's stain (Richardson, Jarett & Fink, 1960) and examined with a Zeiss photomicroscope.

RESULTS

Reorientation of palate shelves in vitro

When the presumptive hard and soft palate are separated and the embryonic heads incubated *in vitro*, the hard palate reorients to a nearly horizontal position within 6 h; the anterior segment was observed to have a PSI of 4·5 and the posterior 3·7. Reorientation of the separated soft palate was much slower, moving less than 1 PSI unit during this time (Bulleit & Zimmerman, 1985). Moreover, the anterior and posterior hard palate reorient rapidly during the earlier period of time. First, when the tongue is removed, there is immediate movement of the shelves toward the horizontal position. The anterior hard palate moves to a PSI of 2·4 and the posterior hard palate to a PSI of 1·9, which is recorded as the zero time (Table 1). In the following 1–2 h of culture there is pronounced movement toward the horizontal position with the anterior hard palate reaching more than 80 % of full reorientation (see below).

Table 1. *The effect of inhibitors of oxidative phosphorylation and glycolysis on palate shelf reorientation* in vitro

Culture conditions	Duration of culture	Palate segment measured (PSI ± S.E. (n))	
		Anterior hard	Posterior hard
No culture	0	2·4 ± 0·09 (28)	1·9 ± 0·06 (28)
DMEM, 10 % FBS 95 % O_2–5 % CO_2	2	4·0 ± 0·06 (58)	3·2 ± 0·08 (58)
PBS + Ca^{2+} and Mg^{2+} 95 % O_2–5 % CO_2	2	4·0 ± 0·05 (30)	3·2 ± 0·08 (30)
DMEM, 10 % FBS 5 mM-DNP, 100 % N_2	2	4·1 ± 0·08 (30)	3·3 ± 0·09 (30)
DMEM, 10 % FBS 10 mM-KCN, 100 % N_2	2	4·0 ± 0·06 (30)	3·1 ± 0·08 (30)
PBS + Ca^{2+} and Mg^{2+} 20 mM-2-DG, 100 % N_2	2	4·0 ± 0·07 (30)	3·3 ± 0·17 (30)
DMEM, 10 % FBS 95 % O_2–5 % CO_2	3	4·2 ± 0·06 (36)	3·3 ± 0·11 (36)
PBS + Ca^{2+} and Mg^{2+} 20 mM-2-DG, 100 % N_2	3	4·2 ± 0·10 (20)	3·4 ± 0·12 (20)

The tongue remained between the palate shelves for the first hour of culture under the various conditions. Following this period the tongue was removed, the hard and soft palate separated and the heads cultured for an additional 1 or 2 h under the various conditions. PSI was measured following culture.

Effect of inhibitors of oxidative phosphorylation and glycolysis on palate shelf reorientation in vitro

To determine if the rapid movement of the hard palate towards the horizontal position was dependent on metabolic energy, we measured palate shelf reorientation in the presence of inhibitors of oxidative phosphorylation and glycolysis. In these experiments embryonic heads were cultured in the presence of inhibitors for 1 h with the tongue remaining between the palate shelves. Reorientation was measured following removal of the tongue and then an additional hour of culture. This protocol allowed a maximal inhibition of ATP prior to movement of the palate shelves *in vitro*. Embryonic heads cultured in oxygenated serum supplemented media or in PBS containing Ca^{2+} and Mg^{2+} were able to reorient to the same degree reaching a PSI of 4·0 in the anterior hard palate (Table 1). This same degree of reorientation was also reached in heads cultured only under anoxic conditions (results not presented). If heads were cultured under anoxia in combination with 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, or potassium cyanide, an inactivator of electron transport, reorientation was again unaffected. The same observation was made when heads were cultured under conditions that would inhibit both oxidative phosphorylation and glycolysis. Reorientation following treatment with 2-deoxy-D-glucose and anoxia was not different from controls reaching a PSI of 4·0 in the anterior hard and 3·3 in the posterior hard palate (Table 1). Transverse sections cut at the border between the anterior and mid hard palate and examined by light microscopy again showed no difference in the degree of movement toward the horizontal position between the various treatment groups, as well as no striking difference in the morphology of the tissue (Fig. 1). Sections from the posterior hard palate also showed no difference in morphology (results not presented).

To determine if alterations in the pattern of reorientation would occur following longer periods of culture, we pretreated embryonic heads in 2-deoxy-D-glucose under anoxia for 1 h with the tongue remaining between the palate shelves. Following this pretreatment the tongues were removed and the heads cultured for an additional 2 h of culture in the presence of inhibitors. Reorientation was measured after this additional 2 h of culture. Again no effect on the degree of reorientation was observed as compared to controls cultured in oxygenated serum-supplemented media (Table 1). Following longer periods of culture increasing necrosis was observed in heads treated with 2-deoxy-D-glucose and anoxia. This prevented accurate measurement of PSI and so heads were not cultured longer than 3 h.

Effect of inhibitors of oxidative phosphorylation and glycolysis on levels of ATP

To confirm that inhibitors of oxidative phosphorylation and glycolysis could significantly reduce metabolic activity, we measured nucleotide levels by HPLC and quantified the amount of ATP in embryonic heads following the various treatments. When heads were cultured for 1 h in oxygenated serum-supplemented

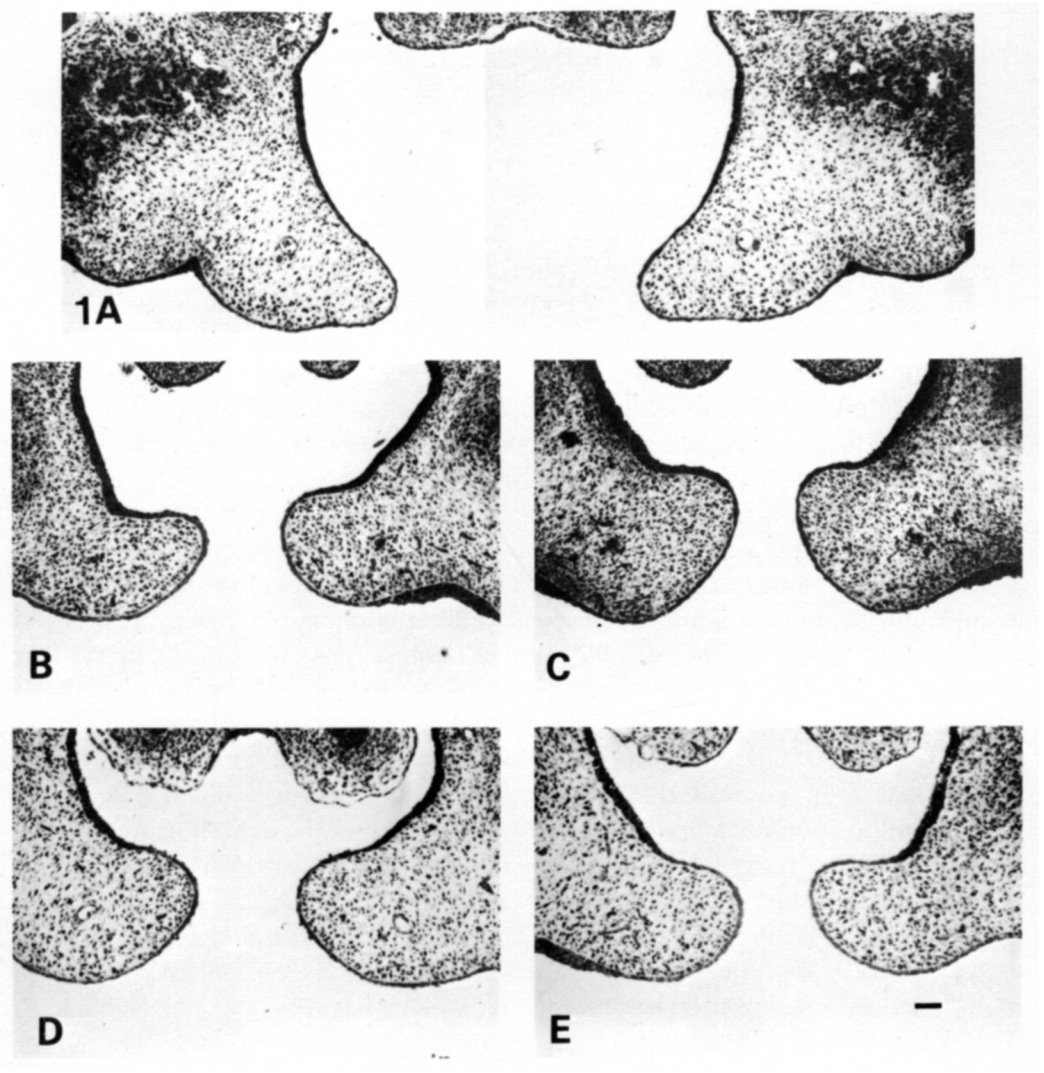

Fig. 1. Histology of anterior/mid hard palate from embryonic heads not cultured (A); and cultured under an atmosphere of 95 % O_2–5 % CO_2 in DMEM + 10 % FBS (B); 95 % O_2–5 % CO_2 in PBS + Ca^{2+} and Mg^{2+} (C); 100 % N_2 in DMEM + 10 % FBS containing 5 mM-2,4-dinitrophenol (D); or 100 % N_2 in PBS + Ca^{2+} and Mg^{2+} containing 20 mM-2-deoxy-D-glucose (E); for 1 h with the tongue between the palate shelves. Following this culture period the tongue was removed, the hard and soft palate separated and the heads cultured for an additional hour under the same conditions. Bar, 100 μm.

media or PBS plus Ca^{2+} and Mg^{2+} there was a small decrease in the level of ATP (10–20 %) compared to the levels in heads prior to culture (Table 2). If heads were cultured under anoxic conditions in the presence of various inhibitors there was a much greater decline in the levels of ATP after the first hour of culture. 2,4-Dinitrophenol reduced ATP levels by 71 %, KCN by 62 %, and 2-deoxy-D-glucose by 92 % (Table 2). The reduction in ATP by 2-deoxy-D-glucose and anoxia

remained constant over an additional 2 h of culture reaching 94 % (Table 2, Fig. 2H). Over this same culture period, levels of ATP in control heads were reduced by only 12 % (Table 2). Treatment of embryonic heads with the various inhibitors also reduced levels of other nucleotide triphosphates including CTP, GTP, and UTP (Fig. 2). ADP was relatively unaffected during the first hour of culture. The concentration of ADP was $1{\cdot}7 \pm 0{\cdot}36\,\mu g\,mg^{-1}$ protein in control embryonic heads prior to culture and was $1{\cdot}4 \pm 0{\cdot}09\,\mu g\,mg^{-1}$ protein following treatment with 2-deoxy-D-glucose and anoxia (Fig. 2B,G). However over the next 2 h, 2-deoxy-D-glucose and anoxia caused some decline in ADP reaching $0{\cdot}87 \pm 0{\cdot}01\,\mu g\,mg^{-1}$ protein (Fig. 2H). The other nucleotide diphosphates appeared relatively unaffected. The nucleotide monophosphates were variably affected with an apparent increase in GMP. AMP was difficult to delineate because of peak spreading. However, no consistent change in this peak could be observed.

To determine if ATP levels were similarly reduced in palate tissue, we measured nucleotides in palate shelves dissected from heads treated with 2-deoxy-D-glucose and anoxia for 1 h. The degree of reduction in ATP as well as other nucleotide triphosphates was similar to that observed in embryonic heads (Fig. 3). However loss of ATP by hydrolysis may have occurred during the prolonged dissection. ATP in control shelves prior to culture was $14{\cdot}5 \pm 0{\cdot}63\,\mu g\,mg^{-1}$ protein and in palates from heads treated with 2-deoxy-D-glucose and anoxia $1{\cdot}21 \pm 0{\cdot}09\,\mu g\,mg^{-1}$ protein (92 % inhibition).

Table 2. *The effect of inhibitors of oxidative phosphorylation and glycolysis on ATP concentrations in embryonic heads*

Culture conditions	Duration of culture	ATP ($\mu g\,ml^{-1}$ protein ± S.E.)	Percent inhibition
No culture	0	13·11 ± 1·29	0
DMEM, 10 % FBS 95 % O_2–5 % CO_2	1	10·71 ± 0·74	18
PBS + Ca^{2+} and Mg^{2+} 95 % O_2–5 % CO_2	1	11·77 ± 1·11	10
DMEM, 10 % FBS 5 mM-DNP, 100 % N_2	1	3·77 ± 0·11*	71
DMEM, 10 % FBS 10 mM-KCN, 100 % N_2	1	4·96 ± 0·06*	62
PBS + Ca^{2+} and Mg^{2+} 20 mM-2-DG, 100 % N_2	1	1·06 ± 0·09†	92
DMEM, 10 % FBS 95 % O_2–5 % CO_2	3	11·49 ± 0·31	12
PBS + Ca^{2+} and Mg^{2+} 20 mM-2-DG, 100 % N_2	3	0·76 ± 0·04	94

ATP concentrations were measured by HPLC after 1 or 3 h of culture under the various conditions. ATP concentrations represent the mean of three samples. Each sample contained three embryonic heads.

Different from uncultured value: * $P < 0{\cdot}005$; † $P < 0{\cdot}001$.

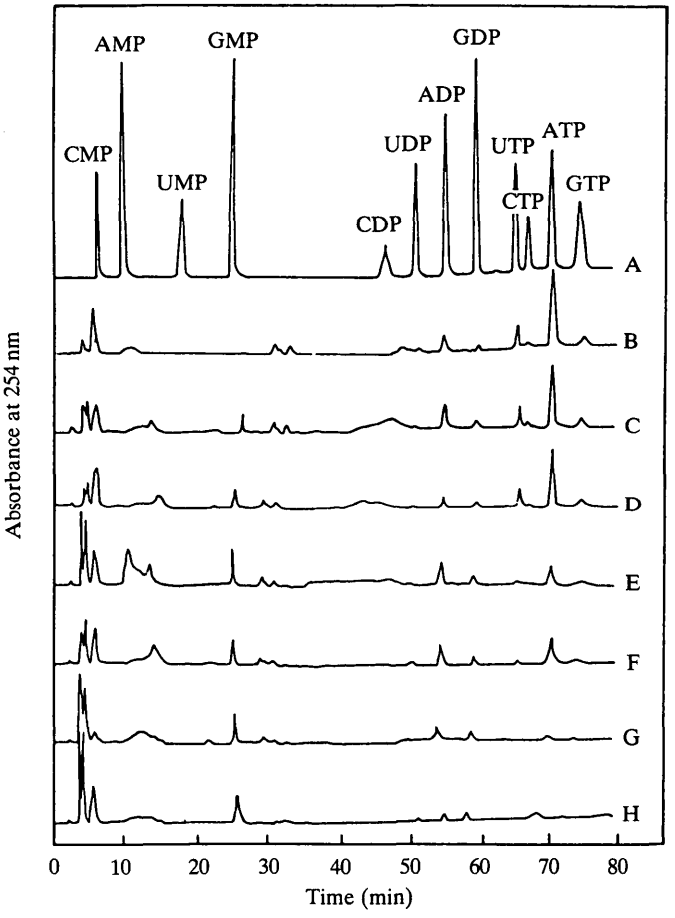

Fig. 2. HPLC analysis of nucleotide standards (A), or extracts from embryonic heads (B–H) separated on a partisil 10 SAX anion exchange column. Nucleotides were extracted from heads not cultured (B); cultured for 1 h under an atmosphere of 95 % O_2, 5 % CO_2 in DMEM + 10 % FBS (C); 95 % O_2, 5 % CO_2 in PBS + Ca^{2+} and Mg^{2+} (D); 100 % N_2 in DMEM + 10 % FBS containing 5 mM-dinitrophenol (E); 100 % N_2 in DMEM + 10 % FBS containing 10 mM-KCN (F); 100 % N_2 in PBS + Ca^{2+} and Mg^{2+} containing 20 mM-2-deoxy-D-glucose (G); or cultured for 3 h in an atmosphere of 100 % N_2 in PBS + Ca^{2+} and Mg^{2+} containing 20 mM-2-deoxy-D-glucose (H).

DISCUSSION

The force necessary for directing reorientation of the secondary palate appears to be intrinsic to the palate shelves (Walker & Fraser, 1956; Brinkley, Basehoar, Branch & Avery, 1975; Brinkley *et al.* 1978), particularly the anterior two-thirds or presumptive hard palate (Brinkley & Vickerman, 1979; Bulleit & Zimmerman, 1985). The elements that mediate this force may require metabolic activity for their expression. However, the results presented in this paper cannot support this contention and show no correlation between ATP levels and the ability of the hard palate to reorient *in vitro*.

Reduction of ATP by 92–94 % had no effect on reorientation as measured by PSI. Reduction of ATP to these levels required that 2-deoxy-D-glucose treatment be combined with anoxia, inhibitors of glycolysis and oxidative phosphorylation, respectively. Previously Marden, Crawford & Bryant (1982) had made a similar observation in V79 cells. They showed that a combined treatment of 15 mM-potassium cyanide, an inhibitor of oxidative phosphorylation, and 15 mM-potassium fluoride, an inhibitor of glycolysis, was necessary to completely reduce levels of ATP. Similarly, Doorey & Barry (1983) employing 0·1 mM-KCN and 20 mM-2-deoxy-D-glucose (to inhibit glycolysis) could not decrease ATP levels below 50 %, although contractile amplitude in cultured embryonic chick ventricular cells could be completely abolished. These results suggest that only a partial decrease of ATP levels will suppress active contractility. In our experiments we also employed high millimolar concentrations of inhibitors of glycolysis and oxidative phosphorylation. Only when we employed 2-deoxy-D-glucose to inhibit glycolysis and anoxia to inhibit oxidation phosphorylation could we reduce ATP levels 92 % in embryonic heads and palates. These observations suggested that the embryonic head and its palate were able to use anaerobic as well as aerobic metabolism to produce energy. Nevertheless, when nearly complete suppression of ATP was achieved with 2-deoxy-D-glucose and anoxia, we showed no effect on palate reorientation *in vitro*.

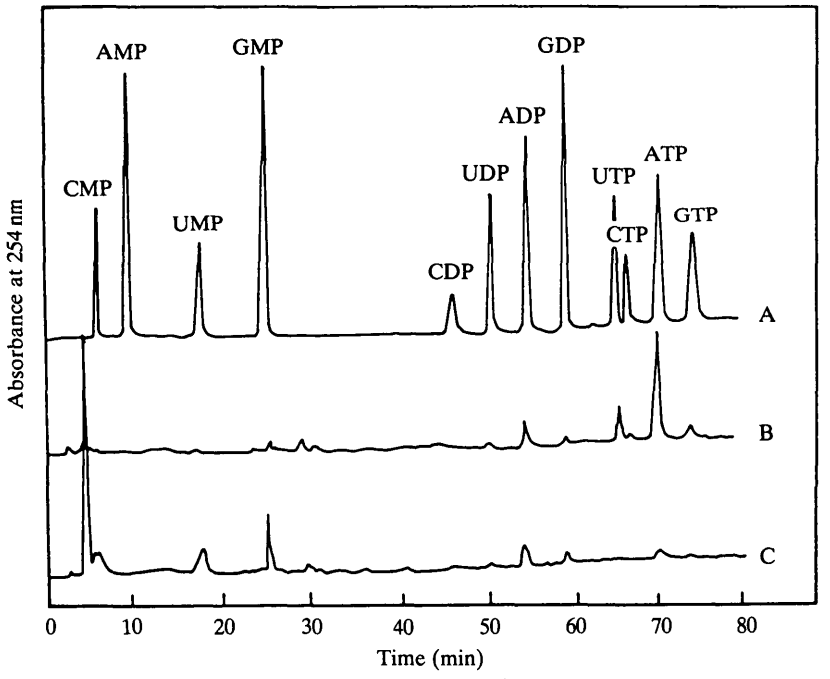

Fig. 3. HPLC analysis of nucleotide standards (A) or extracts of palate shelves (B,C) separated on a partisil 10 SAX anion exchange column. Nucleotides were extracted from palate tissue isolated from heads not cultured (B) or cultured for 1 h under an atmosphere of 100 % N_2 in PBS + Ca^{2+} and Mg^{2+} containing 20 mM-2-deoxy-D-glucose (C).

When embryonic heads were cultured with 2-deoxy-D-glucose and anoxia for longer than 3 h, increasing necrosis was noted; for this reason most experiments were performed in 2 h. Nearly complete reorientation of the anterior hard palate could be achieved (about 80 %) in this time period when the hard and soft palate were separated surgically. Histological examination revealed that necrosis was not evident in any part of the hard palate at that time. It is not known whether palatal cells were killed or not. Marden *et al.* (1982) showed that viability of cultured V79 cells, monitored by trypan blue exclusion, was not decreased in the presence of 5–15 mM-KCN and KF. Alternatively, 5 mM-KCN and iodoacetate markedly decreased cell viability. However even if palate cells are metabolically dead following treatment with 2-deoxy-D-glucose and anoxia for 2 h, palate shelf reorientation can still occur rapidly.

ATP is required for most cellular functions including active contractility involving actin and myosin (Korn, 1978). Since almost complete suppression of ATP levels in the palate did not affect its reorientation, this result suggests that reorientation of the hard palate is not dependent on an *active* contractile mechanism (Babiarz *et al.* 1975). Reorientation also appears independent of biosynthetic processes since all biosynthetic events require at least one of the nucleotide triphosphates (Atkinson, 1977); we showed that UTP, CTP and GTP levels were decreased in the embryonic head and palate by treatment with 2-deoxy-D-glucose and anoxia. These observations would also imply that cell division is not necessary for reorientation *in vitro* since many biosynthetic processes as well as other ATP-dependent events are necessary for mitosis. Also reorientation appears independent of serum factors or nutritive factors, such as amino acids and vitamins. Reorientation can proceed normally when heads are cultured in phosphate-buffered saline. Wee *et al.* (1979) had previously made a similar observation.

Thus the observation that palate shelf reorientation is independent of metabolic activity provides support for the hypothesis that the force for reorientation resides in a predetermined infrastructure of the palatal tissue (Brinkley & Vickerman, 1979; Bulleit & Zimmerman, 1985). The components of the palate shelves that are important in defining the infrastructure may include the epithelium, mesenchyme or extracellular matrix. We have suggested that the development of the oral epithelium plays an integral role in this process (Bulleit & Zimmerman, 1985). Thus the force generated in the oral epithelium may derive from horizontal growth of the palate and subsequent deflection of the shelves downward by the presence of the tongue producing a compliant force to reorient the shelves horizontally. This force may accumulate by (1) differential growth of the oral epithelium including rugae present in the hard palate (Luke, 1984), or (2) movement and rearrangement of epithelial architecture *before* the time of reorientation. Both differential growth and epithelial rearrangement would need metabolic energy during a previous time of palate development (day 12–13) although not during the time of expression of the motive force (day 14–15) in the A/J mouse strain.

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