Effect of Sodium Fluoride and Sodium Pyruvate on Palatal Development *In Vitro* [1]

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ABSTRACT Explants of the embryonic rat palate have been treated by adding sodium fluoride and sodium pyruvate to the culture medium. Fluoride, at specific concentrations, causes retardation of palatal shelf growth so that fusion does not occur during the culture period. Partial or complete fusion does occur if fluoride levels are reduced.

Sodium pyruvate added to the medium advances the time of fusion of explants over that found in controls. When combined with fluoride in the medium, pyruvate can reverse the effects of fluoride on shelf growth and permit complete fusion to take place in a large percentage of explants.

The mode of action of fluoride or pyruvate under these experimental conditions has not been determined. However, the known effects of fluoride as an enzyme inhibitor must be considered.

Development of the secondary palate of mammalian embryos can be adversely affected by the administration of chemical agents (Murphy, '65; Steffek, King and Derr, '66), vitamin antagonists (Kalter and Warkany, '59), hormones (Walker and Fraser, '57; Pinsky and DiGeorge, '65) and by physical methods (Gulienetti, Kalter and Davis, '62). Results from these studies suggest that interference with one of three primary morphological events is the usual cause of abnormal development, i.e., interference with shelf movement, retardation of shelf growth toward the midline, or failure of fusion between the shelves. The conclusion may be reached that many environmental factors can act on these three events to induce palatal cleft. It seems possible, however, that some of these factors may be influencing common metabolic pathways, such as protein synthesis or carbohydrate metabolism, which are fundamental to development of the embryo as a whole. For example, cell proliferation and differentiation and mucopolysaccharide synthesis undoubtedly are dependent on both these metabolic processes and have been implicated in development of the palate (Pourtois, '66; Larsson, '61, '62).

There is little knowledge of the biochemical reactions involved in normal palatogenesis and, therefore, it is difficult to explain the occurrence of anomalous palatal development under experimental conditions or in populations. In order to gain more knowledge in this area, we proposed studies of one metabolic function likely to be involved, i.e., carbohydrate catabolism and energy production, by the administration of compounds which are known to affect this pathway. Because of the physiological difficulties that use of these compounds present in the whole embryo, we felt that application of the *in vitro* technique used successfully in earlier investigations would be warranted in this instance. This report deals with the results of initial studies involving the addition of sodium fluoride to the medium on which palatal explants are grown. In addition, the effects of sodium pyruvate on explants treated with fluoride and on non-treated palates is described.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 150–200 gm each were used for all experiments. A male of the same species was placed in each cage containing three females for a four hour period, from 8:00 AM to 12 noon, in a room in which the

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light-dark sequence was reversed. At the end of the period, positive matings were verified by the vaginal smear method and mating was assumed to have occurred at 10:00 AM of day 0. The mated females were segregated and given commercial pelleted rat chow and water *ad libitum* until used for experimentation.

Culture methods

Fusion of the palate in this species normally occurs during the sixteenth day after mating when this method of pregnancy timing is used. At 10:00 AM on the sixteenth day, the pregnant female was killed by cervical dislocation, the uterine horns removed, the embryos dissected and washed thoroughly in cold Tyrode's solution. Details of the methods used for dissection of the palate and for the culture technique have been described elsewhere (Myers, Petrakis and Lee, '68). The culture method was modified slightly in that lens-paper rafts were used rather than rayon acetate rafts and the volume of fetal calf serum added was reduced to 10% of the total volume of culture medium.

The palatal explants were cultured for 72 hours at 37°C, with air as the gas phase. At the end of the incubation period the palates were examined at low magnification to determine the extent of fusion and the condition of the tissues. They were then fixed in 10% buffered formalin or Bouin's solution, paraffin-embedded, serially sectioned in a transverse plane at 7 μ and stained for histological examination. The stain used was either hematoxylin-eosin, Alcian blue or Mallory's connective tissue stain.

Preparation of media

The defined medium used for culture was Leibovitz Medium L-15 (Leibovitz, '63) which was shown in previous work to permit complete palatal fusion in a large percentage of explants. To this was added, at the time of use, 10% by volume fetal calf serum. Leibovitz Medium utilizes a phosphate buffer system and contains 5×10^{-2} M sodium pyruvate.

The experimental culture media were prepared by adding sodium fluoride, sodium pyruvate, or a combination of fluoride and pyruvate to the semi-defined medium. A concentrated stock solution of fluoride or pyruvate was first prepared by dissolving the compound in defined medium. A small volume of the stock solution was then added to the semi-defined medium immediately prior to use to provide the final concentrations desired. The final concentrations of sodium fluoride used were 2.0-, 2.5-, 3.0-, 3.5-, 4.0-, and 4.5×10^{-3} M. Higher concentrations caused extensive tissue necrosis which made it impossible to attain valid results. Excess pyruvate concentrations in the medium ranged from 10^{-3} M to 10^{-1} M. Osmolarity of the media was tested before use in any experiment.

Palate transfers

In order to determine the ability of the explant to recover from fluoride treatment and to observe the effect of pyruvate on recovery when fluoride was not simultaneously present, a number of palates were transferred from the initial medium to a second medium during the culture period. Transfers were done either 24 or 30 hours after the start of incubation and were performed by removing the explant from the first medium, washing thoroughly in balanced salt solution, and replacing it in a fresh Petri dish containing the second medium for the remainder of the 72 hour incubation period.

RESULTS

Controls

The appearance of the palate when freshly dissected is shown in figure 1. Figure 2 illustrates the extent of shelf fusion attained following growth on the semi-defined medium. In transverse section (fig. 3) the pre-incubation palate was found to include portions of the nasal septum and capsule and the widely separated palatal shelves which are made up of undifferentiated mesenchyme covered by cuboidal epithelium. The shelves of the palate after culture (fig. 4) were fused, breakdown of the midline epithelial lamina had occurred, and mesenchyme was continuous across the midline. Tissues of the explants closely resembled newly-dissected palates in the distribution of cells and intercellular material. Growth of palatal explants on semi-

defined medium resulted in complete fusion of 84% of the palates, partial fusion of 11% and non-fushion of 5%. Explants which fused less than 15% of the total length of the shelves within 72 hours were classified as non-fused for the purpose of these experiments because previous studies had shown that further development of these explants was minimal even though the incubation time was extended.

Sodium fluoride

The addition of several levels of sodium fluoride to the culture medium produced the results summarized in table 1.

The extent of fusion found in individual explants was directly related to the concentration of fluoride added, ranging from complete fusion of all palates at the lowest level of fluoride (2×10^{-3} M), through all degrees of partial fusion at intermediate levels, to an incidence of 90% non-fusion at the highest level (4.5×10^{-3} M). The type of fusion occurring, i.e., epithelial or mesenchymal, was also noted and the sequence of events of fusion appeared normal in this respect.

An explant grown on medium containing 3.5×10^{-3} M fluoride is shown in figure 5. Although the shelves were not noticeably distorted in shape, they appeared to be under-developed and remained widely separated from each other except at the extreme posterior end. This area of contact probably does not represent *in vitro* shelf growth because near-contact at this location was often observed when the explant was first placed in culture and the inverted position of the palate tended to accentuate the contact. Transverse sections of a fluoride-treated palate (fig. 6), taken from near the midpoint of the explant, demonstrate the extent of shelf growth toward the midline and show the histological appearance of shelf epithelium and mesenchyme. Except for a slight increase in staining intensity of intercellular material in the reduced shelves, there was no obvious difference in cell or tissue structure between fluoride-treated and untreated palates under microscopic examination.

Sodium pyruvate

The effect of sodium pyruvate on the explant was most pronounced at a concentration of 5×10^{-2} M. These explants were noticeably larger than controls and fusion occurred within the first 24 hours of incubation. The usual minimum time for complete fusion of controls without excess pyruvate is 36–48 hours. Pyruvate levels of 10^{-1} M delayed the time of fusion and lowered incidence of fusion in all cases. Low to intermediate pyruvate concentrations produced explants which retained more normal morphological and histological characteristics after culture than did control explants. High concentrations (10^{-1} M), on the other hand, produced palates in which fusion was rarely evident and the shelves exhibited reduced cell density and decreased staining intensity of intercellular material (fig. 7).

Sodium fluoride — sodium pyruvate

The concentrations used and the results obtained when sodium fluoride and sodium pyruvate were added to the culture medium simultaneously are given in table 2. A graphic representation of the effects of all combinations of fluoride and pyruvate on incidence and degree of fusion is shown in figure 8. Very low pyruvate levels (10^{-3}–10^{-2} M) had no effect on fusion. How-

TABLE 1

Effect of sodium fluoride on palatal fusion in vitro

Concentration of Na F (M.)	No. palates cultured	Fused	Partially fused	Not fused
		%	%	%
0	64	54 (84)	7 (11)	3 (5)
2.0×10^{-3}	10	10 (100)	—	—
2.5×10^{-3}	14	12 (86)	2 (14)	—
3.0×10^{-3}	40	7 (18)	26 (64)	7 (18)
3.5×10^{-3}	35	—	18 (51)	17 (49)
4.0×10^{-3}	15	—	2 (13)	13 (87)
4.5×10^{-3}	10	—	1 (10)	9 (90)

ever, 5×10^{-2} M pyruvate reversed the effect of 3.0–3.5 $\times 10^{-3}$ M fluoride and permitted a large percentage of fusions to occur.

Previous experiments had shown that either high fluoride or high pyruvate levels in the medium were detrimental to shelf growth and fusion and could cause extensive tissue degeneration. However, if maximum fluoride concentrations and intermediate or high pyruvate levels were present simultaneously in the medium, some explants were capable of complete fusion and the incidence of partial fusions was somewhat greater. A number of explants grown on media containing levels of both fluoride and pyruvate higher than those shown in the table exhibited complete retardation of growth and severe tissue necrosis following incubation.

In histological appearance, the explants varied greatly. Those subjected to low and intermediate concentrations of both compounds closely resembled control cultures (fig. 9). High levels of fluoride and pyruvate usually caused areas of separation of epithelium from mesenchyme. Loss of continuity of mesenchyme across the midline was also observed, even though the palate appeared to be fused in gross aspect (fig. 10).

Palate transfers

Table 3 summarizes the results obtained from a series of experiments in which the explants were transferred from a medium containing fluoride to a fluoride-free medium during incubation. Palates transferred from medium containing 3.5 $\times 10^{-3}$ M fluoride demonstrated a limited ability

TABLE 2

Effect of simultaneous administration of sodium fluoride and sodium pyruvate on palate explants

Concentration of Na F (M.)	Concentration of Na Pyr. (M.)	No. palates cultured	Fused	Partially fused	Not fused
			%	%	%
3.0×10^{-3}	0	40	7 (18)	26 (64)	7 (18)
	10^{-3}	8	1 (12)	5 (63)	2 (25)
	10^{-2}	19	3 (16)	10 (52)	6 (32)
	5×10^{-2}	28	26 (93)	2 (7)	—
	10^{-1}	13	10 (77)	3 (23)	—
3.5×10^{-3}	0	35	—	18 (51)	17 (49)
	10^{-2}	15	—	11 (73)	4 (27)
	5×10^{-2}	26	22 (85)	4 (15)	—
	10^{-1}	17	10 (59)	6 (35)	1 (6)
4.0×10^{-3}	0	15	—	2 (13)	13 (87)
	10^{-2}	14	—	2 (14)	12 (86)
	5×10^{-2}	20	4 (20)	2 (10)	14 (70)
	10^{-1}	15	4 (27)	6 (40)	5 (33)
	5×10^{-1}	10	—	4 (40)	6 (60)

TABLE 3

Fusion of palatal explants when transferred from sodium fluoride to recovery medium [1]

Concentration of Na F (M.) in primary medium	Concentration of Na Pyr. (M.) in recovery medium	No. palates cultured	Fused	Partially fused	Not fused
			%	%	%
3.5×10^{-3}	0	24	4 (17)	8 (33)	12 (50)
	5×10^{-2}	34	29 (85)	5 (15)	—
	10^{-1}	37	16 (43)	21 (57)	—
4.0×10^{-3}	0	20	—	10 (50)	10 (50)
	5×10^{-2}	38	14 (37)	24 (63)	—
	10^{-1}	24	—	—	24 (100)

[1] At 24–30 hours incubation time.

to recover and eventually fuse even when excess pyruvate was not present in the recovery medium. However, the addition of 5×10^{-2} M pyruvate to the second medium greatly enhanced the incidence and extent of fusion. The percentage of complete fusions found was equal to that occurring when fluoride and pyruvate were used in combination and some degree of fusion occurred in all explants.

The effects of a higher initial fluoride concentration (4.0×10^{-3} M) were not as readily reversed by transfer. If no excess pyruvate was present in the recovery medium, evidence of fusion was slight and consisted only of a short area of partial fusion. An excess pyruvate level of 5×10^{-2} M again proved most effective in that instances of complete fusion were found and total non-fusion did not occur. In this case, transfer seemed to increase the possibility of fusion over that found when fluoride and pyruvate were used simultaneously.

The time of transfer, i.e., whether at 24 or at 30 hours, appeared to affect the success of fusion only when excess pyruvate was not added to the recovery medium. Because the duration of culture was limited to 72 hours for all explants, differences found in incidence of fusion may reflect merely a delay in the fusion process which could not be overcome by the 30 hour transfers within the limited period available, rather than total loss of the ability of the shelves to grow and fuse.

Transferred palates were generally similar in microscopic appearance to those grown on combined fluoride and pyruvate. However, none of the transferred palates demonstrated the changes in cell density characteristic of explants in contact with high pyruvate levels for the complete 72 hour incubation period.

DISCUSSION

The experiments reported here demonstrate an effect of fluoride on embryonic palatal explants which is characterized by retardation of shelf growth toward the midline, precluding normal fusion. The effect can be reversed by addition of pyruvate to the medium and the explant will then develop in a normal manner. The mechanism of action of fluoride and pyruvate under these conditions is not clear and will require further study. Several explanations, based on previously reported actions of fluoride, are possible.

Fluoride may affect a number of biological reactions associated with embryonic growth and differentiation. At high concentrations, cell destruction, tissue necrosis and death of the whole organism may occur (Hodge and Smith, '65). Lower fluoride levels have been shown to inhibit activity of specific enzymes, including those concerned with glycolysis and cell respiration. Localized morphogenetic effects of such inhibition have been suggested by the work of Spratt ('50), Duffey and Ebert ('57), and others.

In experiments reported upon here, widespread death of cells or degeneration of tissues does not appear to be a major factor in the lack of palatal shelf growth *in vitro*, except when relatively high levels of fluoride are present. Microscopic examination of serial sections from treated palates indicate that both epithelial and mesenchymal cells making up the shelves are similar to those of control explants in size, shape and staining quality. More extensive histochemical studies or electron microscopy might reveal changes not made apparent by the methods used. Apparent differences found in staining intensity between the shelves of fluoride-treated and control explants may be explained by increased cell density and concentration of intercellular material in the retarded shelves when compared to the larger shelves of normal explants. Tissue necrosis, when it does occur, is confined to circumscribed areas and is located in the same centralized areas in both experimental and control palates. It seems most likely due to nutritional inadequacy deriving from the *in vitro* method. The fact that the shelves recover their ability to grow and fuse if explants are transferred to a fluoride-free medium also indicates that tissue necrosis is not responsible for the lack of shelf development. The active proliferation of cells which would be required in that circumstance was not observed.

Inhibition of enzyme systems involved in the growth process is an alternative explanation for the results obtained in these studies. The need for an intact glycolytic

pathway for palate development has been suggested by other investigators. DeAngelis ('69) has associated a diminution of glycogen in mouse palate mesenchymal cells with cortisone treatment and indicates that loss of this potential energy source could be a cause for lack of shelf movement with resulting clefts in these animals. Depletion of glycogen by fasting may also cause palatal cleft in mice (Runner and Dagg, '60). The results obtained in the present study could be a consequence of blockage of specific sites in glycolysis or in oxidative phosphorylation which retard cell proliferation or synthesis of cell products. However, fluoride inhibition not directly associated with energy metabolism might also account for retardation of shelf growth.

It is necessary to distinguish between action of fluoride on shelf growth and on palatal fusion. Fluoride inhibition does not appear to affect the fusion process itself but rather to prevent shelf contact which precedes fusion. The short area of shelf contact at the posterior border of the explant which is present prior to incubation subsequently fuses in the majority of explants even in the presence of relatively high fluoride concentrations. The appearance of partially fused palates also suggests that normal mesenchymal fusion can take place in the presence of fluoride if the shelves come into contact with each other.

The action of excess pyruvate, when added to the medium alone or in combination with fluoride is unknown. The effect of pyruvate in increasing the rate of fusion may be a consequence of improved energy utilization by the explant which results in faster growth. Alternatively, the increase in size of the explant and in rate of fusion could be due to an edematous state of the explant which serves to bring the shelves into contact at an earlier time than would otherwise occur. The tissue changes found at high pyruvate levels would tend to support this explanation. However, pyruvate levels most effective in promoting fusion cause little or no edema in the explant. Conversely, the explants exposed to high pyruvate concentrations and which exhibited tissue changes did not generally fuse.

The method involving transfer of palates during incubation provides some confirmation that retarded shelf growth is not a result of explant death. The possibility of interaction between fluoride, pyruvate and other medium components to give misleading data is also reduced. Results from these experiments suggest that the action of fluoride is not one of absolute inhibition of shelf development but rather a retardation of growth which can be reversed if certain limitations as to fluoride level and time are met. The increase in shelf size produced by the use of pyruvate in the second medium may compensate for delayed growth due to fluoride and permit normal fusion to occur, even though the growth period is limited.

Although no evidence of the primary site of fluoride action under *in vitro* conditions has been presented, the influence of fluoride on palatal shelf growth is evident. The developing palate demonstrates a sensitivity to fluoride inhibition, emphasized by the fact that the dosage levels used here are of the same magnitude commonly used to inhibit enzyme activity or cause morphological changes in other systems (Hodge and Smith, '65; Papaconstantinou, '67). If the effects noted for palatal explants can be shown to be due to variations in specific metabolic pathways, explanation for some of the results obtained in other studies of normal and abnormal palate development may be possible.

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Abbreviations

L, lip
PP, primary palate
SP, secondary palate
NS, nasal septum
PS, palatal shelves
FL, fusion line
N, nasopharynx

PLATE 1

EXPLANATION OF FIGURES

1 Photograph of a gross palatal explant from a rat embryo 16 days ± two hours old. The extent of palatal development prior to culture is demonstrated. × 45.

2 Gross palatal explant after growth for 72 hours on semi-defined medium. Note the extent of fusion achieved. × 28.

3 Transverse section of the embryonic rat palate at 16 days ± two hours. The palatal shelves (PS) are in a horizontal position but widely separated. × 28.

4 Transverse section of the fused palate after culture showing loss of the epithelial lamina between the shelves and continuity of palate mesenchyme. × 36.

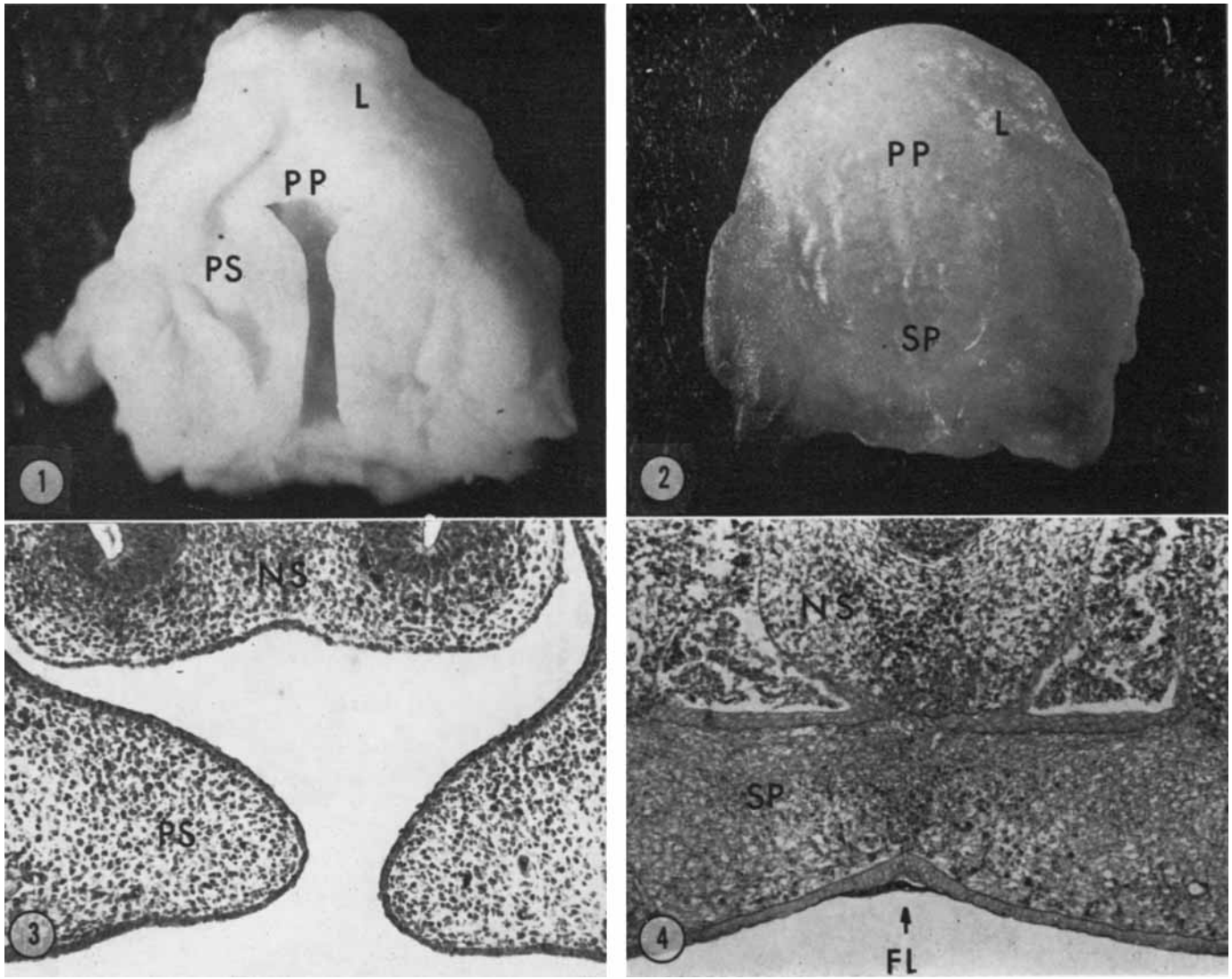
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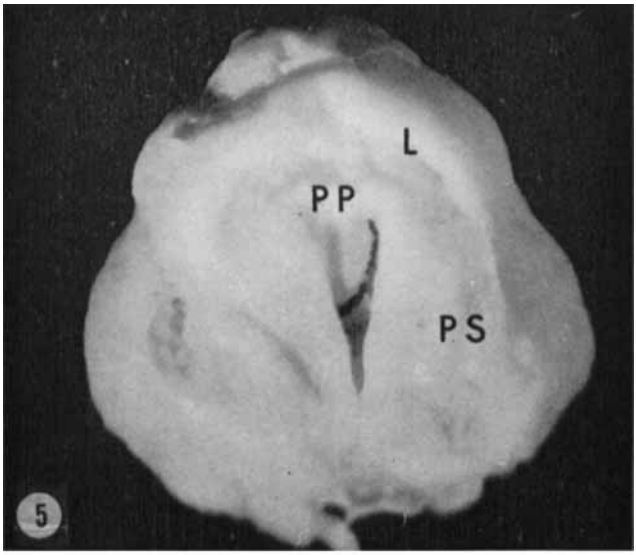
PLATE 2

EXPLANATION OF FIGURES

5 Gross palatal explant grown for 72 hours on semi-defined medium containing 3.5×10^{-3} M NaF. Shelf configuration is typical of fluoride-treated palates. $\times$ 27.

6 Transverse section of a palate treated with 3.5×10^{-3} M NaF in culture medium. Compare the shelf development and tissues to the pre-culture control in figure 3. $\times$ 13.

7 Transverse section of a palatal explant exposed to 10^{-1} M sodium pyruvate in the medium, showing the decreased cell density in the shelves, (PS). $\times$ 27.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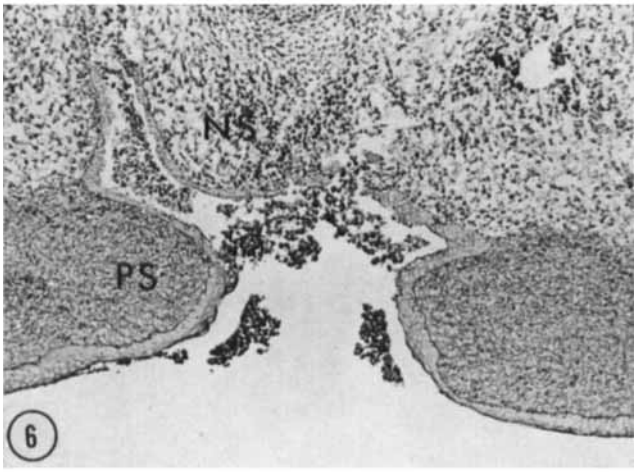
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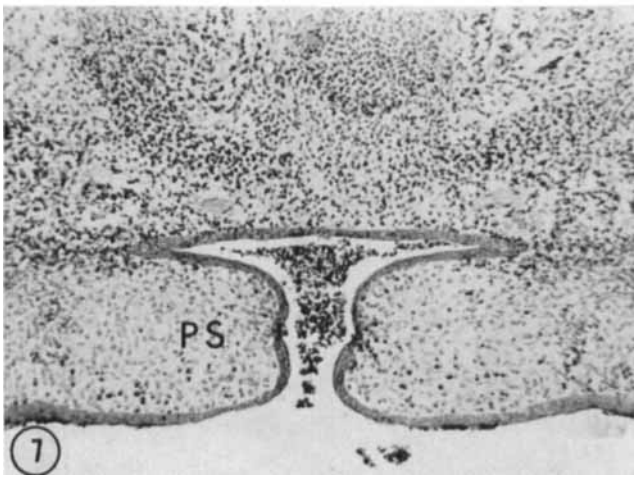
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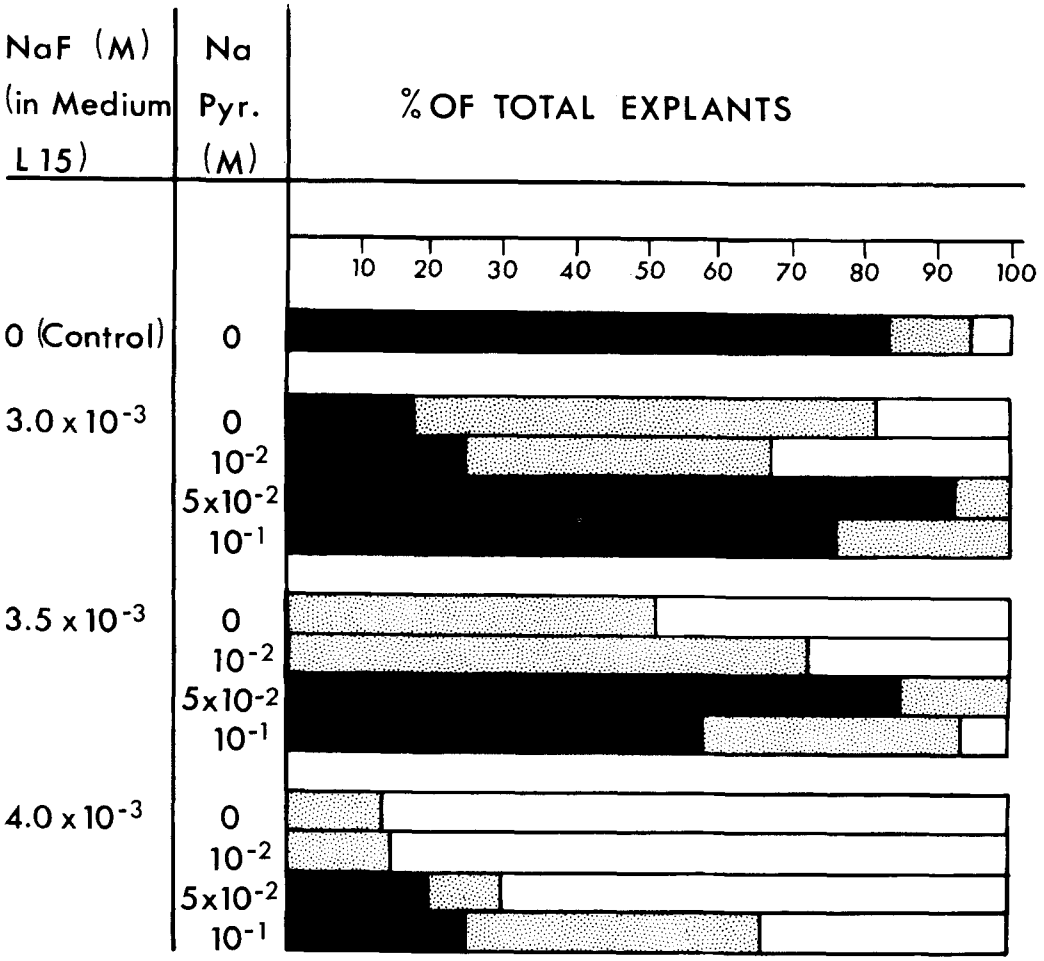
PLATE 3

EXPLANATION OF FIGURE

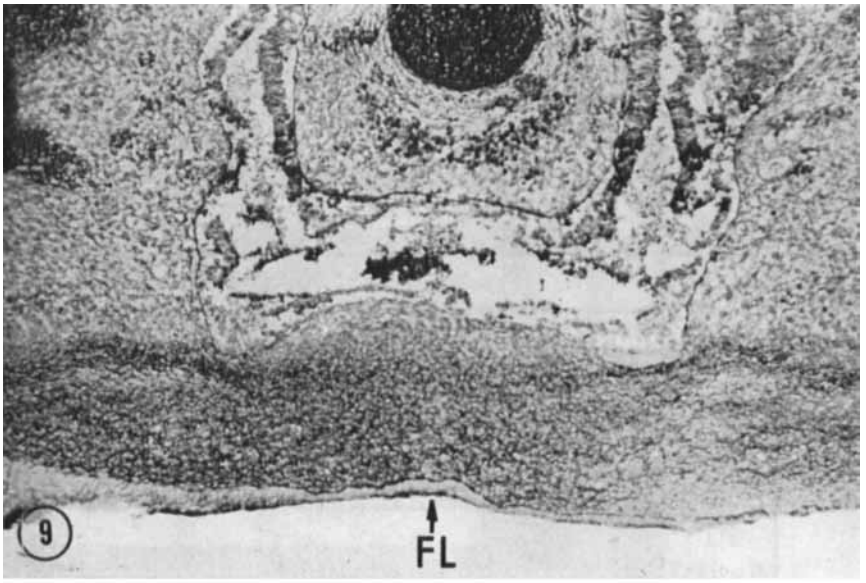
8 Graphic representation of the effect of sodium fluoride and sodium pyruvate on incidence and type of fusion of explants.

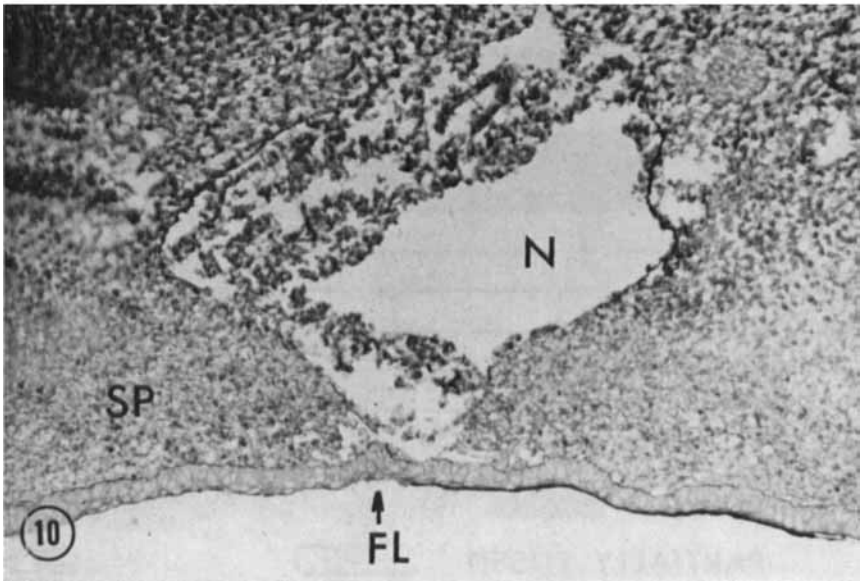


LEGEND – FUSED

PARTIALLY FUSED

NOT FUSED





EXPLANATION OF FIGURES

9 Transverse section of an explant grown on medium containing 3.5×10^{-3} M NaF and 5×10^{-2} M Na pyruvate. × 32.

10 Transverse section of an explant demonstrating tissue damage caused by 4.0×10^{-3} M NaF plus 10^{-1} M Na pyruvate. × 32.