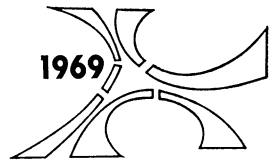
Cleft Lip Induced in the Rat



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Normal and abnormal development of the primary palate are still the subject of much controversy in the literature. While numerous publications appeared in the last years on closure of the secondary palate, no important study on pathogenesis of cleft lip has been published since the work of Töndury (*23*). This author, like his predecessors, analyzed a few young human cleft emroyos. Such a study needs numerous well-fixed and well-sectioned embryos, but human material is too scarce to enable a full description of the formation of clefts, and conclusions must remain hypothetical. This is true also for the work of those authors who described normal development of human embryos or old cleft embryos and tried to explain abnormal development by their observations.

Because of the lack of human material, some authors studied congenital clefts in mice (*14, 15, 18, 19, 20*). Höchstetter (*7*) already emphasized that the main developmental aspects of the primary palate are similar in all mammals. Unfortunately, the descriptions of Reed and Steiniger (*14, 15, 18, 19, 20*) were not embryological analysis, stage by stage, of abnormal development. Reed (*15*) concluded from his study that a cleft is mainly due to an insufficient growth of the maxillary process. Steiniger (*19*) observed cysts in the fusion line of the processes and assumed that a cleft may result from rupture of a huge cyst.

The French surgeon Veau, who operated more than 1,000 clefts, was the first to insist on the fact that there are 20% of bridges in cleft cases, which are not explained by the classical hypothesis on the pathogenesis of clefts. He studied a few human and dog clefts (*24, 25, 26*) but was unable to demonstrate his personal views on cleft formation.

It is now possible to create malformations in laboratory animals.

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We chose hadacidin to induce clefts in the rat. Hadacidin is an antibiotic isolated from broth cultures of Penicillium frequentans by Gitterman and associates (*4*). It is an inhibitor of purine synthesis (*16*), and retards RNA synthesis. Its teratogenic effect was described by Chaube and Murphy (*2*).

In previous works, we first studied normal development of the nasal region in the rat, by morphological and histochemical techniques (*9, 10, 11*).

The aim of the present study is to analyze, stage by stage, abnormal development of the primary palate and formation of clefts.

Materials and Methods

One hundred Wistar female rats, 4 to 6 months old, were selected at the beginning of gestation. They were injected intraperitoneally with fractionated doses of hadacidin at 6 hour intervals (on the 11th day), or at 24 hour intervals (on the 10th and 11th days), in order to decrease the toxicity of the drug.

The dose of hadacidin was 2 gm/kg in the majority of experiments. Lower doses have no teratogenic effect, and higher doses have a more toxic effect and induce a higher percentage of resorptions.

The embryos were removed by Cesarean section during the 11th day (6 cases), 12th day (8 cases), 13th day (23 cases), 14th day (32 cases), 15th day (11 cases), 16th day (4 cases), 17th day (8 cases), 18th day (11 cases), 19th day (4 cases), 20th day (1 case), and 21st day (1 case). One litter was allowed to reach birth.

The fresh embryos were examined in Locke's solution. Some of them were placed in a Nil's blue solution (1/40,000 in Locke's solution) during 20 minutes. Nil's blue staining demonstrates superficial dying cells in the whole embryo and is very useful to give a three-dimensional view of necrotic areas in the fusing epitheliums of the nasal processes.

All embryos were dissected, and the head (or the palate only in the old ones) was generally photographed, then fixed. The nasal region was studied in 10 microns' frontal sections in 298 embryos. 202 were stained by Unna-Brachet method, which demonstrates RNA. RNA increases in the future olfactory epithelium and also characterizes the growing mesoderm of the nasal processes. In addition, this technique clearly demonstrates dying cells, very scarce in the normal embryo, but numerous and highly significant in abnormal embryos. Eighteen embryos were stained by McManus-Hotchkiss method for the demonstration of glycogen, 52 were submitted to histoenzymatical methods for dephosphorylating activities, and 23 were simply stained with hematoxylin-eosin. The details of these techniques have been published elsewhere (*9, 11*).

Results

General Observations. 772 embryos were obtained in one hundred experiments; 317 of them were dead (41%).

Embryonic growth was retarded in the young embryos, which often looked one day younger than their actual age. Some of the fetuses showed generalized edema in the late stages. All 11-day embryos had abnormalities of the nasal region, but, after 14 days, only 34% of the non-resorbed embryos had clefts. This means that all embryos undergo the action of the drug. Some of them die, some recover completely, and some develop clefts. In the 109 clefts, there were more unilateral than bilateral cases, and many more right than left clefts.

Some associated malformations were observed: microcephaly, omphalocele, poly- and syndactyly. Cysts are often observed in the Gasser ganglions. They sometimes expand in the maxillary processes, but have no relation to clefts.

No relation was noted between intrauterine location of embryos and occurrence of malformations.

Description of Abnormalities. The description will be based on morphological stages rather than on the age of the embryos, because growth retardation varies from one embryo to the other.

Placode stage. In normal embryos, olfactory placodes appear at the beginning of the 11th day, and invaginate after 10 to 12 hours.

In hadacidin-treated embryos, placodes were observed until the end of the 12th day. Some embryos are completely disorganized. Frontal sections show hydrocephaly with necrotic cells falling from the roof of the prosencephalon. There is edema dissociating mesodermal cells, and dying cells are obvious in the olfactory placodes and the underlying mesoderm. Such embryos cannot recover from the action of the drug and certainly die quickly. Others are less affected and their frontal sections reveal only a thickened nasal placode containing dying cells, mostly in its middle portion (Figures 1 and 2). The carotid branch which brings blood to the placode is dilated and surrounded by necrotic cells and phagosomes. These are seen more in the upper part of the mesoderm (the future lateral nasal process) than in the lower part (the future medial nasal process).

Invagination stage. In normal embryos, this stage is so quickly followed by fusion of the nasal processes that it is difficult to isolate. In hadacidin-treated embryos, we find invagination from the 12th to the 14th day.

Invagination means that the placode sinks in the head mesoderm. In fact, there is no real invagination, but a quick growth of the lateral borders of the nasal placode, changing the flat placode into a groove, by growth of its sides, the medial and lateral nasal processes. During the normal process of fusion, the actual coalescence of the epithelial

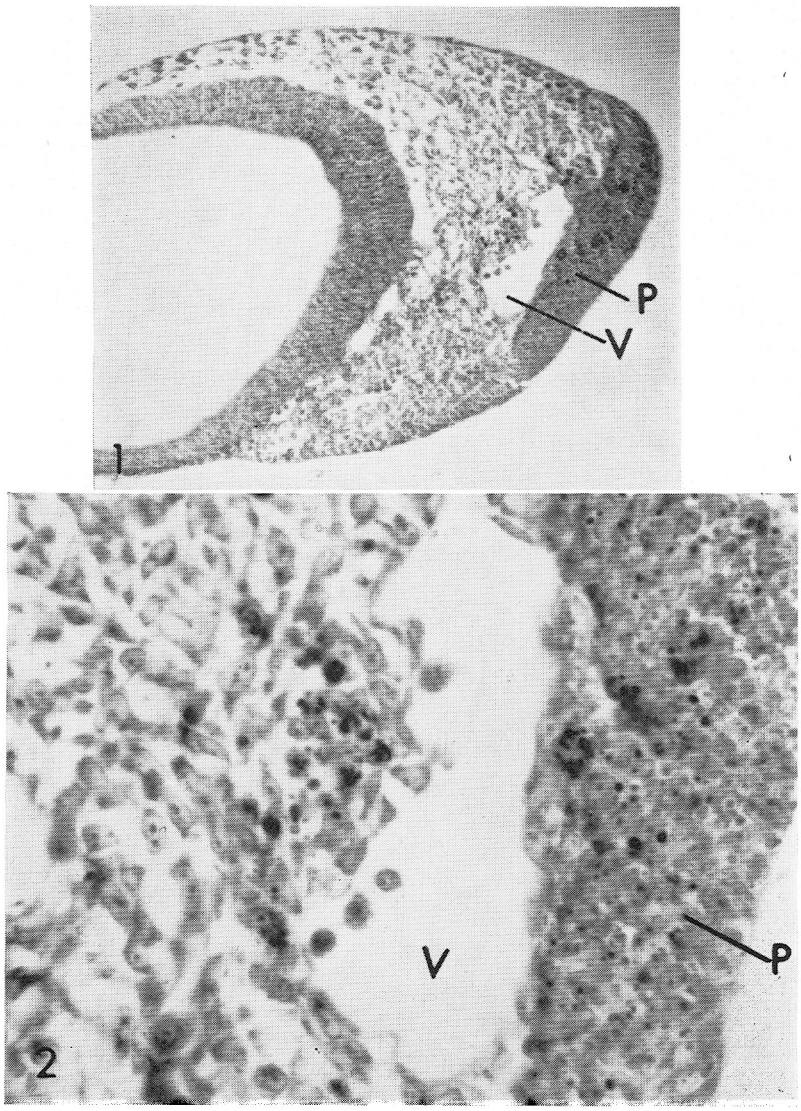


FIGURE 1. Hadacidin-treated 11-day embryo. Frontal section of a thickened left nasal placode (P). Unna-Brachet. Dilated vessel (V) near the placode. Dying cells and phagosomes in the medial part of the placode and the underlying mesoderm. FIGURE 2. Same slide as Figure 1. Detail of the placode at a higher magnification.

edges of the groove is preceded by the occurrence of some necrotic changes in the areas ready to fuse. These changes are particularly evident with Nil's blue staining.

Hadacidin-treated embryos which resisted the toxic effect of the drug invaginate slowly their nasal placodes (Figure 3). In the posterior

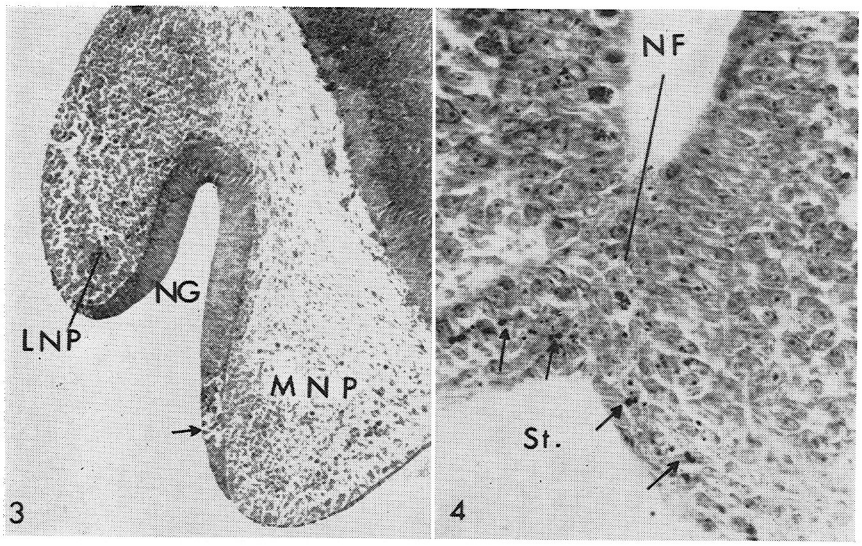


FIGURE 3. Invagination stage in a hadacidin-treated embryo 13 days old. Unna-Brachet. Frontal section. Epithelium of the medial nasal process (MNP) shows dissociated and dying cells at the location where fusion is prepared. LNP: lateral nasal process. NG: nasal groove.

FIGURE 4. Fusion of the edges of the groove in a normal embryo. Unna-Brachet. Frontal section. A few cellular disintegrations are obvious in the inferior portion of the nasal fin (NF). St: stomodeum.

part of the olfactory groove, drastic necrotic changes affect both epithelial edges, which demonstrates that, as in normal embryos, degeneration occurs in the presumptive area of epithelial fusion, but to a much greater extent. It may thus be assumed that the preparatory changes of epithelial fusion occur in all hadacidin-treated embryos, even in those which will develop a cleft.

Beginning fusion. Fusion of the edges of the nasal pit starts in normal embryos at the caudal extremity of the groove at day 11¾. In hadacidin-treated embryos, three possibilities were observed, but fusion usually does not start before the 14th day.

a) The groove may close in a separate tube as in normal embryos, but with a variable delay. In the region of fusion, the so-called "nasal fin", there are many more dying cells and phagosomes than in normal embryos (compare Figure 4 to Figure 5).

b) The groove may close and then immediately break. Such a case is obvious in Figure 6. While the left side of this embryo is fusing, the right side is reopening. Nil's blue reveals the same necrotic pattern on both sides (Figure 7), which means that epithelium is not responsible for the fact. In frontal sections (Figure 8), the left side is fusing slowly and shows necrotic epithelial cells in the line of fusion (Figure 9). The right side has just broken open (Figure 10), as shown by the

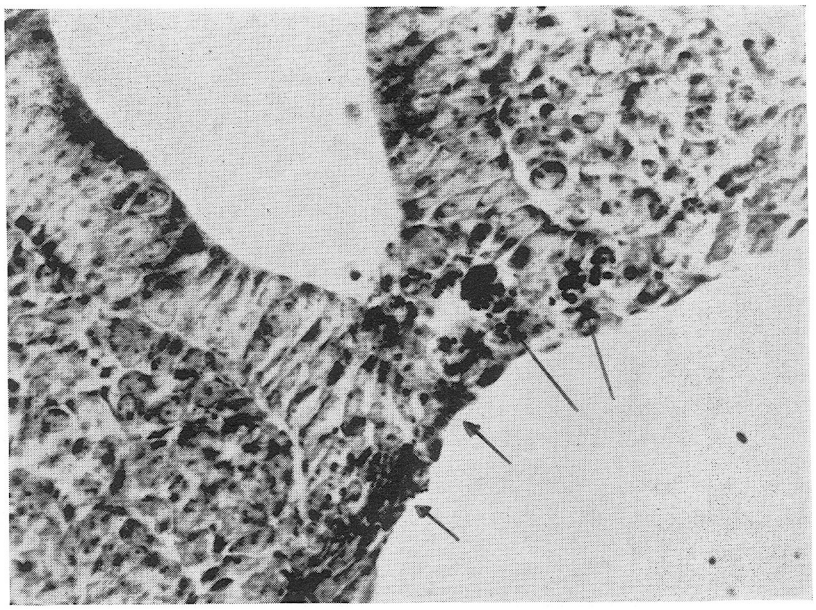


FIGURE 5. Small fusion in a 13-day hadacidin-treated embryo. Unna-Brachet. Many phagosomes and dying cells may be observed in the contact.

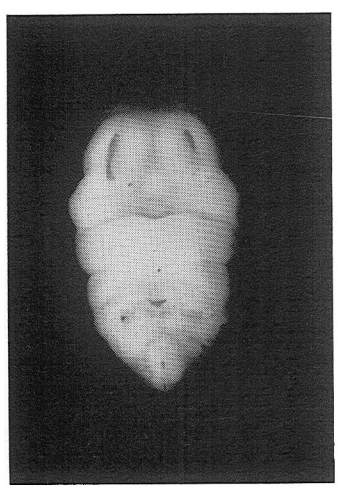
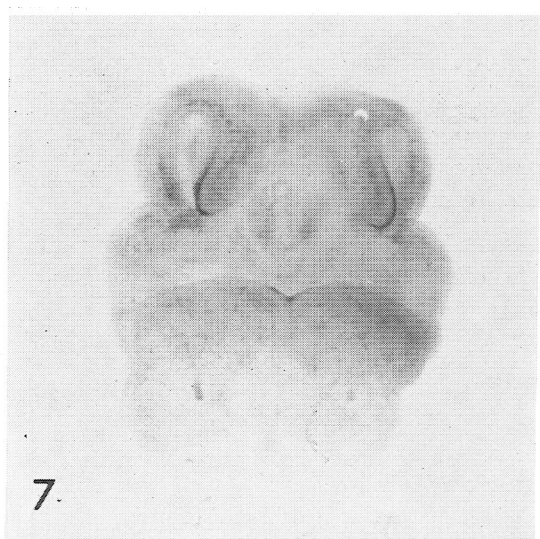


FIGURE 6. Ventral view of the head of a 13-day hadacidin-treated embryo. Serra fixation. The left nasal groove is fusing, the right is open.

irregularities of its bordering epithelium. Here a complete cleft is developing.

c) There is no fusion at all. This case is scarce and will be better explained by the following stage.

Progressing fusion, or cleft formation. This stage corresponds grossly, on our embryos, to the 14- and 15-day stages, but in normal embryos,



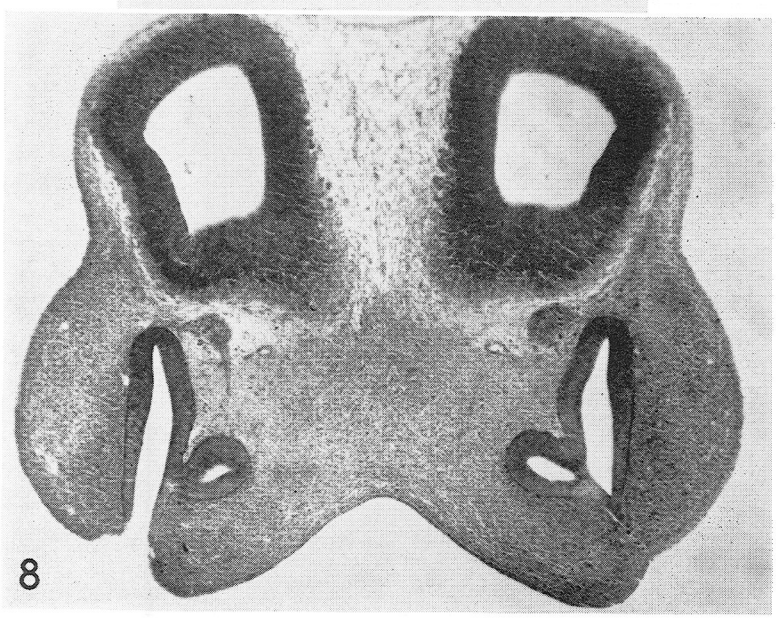


FIGURE 7. Nil's blue staining of the same embryo, before fixation. Dying cells, dark stained, are as numerous on the right side as on the left side.

FIGURE 8. Frontal section of the same embryo. Unna-Brachet. The right side is reopened by rupture of its edges.

fusion proceeds from 11¾ to 14 days. During this stage, abnormalities become evident and the various changes of the nasal region are more obvious. Fusion may reach the nares, or a short primary palate may form, or a cleft, unilateral or bilateral, with or without a bridge, may occur.

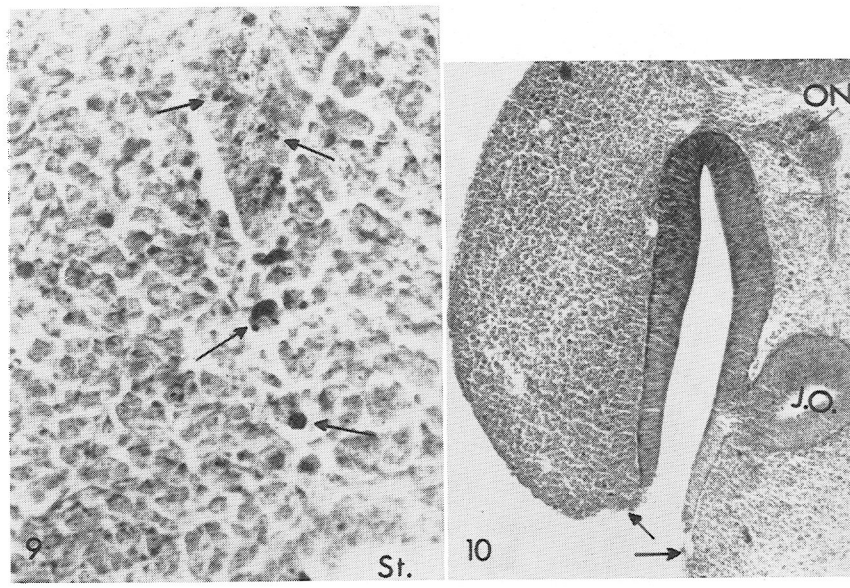


FIGURE 9. Detail of the fusion on the left side of the same embryo. Phagosomes are still obvious in the disintegrated nasal fin, and cellular remnants in the nasal epithelium.

FIGURE 10. Detail of the right side of the same embryo. Recent rupture is evident in the epithelial edges of the groove. JO: Jacobson organ. ON: olfactory nerve.

a) *Normal fusion.* Retarded but normal fusion is frequent in hadacidin-treated embryos. It leads to newborns with a normal lip and a normal maxillary arch (66%).

b) *Short primary palate.* Sixteen of the 109 embryos 14 days old or more which have abnormalities of the nasal region show a short primary palate. They have a fusion of the nasal groove. The nasal fin is partly penetrated by mesoderm, but the choana, produced by rupture of the bucco-nasal membrane, is longer than normal, and the naris is also too long (Figure 11). In a 14-day embryo (Figure 12), the ATPase reaction clearly shows on the right side that though the nasal fin has disappeared its previous location is still visible. The mesoderm of the medial nasal process has expanded laterally, the nasal fossa is too long inferiorly, and the maxillary process seems strangulated at its base.

c) *Evident cleft formation.* In some cases, the *nasal fin is formed, but not destroyed,* by the contiguous growing mesoderm (Figure 13, on the left side). The caudal part of the same nasal fin is already reopening. Such a case must lead to a complete cleft. In most cases, the *cleft is already constituted by reopening of the groove.* This may occur on one or both sides, as previously stated. Figure 14 shows a 14-day embryo with a short primary palate on the left side and a complete cleft on the right side. In a frontal section of this embryo

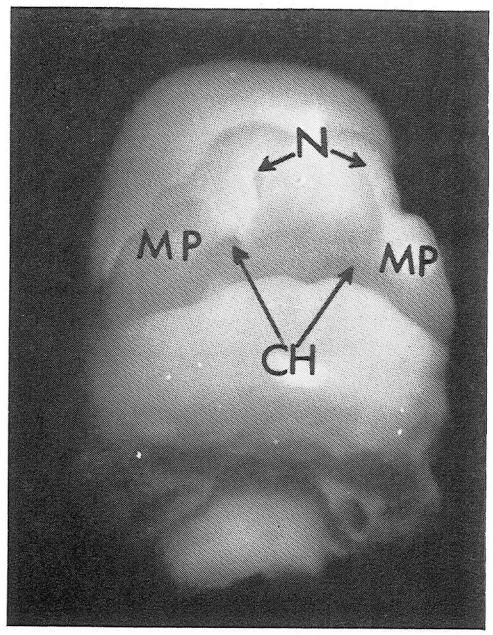


FIGURE 11. Short primary palate in a 14-day hadacidin-treated embryo. Serra fixation. On both sides, the nares (N) and the primary choanae (CH) are too long. The maxillary processes (MP) show a retarded growth.

(Figure 15), the place where the nasal fin ruptured is obvious on the inner side of the cleft. As soon as the contact has ruptured, the inner side is propulsed rostrally and inferiorly, probably by the growing vomerine center. As a consequence, both sides of the rupture cannot be seen on the same section, unless it is slightly oblique, in a bilateral case, as shown in Figure 16. Such epithelial lesions are observed up to the 15th day. Then, the epithelium seems to heal and does not show any further irregularities. In such cases, there is always a bucco-nasal membrane at the 14-day stage. This gives another proof that the nasal fin was first constituted.

In a few clefts, however, which are always bilateral and broadly open (Figure 17), we do not observe a bucco-nasal membrane nor epithelial irregularities bordering the cleft (Figure 18). Consequently, we must admit that there has been *no fusion at all* and that no nasal fin has been formed. In some 14-day embryos with unilateral and bilateral clefts, we find slight irregularities of the epithelium (usually blebs) where it should have fused, and no bucco-nasal membrane. In these cases, we may admit that the blebs correspond to the place where epithelium was prepared to fuse during the invagination stage, even if no fusion occurred in fact.

Constituted cleft. In older embryos, we observe the same clefts as

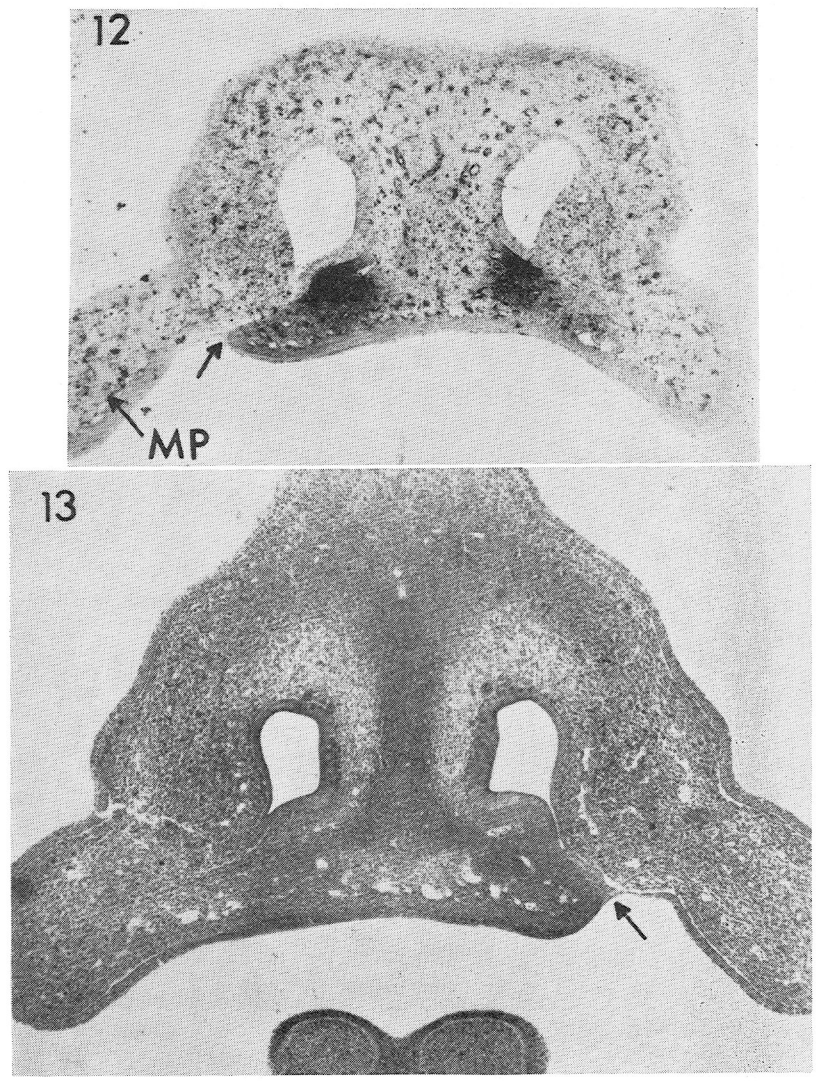


FIGURE 12. Frontal section of a 14-day hadacidin-treated embryo. ATPase reaction. On the left side, the primary palate is formed. On the right side, fusion has occurred between medial and lateral nasal processes, and the location of the nasal fin is still obvious. The maxillary process is thinned at its base.

FIGURE 13. Frontal section of a 14-day hadacidin-treated embryo. Unna-Brachet. The primary palate is constituted on the right side. On the left, the nasal fin was not disintegrated and both sides of the groove begin to separate.

in human cases. Figures 19 and 20 show 17-day embryos with typical unilateral and bilateral clefts. Alizarine technique reveals that, on the side of the cleft, the inferior part of the premaxillary bone has not developed and is not ossified (Figure 21).

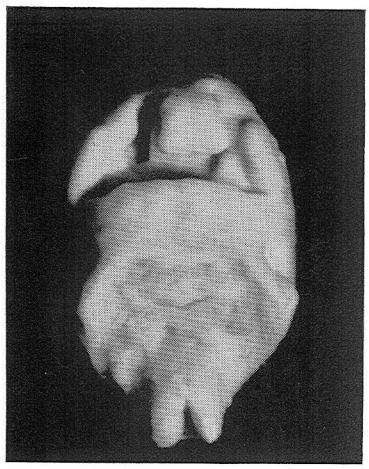


FIGURE 14. Ventral view of a 14-day hadacidin-treated embryo. Serra fixation. Short primary palate on the left side, open groove on the right.

An anterior bridge may be observed (Figures 22 and 23) in the region of the maxillary arch. We explain such cases by the progressive shrinkage of the short primary palate which may be observed in younger embryos.

Discussion

Different hypotheses have been developed to explain the various types of clefts of the primary palate in the embryos, which lead to clefts of the lip and the maxillary arch in the newborns. We will discuss them in the light of our observations on normal development and cleft formation induced by hadacidin in the rat.

All authors now admit that a cleft of the primary palate is the result of an abnormal development of the nasal fossae.

NORMAL DEVELOPMENT. Most of the modern authors describe the normal development as we observed it in the rat embryos. The nasal placode is surrounded by 2 swellings, the medial and the lateral nasal processes, forming the nasal groove. The edges of the groove fuse together caudally, often with the help of the maxillary process which reinforces the lateral nasal process. Fusion progresses forward, constituting the "nasal fin", already described by Höchstetter in 1891 (*7*). The same author first demonstrated that the posterior part of the nasal fin becomes the bucco-nasal membrane, which soon perforates, opening the primary choana. The cleft is due to an abnormal evolution of the nasal fin, which normally soon disappears when the primary palate forms, and separates nasal and buccal cavities.

This normal development was admitted by Pohlmann (*13*) and Fleischmann, referred to in Veau (*24*), Reed (*14, 15*), Veau and Politzer

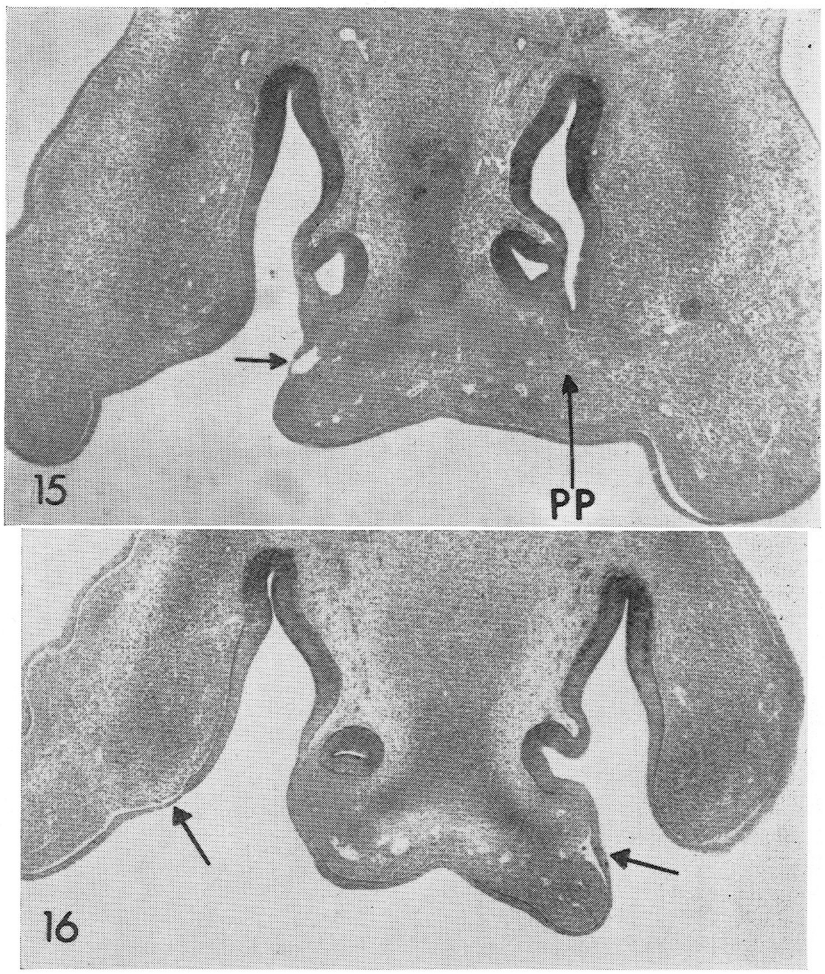


FIGURE 15. Frontal section of the same embryo. Unna-Brachet. Primary palate (PP) on the left side. Open groove on the right, with an irregular epithelium on the inner side, where rupture took place.

FIGURE 16. Frontal section of another embryo of the same stage. Unna-Brachet. Bilateral cleft. The section is slightly oblique, the left side being more rostral than the right. Signs of rupture are shown on both sides.

(*25*), Stroer (*22*), Steiniger (*19, 20*), Töndury (*23*), Streeter (*21*), Stark and Ehrmann (*17*), Warbrick (*27*), and Kraus and associates (*8*).

It must be noted also that the old classical theory of normal development, which was developed by His (*6*), assumed that the nasal groove is closed only by growth of the maxillary process, which bridges its edges and which does not constitute a nasal fin. This theory was later defended by Peter (*12*), and prevailed a great while in well-known textbooks (*5*).

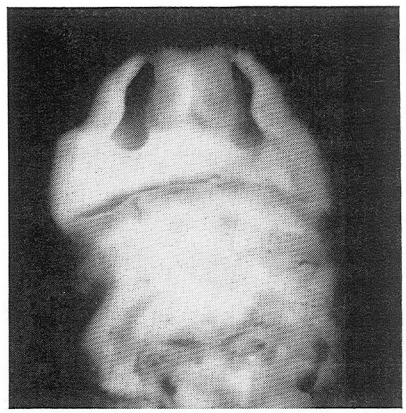


FIGURE 17. Wide open bilateral cleft in a 14-day hadacidin-treated embryo. Serra fixation.

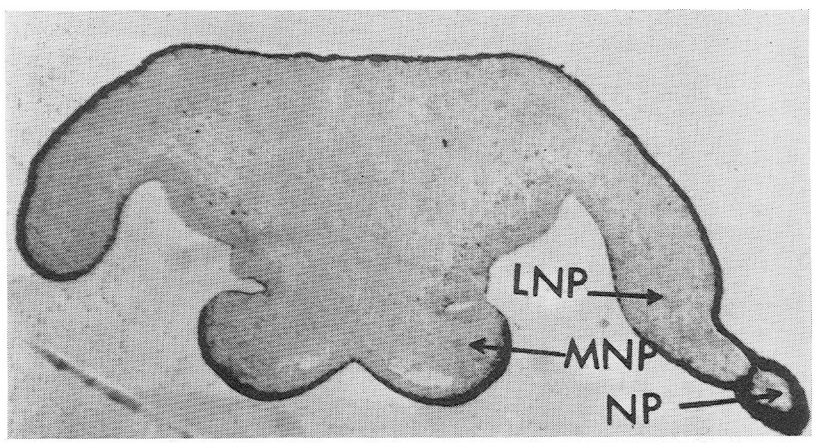


FIGURE 18. Frontal section of a 14-day hadacidin-treated embryo with a bilateral wide open cleft. AMPase reaction is regular in the epithelium of the edges of the clefts.

Recently, Andersen and Matthiessen (*1*), studying the normal development in human fresh embryos, stated that there is no nasal fin, as they could not demonstrate dying cells in its location by histochemical techniques. For them, the nasal groove grows posteriorly towards the epithelium of the primitive oral cavity. It bridges and cuts off a narrow rim of mesoderm, which they call "maxillary isthmus", and which later widens, forming the primary palate.

This theory reminds one of that of Fischel (*3*), who did no personal experiments and elaborated his hypothesis by comparison to the development of amphibians.

We completely disagree with such a hypothesis, as our numerous observations on well-fixed, well-stained embryos quite clearly demon-

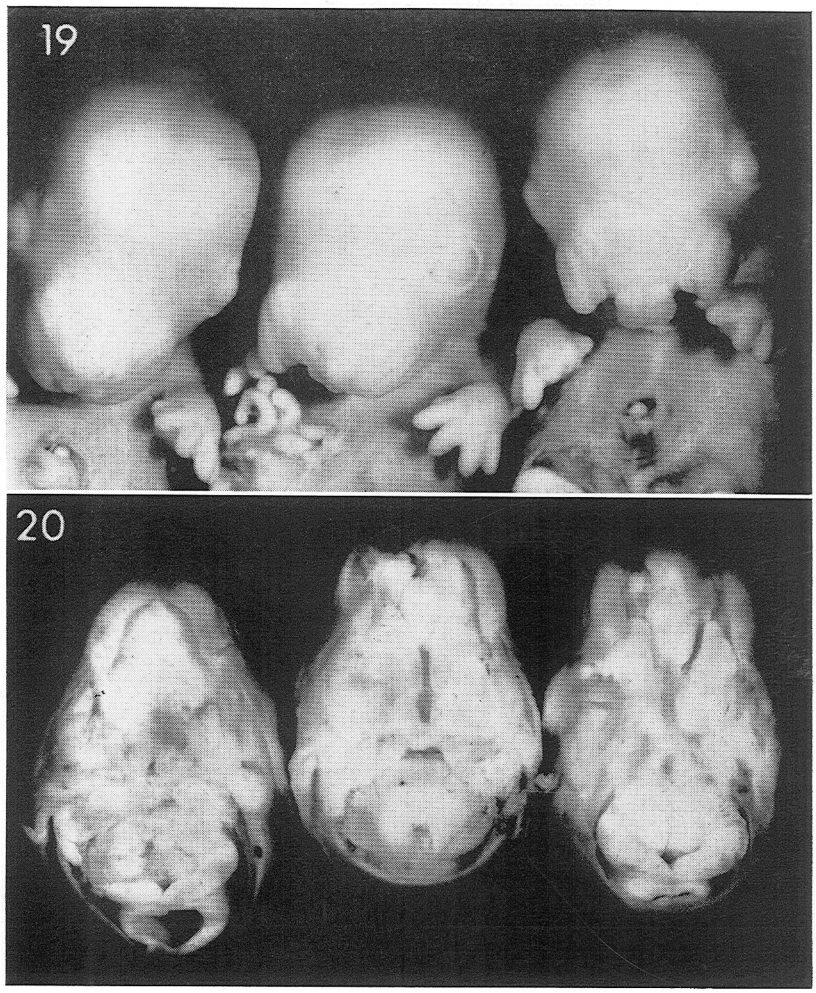


FIGURE 19. 17-day old hadacidin-treated embryos. Serra fixation. Normal embryo, right and bilateral clefts.

FIGURE 20. The palates of the same embryos.

strate the formation of the nasal fin and the apparition of dying cells in it. Andersen and Matthiessen (*1*) studied 50 normal embryos, among which only 6 were young enough to analyze the first stages of development. Our studies have convinced us that numerous young embryos are necessary to understand these stages, as changes occur so quickly at the beginning of development that important facts may otherwise be overlooked.

ABNORMAL DEVELOPMENT. *Formation of a Complete Cleft.* According to the classical hypothesis, a complete cleft appears when the edges of the nasal groove do not fuse because they never reach each other. In our

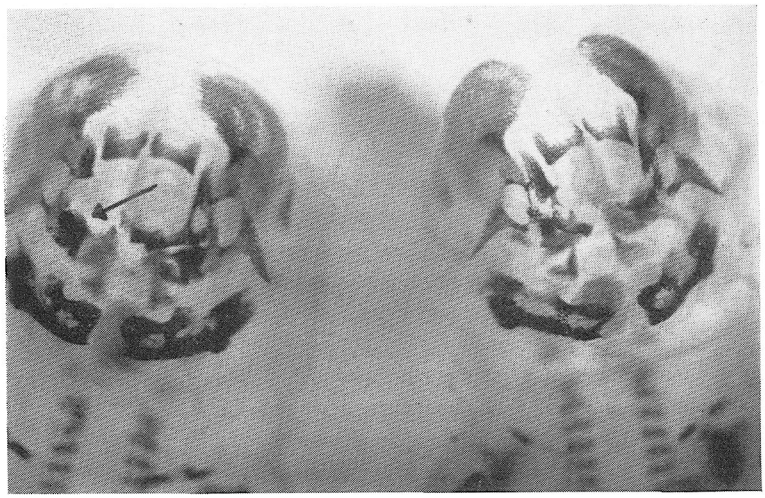


FIGURE 21. Alizarine technique in 19-day hadacidin-treated embryos. The one on the right is normal; the one on the left has a right cleft and shows an absence of the inferior portion of the premaxillary bone.

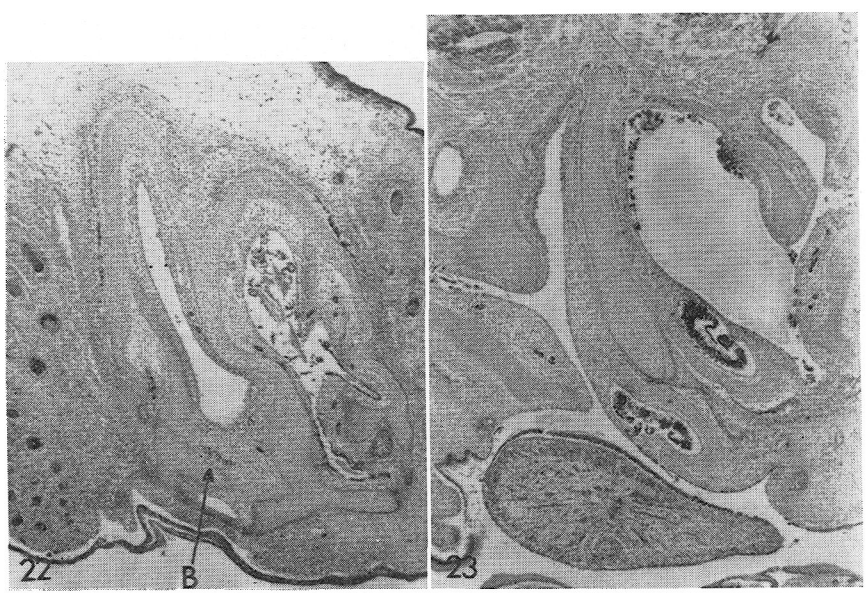


FIGURE 22. 19-day hadacidin-treated embryo. Frontal section. Right cleft with an anterior bridge (B). Hematoxylin-eosin.

FIGURE 23. Frontal section more caudally in the same embryo. The cleft is here complete, and the distortion of the vomer obvious.

observations, this occurs infrequently. We have seen it only in a few bilateral clefts. In such cases, no bucco-nasal membrane and no irregularities of the epithelium bordering the cleft were noted at the 14-day stage.

Pohlmann (*13*) was the first to imagine, without demonstration of his hypothesis, that a cleft may be the result of the reopening of the nasal groove, when the nasal fin does not disappear soon enough. His ideas were strongly defended by Veau (*24, 26*). The truth of this hypothesis was demonstrated by our observations in numerous embryos 13 to 15 days old. We observed irregularities of the epithelium bordering the cleft during one or two days after the rupture, and a bucco-nasal membrane at the 14-day stage. It must be well understood that, after the 15th day, this has disappeared and it is impossible, in an old fetus, to see any difference between a cleft by absence of fusion and a secondary cleft.

Rupture of a huge cyst in the line of fusion as assumed by Steiniger (*20*) has not been encountered in our experiments.

Formation of a Partial Cleft. The classical hypothesis of incomplete fusion of the edges of the groove was not denied by recent authors. It seems more understandable in the light of our observations, which showed that fusion is a common phenomenon even in complete clefts. In incomplete clefts, fusion also occurs, but more slowly than in normal embryos. It does not reach the naris, and produces a partial cleft.

Formation of a Cleft with a Bridge. Töndury (*23*) assumes 3 possibilities to explain such cases. a) The nasal fin is partly destroyed by mesoderm, but the bucco-nasal membrane is too long, and the anterior fusion of the processes is incomplete. b) The nasal fin remains too late and is then partly penetrated by mesoderm. Penetration occurs after the formation of the bony maxillary arch, and therefore never brings bone into the bridge. c) According to Steiniger (*20*) a cyst might break in the line of fusion of the nasal processes and partly reopen it.

In our observations, a complete cleft with a bridge occurs when the nasal fin is formed, and then partly destroyed. In such cases, we observe at the 14-day stage a short primary palate, with a long bucco-nasal membrane (ruptured or not), and a long naris. This corresponds to the first hypothesis of Töndury.

Summary

In 100 experiments, hadacidin, a drug isolated from cultures of Penicillium frequentans, was injected intraperitoneally into pregnant rats during the 10th and 11th days of gestation. Among 772 embryos obtained by Cesarean section at the different stages of development of the primary palate, 34% had abnormalities of the nasal region. In the embryos old enough to show the malformation, 109 clefts were observed. They were identical to human clefts. A complete cleft is more often the result of a rupture of the contact between the edges of the

nasal groove than of an absence of fusion, as usually admitted. Clefts with bridges are produced when the "nasal fin", the epithelial contact between the edges of the groove, is partly penetrated by mesoderm in the region of the maxillary arch.

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