

Analysis of upper beak defects in chicken embryos following with retinoic acid

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SUMMARY

Implanting inert carriers soaked in retinoic acid into the anterior margin of the developing limb of chicken embryos leads to orofacial malformations as well as affecting pattern formation in the limb. Using anion-exchange beads as carriers, and soaking solutions of 1–10 mg/ml retinoic acid, almost 100% of the embryos have malformations of the face. The effects on the treated limbs range from symmetrical patterns of duplicated digits (maximum number of digits being four) to truncations in which no digits were formed at all.

Typically, in the malformed faces the upper beak is completely absent, no nostrils are present and the front of the face forms a scalloped rim of tissue above the mouth. By reference to normal beak development, the seven bulges of tissue that make up the rim can be identified as derivatives of the masses of tissue that normally would fuse to form the upper beak. The roof of the mouth consists of three bulges of tissue flanked by widely separated palatal shelves. The defect can thus be classified as severe bilateral clefting of the primary palate.

By examining the morphology of the faces of treated embryos, the origin of the defect can be traced to failure of the frontonasal mass to enlarge. Thus, the oronasal fissures are very wide and fusion across them to form the primary palate cannot occur.

The way in which retinoic acid brings about the defect is discussed in relation to possible mechanisms involved in the production of cleft palate. The parallel is noted between the associated effects of retinoic acid on beak and limb morphogenesis and the chick mutation *cpp*, that also affects both face and limbs.

INTRODUCTION

Recently we have found that local application of retinoic acid to chick wing buds leads to the development of additional digits. Associated with this effect of retinoic acid on pattern formation in the treated limb, the embryos frequently had upper beak defects (Tickle, Alberts, Wolpert & Lee, 1982). Using a carrier that releases retinoic acid more effectively to apply the chemical to the developing limb buds (Eichele, Tickle & Alberts, 1984), almost 100% of the embryos exposed to the highest concentrations of retinoic acid also have a beak effect. This provides a good system for the analysis of such beak defects since their abnormal morphogenesis can be followed during development.

Analysis of these upper beak defects can provide insights into mechanisms of orofacial morphogenesis and how such abnormalities may arise, since, at early stages, the development of the face of birds is similar to that of mammals.

The teratogenic effect of vitamin A derivatives on facial morphogenesis of mammalian embryos is well known (Morris, 1973). Embryos of pregnant rats given excess vitamin A during day 8 of gestation show abnormal orofacial morphogenesis including upper jaw defects such as cleft palate. At the time of treatment, cranial neural crest cells are migrating into the tissues that will form the upper jaw and it has been suggested that the way in which vitamin A causes the abnormalities is by interfering with this migration (Thorogood *et al.* 1982). A recent report (Jelenik & Kistler, 1981) describes the teratogenic effect of retinoic acid applied systemically on orofacial morphogenesis of chick embryos on days 3 and 4, i.e. the same age as those treated in our experiments. The orofacial defect produced was bilateral cleft palate but a description of the defect was not given and its incidence was quite low. The development of the limbs was also affected.

In view of the association between orofacial and limb defects following retinoic acid treatment, the chicken mutant discovered by Abbott & MacCabe (1966) and subsequently named 'cleft primary palate' *cpp* (Yee & Abbott, 1978) is of great interest. This mutation also affects both the upper jaw and limb development, although the lower limbs only are affected. In the mutant embryos the upper beak is absent. A study of the morphogenesis of the face of these embryos showed that the defect appears to result because fusion of the median nasal process with the lateral nasal processes does not occur (Yee & Abbott, 1978).

Here, we describe the morphology of the upper beak defects that develop following exposure of chick embryos to retinoic acid applied on a controlled release carrier to the wing bud. To analyse how the defects have arisen we have studied how the normal beak develops. Recent studies dealing with upper beak development in the chick (Yee & Abbott, 1978; Shah & Crawford, 1980; Koch & Smiley, 1981; Will & Meller, 1981; Greene *et al.* 1983) have emphasized cytological aspects and/or the topography of early developmental stages. Since these reports did not follow normal development beyond stage 28 (about 6 days incubation: Hamburger & Hamilton, 1951) we have studied later stages up to the formation of the definitive beak. As a further aid in the analysis of the defects, we have studied the morphogenesis of the abnormal beaks.

METHODS

Chicken embryos (stages 20–21, Hamilton-Hamburger stages) were treated with all-*trans* retinoic acid (Sigma, lot Nos. 41F-0440 or 12F-0598) by implanting AG1-X2 beads (200 μ m in diameter) loaded with this chemical to anterior positions in the right wing buds. The standard procedure for loading the beads

was a 20 min soak in retinoic acid solutions in DMSO, followed by two rinses in tissue culture medium and a final 20 min wash in tissue culture medium at 38 °C. The beads were implanted in slits cut between the apical ectodermal ridge and the mesenchyme over the anterior part of the bud. Fig. 1 shows an embryo fixed 2h after the operation and illustrates the relative positions of the

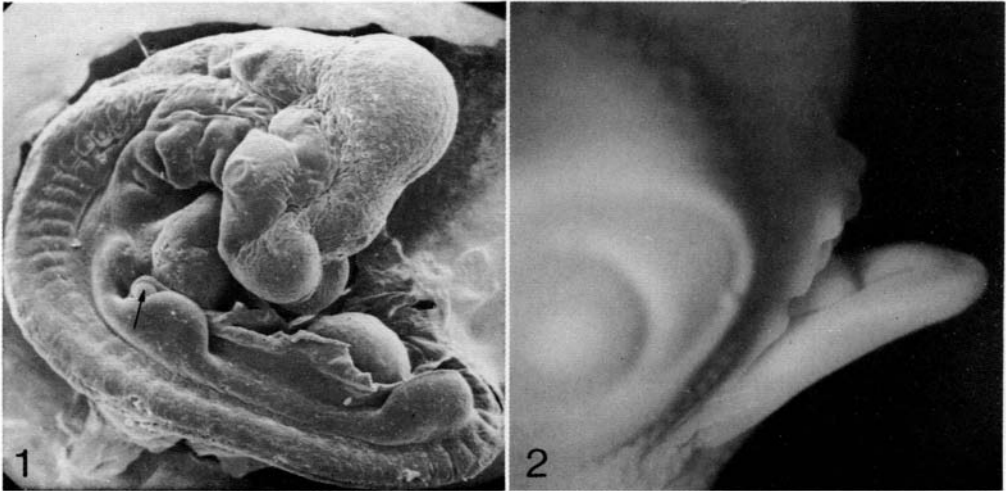


Fig. 1. Right side of whole embryo showing site (arrow) of the bead implanted in the wing bud. Specimen fixed *in situ* two hours after operation. Stage 20. PW = 4.3.

Fig. 2. Profile of experimental specimen at stage 35 (light micrograph). The upper beak has failed to develop and is represented by a series of tubercles (See Figs. 19 and 20). Development of the lower beak is essentially normal. PW = 10.

PW = picture width in mm.

(all figures are scanning electron micrographs except figure 2)

retinoic acid impregnated bead in the right wing bud, and the developing face.

For determining the incidence of beak defects the embryos were reincubated at 38 °C after implanting the bead, and the gross effects of exposure to retinoic acid were observed in 10-day embryos (stage 35–36). The heads were fixed in 5% TCA and stored in 70% alcohol. The associated effect of retinoic acid treatment on the development of the right wing was assessed by fixing the limb in 5% TCA and staining with Alcian Green to show the skeletal pattern.

For the detailed study of the morphology of the abnormal beaks and the development of both the normal and abnormal faces, heads from stage 28 to stage 35 were prepared for scanning electron microscopy. (The bodies of these embryos were fixed in 5% TCA to aid staging of the normal series of embryos and to check right limb development in the series of treated embryos). The heads were fixed in 1% glutaraldehyde made up in a modified Tyrode's

solution (Litze & Low, 1977) at 4 °C for at least 24h. The heads were then washed several times in distilled water and then left in distilled water for at least 12h. Following several changes of absolute alcohol over a period of 24h, the heads were placed in amyl acetate as the transfer liquid (2 changes: 20min each) for critical-point drying in liquid CO₂. The dried specimens were mounted on aluminium stubs with silver conducting paint and coated with gold-palladium alloy. The specimens were viewed using either a Cambridge 410 Stereoscan or a Jeol JSM-35 scanning electron microscope. The SEM data are based on the examination of 25 normal and 16 abnormal specimens representing the relevant stages of development.

RESULTS

Incidence and morphology of beak defects

The incidence of beak defects noted following implantation of beads loaded with retinoic acid to the anterior margins of right wing buds of stage 20 hosts is shown in Table 1 for our first series of experiments. With beads soaked in solutions of 10 mg/ml and 1 mg/ml retinoic acid (Sigma lot: 41F-0440) there is virtually 100% production of beak defects. The defect is immediately recognizable in the 10-day embryos – the upper beak is completely missing, no nostrils form and the front of the face ends in a scalloped rim of tissue bordering the mouth (Fig. 2). In contrast, the lower beak appears relatively unaffected although a shortening cannot be ruled out. In a few cases the lower beak arched downwards suggesting that the upper beak may influence its direction of growth.

The symmetrically truncated upper beak is typical of embryos to which beads soaked in 10 mg/ml retinoic acid have been implanted. With beads soaked in 1 mg/ml the faces of the treated embryos are also truncated but in some cases it is now possible to detect an asymmetry in the effect; the right side

Table 1. *Incidence of beak defects in a series of experiments in which beads soaked in retinoic acid were implanted in the right wing buds of chick embryos*

Concentration of retinoic acid in which bead was soaked	Number of embryos	Number of embryos with beak defect (%)	Right wing morphology
10 mg/ml	9	8(90)	Severe truncation mean number of digits = 0.83
1 mg/ml	10	10(100)	Mostly truncations but less severe mean number of digits = 0.7
0.1 mg/ml	21	4(19)	Symmetrical digit patterns mean number of digits = 3.6

being more affected than the left. Such an asymmetry in the defect and a reduction in its severity is marked in the few affected embryos treated with beads soaked in 0.1 mg/ml retinoic acid. In two of these embryos, the nostril and the beak margin on the left side are present, while on the right, the nostril and the beak margin are rudimentary. In the other two cases of affected embryos at 0.1 mg/ml, nostrils are formed on both sides but the upper beak is very short. One of these short beaks had a well-developed egg tooth.

In the majority of the cases of the first series of experiments (Table 1), the beak defect is associated with truncation of the right wing. The other effects on the treated limbs are a reduction in the number of digits, or symmetrical hand plates made up of three digits in the pattern **434** (see Tickle, Lee & Eichele, in preparation, for more details). It should be noted that the only case in which the face of an embryo was normal following implantation of a bead soaked in 10 mg/ml retinoic acid, the right limb of that embryo was also normal.

Later, experiments were carried out using different batches of retinoic acid either lot No. 12F-0598 from Sigma or Ro 1:5488 from Hoffman la-Roche. The results are qualitatively similar. Thus 100% of embryos receiving beads soaked in 10 mg/ml (11 cases) have a missing upper beak and with beads soaked in 1 mg/ml, the incidence of beak defects in treated embryos (9 cases) is 90%. However, these batches of retinoic acid appear to be less potent (Tickle *et al.*, in preparation). Thus, the faces of the treated embryos resemble those of embryos treated with a ten times lower concentration of the batch of retinoic acid used in the first series of experiments. Thus with beads soaked in 10 mg/ml of these less 'potent' batches of retinoic acid the upper beak is completely absent but in some cases a difference in the effect on the right and left sides of the face can be detected. With beads soaked in 1 mg/ml the beak defects are less severe and in about 50% of the cases, nostrils are present but the upper beak is very short. At 10 mg/ml, the beak defect is still mostly associated with limb truncations but at 1 mg/ml is associated with symmetrical patterns of up to four digits (digit pattern **4334**).

The detailed study of the morphology and morphogenesis of the defective beaks was carried out on embryos into which beads soaked in 10 mg/ml retinoic acid had been implanted. In most cases, retinoic acid from Sigma, lot No. 12F-0598, was used. The effect of the retinoic-acid-impregnated implant on the face appears to be due to exposure to the chemical that diffuses across the extraembryonic space (see Fig. 1). An asymmetrical effect on the face has already been noted, with the right side nearer the bead being more affected than the left. Furthermore, when beads soaked in 10 mg/ml retinoic acid are placed farther away from the face, for example, in posterior positions in the developing wing, the defects are not so severe. Typically in these cases, the nostrils form but the upper beak is short. These effects are consistent with the idea that proximity of the developing face to the implant site leads to the facial defect. We can estimate, from the amount of retinoic acid released from these

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|---|---------------------------------|
| <i>c</i> = primordium of rostral concha | <i>n</i> = medial nasal process |
| <i>e</i> = eye | <i>o</i> = nasolachrymal groove |
| <i>f</i> = frontonasal mass | <i>p</i> = palatal process |
| <i>g</i> = globular process | <i>t</i> = tomium |
| <i>l</i> = lateral nasal process | <i>u</i> = culmen |
| <i>m</i> = mandibular process | <i>x</i> = maxillary process |
| 1 = midrostral unpaired tubercle (from frontonasal mass) | |
| 2 = first tubercle lateral to 1 (from lateral nasal process) | |
| 3 = second tubercle lateral to 1 (from lateral nasal process) | |
| 4 = third tubercle lateral to 1 (maxillary process) | |
| 5 = unpaired tubercle caudal to 1 (from frontonasal mass) | |
| 6 = first tubercle lateral to 5 (from conchal primordium) | |
| 7 = second tubercle lateral to 5 (palatal process) | |

Fig. 3. Normal specimen, stage 28. The broad frontonasal mass (*f*) consists of five regions; the paired elevations of the medial nasal processes (*n*) bordering the oronasal fissures (arrow) a central elevation (frontonasal process) and its paired globular processes (*g*) at the inferior-lateral corners. The maxillary processes (*x*) arise at the corners of the stomodeum adjacent to the mandibular process (*m*) and incline mediorostrally towards the lateral nasal processes (*l*) and the globular processes. The nasolachrymal groove is indicated by 'o'. The palatal processes (*p*) arise as extensions of the maxillary processes within the stomodeum. PW = 3.0

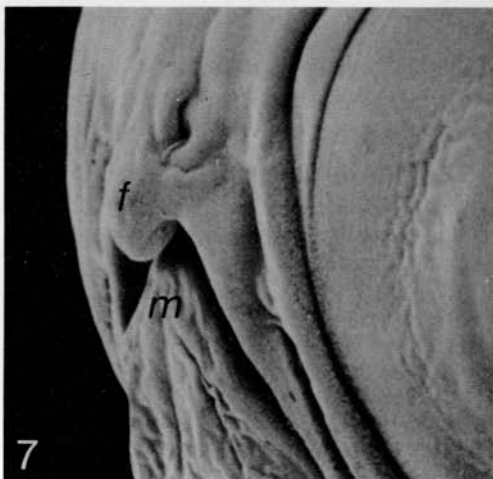
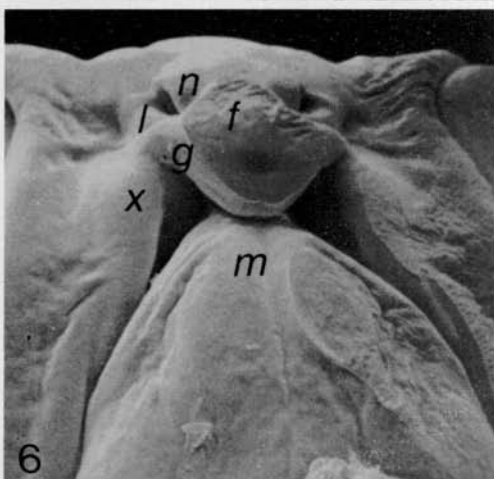
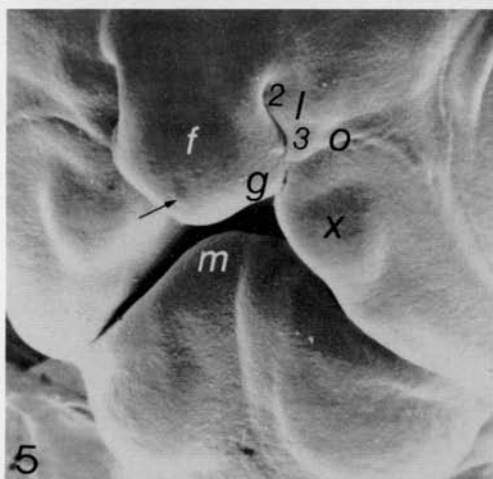
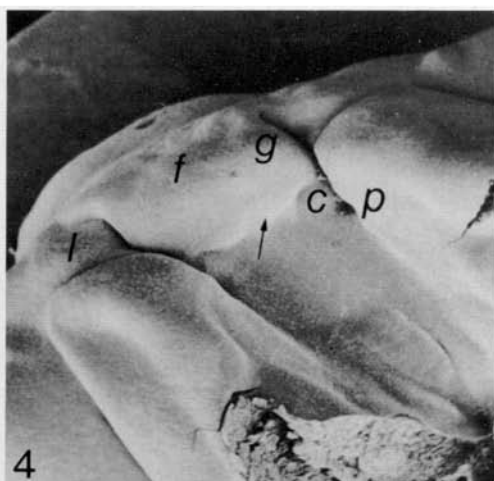
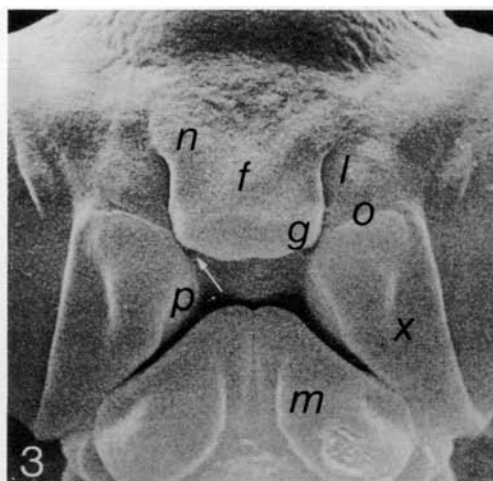
Fig. 4. Stomodeal roof, normal specimen stage 28. The inner aspect of the frontonasal mass (*f*) is indented at the centre (arrow). Primordia of the rostral conchae (*c*) are visible anteriorly between the globular processes (*g*) and the palatal processes (*p*). Note that the lateral nasal processes (*l*) do not border the stomodeal rim. PW = 3.0.

Fig. 5. Normal specimen stage 30. The medial aspect of the lateral nasal processes (*l*) is indented thereby establishing two swellings (2 and 3). The inferior part of the oronasal fissure below the nasolachrymal groove (*o*) is becoming obliterated by the fusion of the maxillary processes (*x*) with the globular processes (*g*). The egg tooth primordium (arrow) has begun to form in the centre of the frontonasal mass (*f*). The mandibular process (*m*) curves into the stomodeum. PW = 3.3.

Fig. 6. Normal specimen stage 31. The frontonasal mass (*f*) overlaps the mandibular processes (*m*) and has begun to assume a beak-like shape. The globular processes (*g*) flare laterally to meet the maxillary processes (*x*). At this point, the stomodeal antrum forms acute angles on either side of the forming upper beak. The definitive medial nasal processes (*n*) form the opercula of the nostrils. The bilobed lateral nasal processes (*l*) form the nostril's lateral borders. PW = 2.5

Fig. 7. Left lateral view of normal specimen stage 31. The centre of the upper beak (*f*) has begun to extend outward but the overall profile of the face is still rather flat. Note that the lower beak (*m*) curves into the stomodeum and its tip is covered by the upper beak. Contributions of the various processes to the developing face are easily discernible. PW = 3.3

Fig. 8. Stomodeal roof, normal specimen stage 31. The inner aspect of the frontonasal mass (*f*) is fan shaped with its bilobed apex (arrow) between the rostral ends of the paired palatal processes (*p*). The 'lip ridge' tomium (*t*) is separated from the facial aspect of the beak by a shallow groove. The conchal primordia are obscured by the palatal shelves. PW = 1.8



beads into tissue culture medium (Eichele *et al.* 1984), that the effective concentration range of retinoic acid to which the face is exposed is rising to about 10^{-7} M over 24h. Preliminary experiments show that at most a 24 h exposure is sufficient to bring about the facial deformity.

Normal development of the upper beak

To be able to interpret the upper beak defects, one needs to understand how the normal beak develops. The early stages in face development have been well documented (Yee & Abbott, 1978; Will & Meller, 1981). To summarize; at the time when retinoic acid is applied (stage-20 embryos), the tissue masses, that will form the face, border the stomodeum in a square-shaped configuration. The masses of tissue that will form the upper beak and the primary palate consist of a median frontonasal mass separated from the lateral nasal processes by the invaginating nasal placodes at each top corner of the square. The lateral nasal processes have merged with the maxillary processes which form the lateral sides of the square.

By stage 28 the combined frontonasal and median nasal processes form a relatively broad, flat rectangular mass (frontonasal mass) (Fig. 3). The

Fig. 9. Right lateral view of normal specimen stage 32. The bill has definitely begun to protrude forward but the upper beak still overlaps the tip of the lower one. PW = 3.6

Fig. 10. Right lateral view of normal specimen stage 33. The bill has greatly extended and the beaks no longer overlap. The anatomical regions of the mature upper beak may be identified with respect to their developmental primordia: the tomium (*t*) is formed from globular process; culmen (*u*), egg-tooth (arrow) and tip arise from the centre of the frontonasal mass; the nostril operculum is formed from medial nasal process (*n*); the midlateral portion of the beak between the tomium and nostril is formed by the lateral nasal process (*l*). The maxillary process (*x*) forms the upper jaw posterior to the post-tomium groove (arrowhead). PW = 3.3

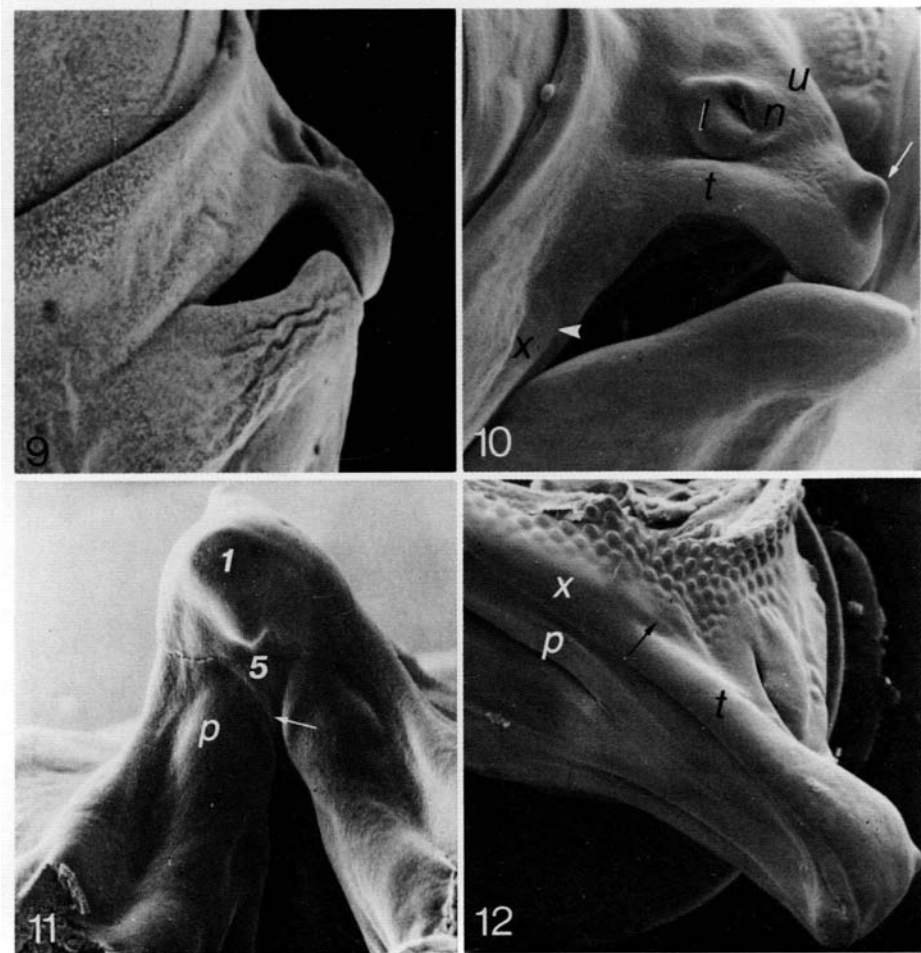
Fig. 11. Palate, normal specimen stage 33. The primary palate forms the rostral portion of the beak and extends caudally in the midline as a wedge-shaped mass inclining towards the stomodeal roof between the rostral ends of the paired shelves of the secondary palate (*p*). The palatal shelves are markedly convex and bulge towards each other at the caudal apex of the primary palate (arrow). The wide rostral part of the primary palate (*l*) is homologous to the tubercle labelled *l* in abnormal specimens while the caudal wedge-shaped part (*s*) is homologous to the tubercle labelled *5* in abnormal specimens. PW = 2.8

Fig. 12. Upper beak of normal specimen stage 35. The tomium (*t*) is separated from the palatal surface by a groove and extends caudally to a depression (arrow) at the margin of the ramphotheca where it meets the maxillary portion (*x*) of the upper jaw. The tip of the beak has enlarged and curves downwards. The choanal slit separates the palatal shelves (*p*) posteriorly. PW = 7.9

globular processes of the frontonasal mass are closely apposed to, and partially fused with the corners of the maxillary processes. The medial nasal processes bulge slightly at the rims of the forming nostrils. The lateral nasal processes fill the triangular area bordered by the oronasal fissures and the nasolacrimal grooves.

Inside the stomodeum (Fig. 4), the primordia of the secondary palate (palatal processes) have begun to form as medial enlargements of the maxillary processes. The primordium of the primary palate appears as a slightly bilobed extension of the frontonasal mass. Two small tubercles at the corners of the stomodeal roof are the primordia of the rostral conchae.

At stage 30 (Fig. 5), the midsaggital part of the frontonasal mass is elevated and the egg-tooth rudiment is visible. The frontonasal mass protrudes into the stomodeal antrum towards the lower beak. The edges of the lateral nasal



processes bordering the developing nostril slits have a sigmoid outline thus establishing two swellings. The lower parts of the oronasal fissures are almost obliterated by fusion of the globular processes with the maxillary processes.

By stage 31 (Fig. 6), the frontonasal mass extends in the midline over the oral antrum and tip of the lower beak. The globular processes are now quite large and flare laterally between the maxillary processes and the inferior borders of the lateral nasal processes. At this stage, it is quite clear that the main protruding part of the upper beak – that region eventually encased by the keratinized epidermis, the ramphotheca – forms primarily from components originating in the frontonasal mass. In profile view (Fig. 7), the upper beak can be seen to be just beginning to protrude from the face surface while the lower beak is depressed into the stomodeal space. The palatal processes (Fig. 8) are well developed, forming cantilevered shelves that separate the nasal from the oral parts of the stomodeum laterally. The shape of the primary palate is fan-like. The rostral part of the primary palate is demarcated from the facial aspect of the developing beak by a shallow groove which is the border of the 'lip ridge' – the tomium. At stage 32 (Fig. 9) the entire bill begins to protrude rostrally although the tip of the upper beak still overlaps the lower one.

At stage 33 (Fig. 10), the upper beak no longer overlaps the lower one and the entire bill extends well forward from the face. Contributions of the various embryonic primordia to the upper beak are easily recognized: the globular processes form the tomium – the lateral borders of the beak, while the midsagittal part of the frontonasal mass forms the beak tip, the egg tooth and the central edge – the culmen. The opercula of the nostrils correspond to the medial nasal processes while the triangular areas between the nostrils and the tomium are formed by the lateral nasal processes.

The palate (Fig. 11) is now further developed. The primary palate forms the rostral portion of the beak. The shelves of the secondary palate now appear as enlarged ridges which bulge into the stomodeum, especially at their rostral ends where they come very close together at the mid line.

Fig. 12 shows the essentially completed upper beak at stage 35. The rostral-medial margins of the shelves of the secondary palate have fused while caudally the shelves remain unjoined to form the choanal slit which connects the oral and nasal chambers (This arrangement is typical of galliform birds). The intra-oral aspect of the tomium is delineated by a sharp groove. It should be noted that the maxillary processes form the border of the upper jaw caudal to the ramphotheca as well as the shelves of the secondary palate.

*Abnormal development of the upper beak in experimental specimens
(Figs. 13–20)*

Since nearly every embryo in which a bead soaked in 10 mg/ml retinoic acid has been implanted develops with an upper beak deformity, we can follow the

way in which such deformities arise by examining embryos at a sequence of stages following bead implantation. In this section we describe the morphogenesis of the face in treated embryos.

At stage 28 (Fig. 13), the maxillary processes and the globular processes have failed to approximate to each other. Hence the oronasal fissures are wide: the lateral nasal processes form the upper boundary and their medial margins are slightly indented.

At stage 29 (Fig. 14), the oronasal fissures are much wider and the lateral nasal processes fill much of the space between the frontonasal mass and the maxillary processes. At this stage, the indentations of the margins of the lateral nasal processes are very marked, thus dividing them into two tubercles (labelled 2 and 3 in Fig. 14). The medial ones (2) are the smaller ones and they slope towards the frontonasal mass (1) but are separated from it by slits which are homologous to the nostrils in normal embryos. The lateral tubercles (3) of the lateral nasal processes extend downwards almost to the margin of the maxillary processes (4) but are separated from them by well-developed depressions. Inside the stomodeum, the palatal shelves have a fairly normal relationship with the maxillary processes.

An asymmetrical specimen was deliberately chosen to illustrate abnormal stage 30 (Figs. 15–16) because the homologies to normal primordia are more easily appreciated in this embryo. Here, the frontonasal mass slopes towards the left side of the embryo and the left oronasal fissure is extremely deep. Tubercles of the left lateral nasal process (2 and 3) and the medial part of the maxillary process (4) form the lateral side of the fissure. The left medial nasal process is a small extension of the frontonasal mass lying above the nostril slit. On the right side of the embryo, the oronasal fissure is relatively shallow and corresponds to the wide space lying between the maxillary process (4) and the right edge of the frontonasal mass. Thus the tubercles of the right lateral nasal process actually border the rim of the stomodeal antrum between the maxillary process and the globular part of the frontonasal mass. The right side shows the morphology most commonly found in the experimental embryos and demonstrates why the nostrils are absent in the abnormal specimens. Fig. 16 shows this specimen in left profile view to emphasize the retarded growth of the frontonasal mass, i.e. it curves into the stomodeum and does not overlap the lower beak.

The facial aspect of stage 31 (Fig. 17) shows the more common, bilaterally symmetrical pattern of abnormal beak development. The upper rim of the stomodeal antrum is formed by seven tubercles: the frontonasal mass with its globular processes projecting laterally to the vestigial nostril slits, the lateral nasal processes in the form of double tubercles and lastly the maxillary processes extending to the corners of the stomodeum on either side. The intrastomodeal aspect of this specimen is shown in Fig. 18. Here the caudal part of the frontonasal mass is seen to form a separate, unpaired tubercle (5)

which lies between a pair of smaller tubercles (6) arising from the anterior part of the stomodeal vault. The latter are homologous to the primordia of the rostral conchae. Laterally, the palatal shelves (7) extend part of the way into the stomodeum but their medial edges remain widely separated.

From the foregoing account, it is seen that the nasomaxillary complex in experimental embryos, undergoes arrested growth and distorted morphogenesis wherein the upper 'beak' is formed by twelve tubercles homologous to normal primordia, i.e. two unpaired tubercles (1 and 5) which form from the frontonasal mass and five sets of paired tubercles representing the lateral nasal processes (2 and 3), the maxillary processes (4), the palatal processes (7) and

Fig. 13. Experimental specimen stage 28: the oronasal fissures (arrow) are quite wide and bounded by the globular process (g) medially, the bilobed lateral nasal process (l) superiorly, and the maxillary process (x) laterally. The bulge lying between the central part of the frontonasal mass (f) and the lateral nasal processes is the definitive medial nasal process. Compare with normal specimen at this stage, Fig. 3. PW = 2.5

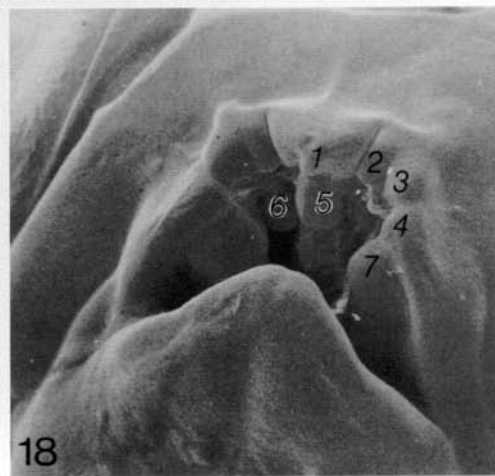
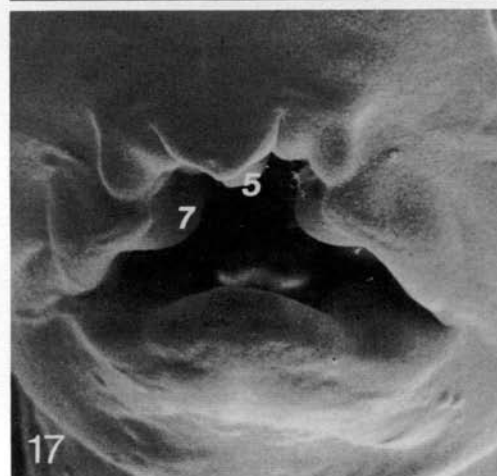
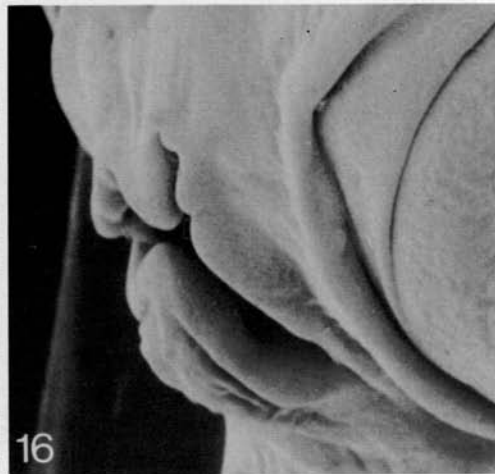
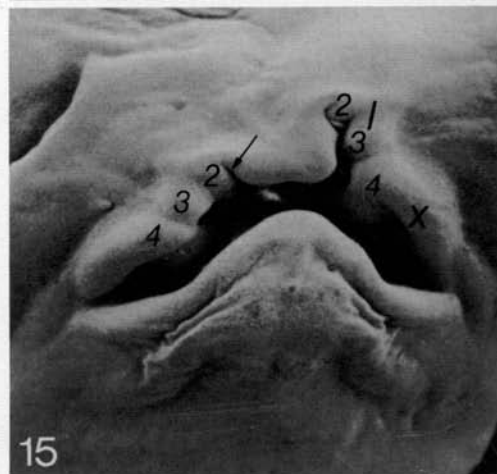
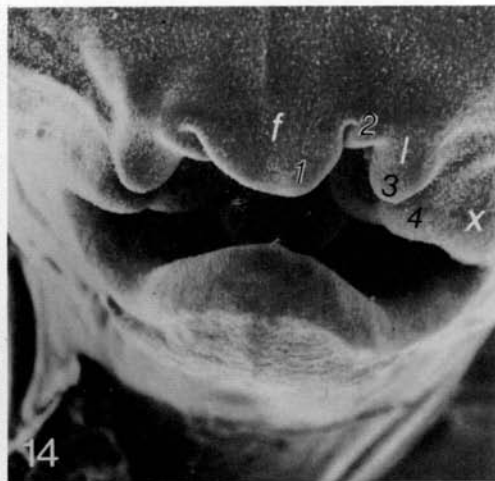
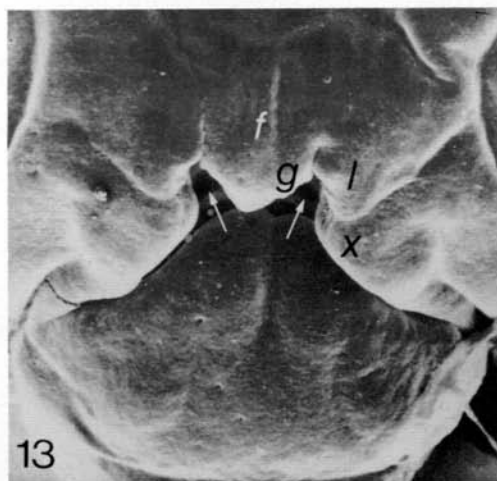
Fig. 14. Experimental specimen stage 29. The bilobed character of the lateral nasal processes (l) is accentuated so as to form two distinct tubercles (2 and 3). The frontonasal mass (f) forms a single large tubercle (1) in the centre while the maxillary processes (x) protrude (4) slightly below the tubercles labelled 3. PW = 2.5

Fig. 15. Experimental specimen stage 30. By comparing the left and right sides of this asymmetrical specimen the homologies of the various tubercles are clarified. The left oronasal fissure is a deep cleft and tubercles 2 and 3 are clearly seen as belonging to the lateral nasal process (l). Tubercle 4 is the medial part of the maxillary process (x). On the right side, the tubercles of the lateral nasal process have advanced towards the stomodeal rim so that only the nostril slit (arrow) separates 2 from the frontonasal mass. PW = 3.6

Fig. 16. Left lateral view of specimen shown in Fig. 15, stage 30. The retarded forward and downward growth of the frontonasal mass is clearly demonstrated. Failure of the upper beak to overlap the lower one has allowed the lower beak to extend forward of the face as compared to normal embryos of the same stage (compare with Fig. 7). PW = 3.3

Fig. 17. Experimental specimen stage 31. Components of the malformed upper beak are arranged in array typical for advanced experimental specimens. Some of the intraoral tubercles (5 and 7) are also seen in this projection: compare with Fig. 18. PW = 2.6

Fig. 18. Intrastomodeal view of specimen shown in Fig. 17, stage 31. The twelve basic tubercles which characterize the upper 'beak' in experimental specimens are visible. Tubercle 1 originates from the frontonasal mass and may show different degrees of contribution from the globular and medial nasal components. Tubercle 5 originates from the palatal portion of the frontonasal mass. Tubercles 2 and 3 arise from the lateral nasal process. Tubercle 4 arises from the maxillary process as does 7, i.e., from the palatal process. Tubercle 6 originates from the primordium of the rostral concha (see Fig. 4). PW = 3.1



the primordia of the rostral conchae (6). Figs 19 and 20 (stage 35) show some of the variations that these elements can assume in different specimens. However, the basic number and arrangement of tubercles in all the observed abnormal embryos followed essentially the same pattern.

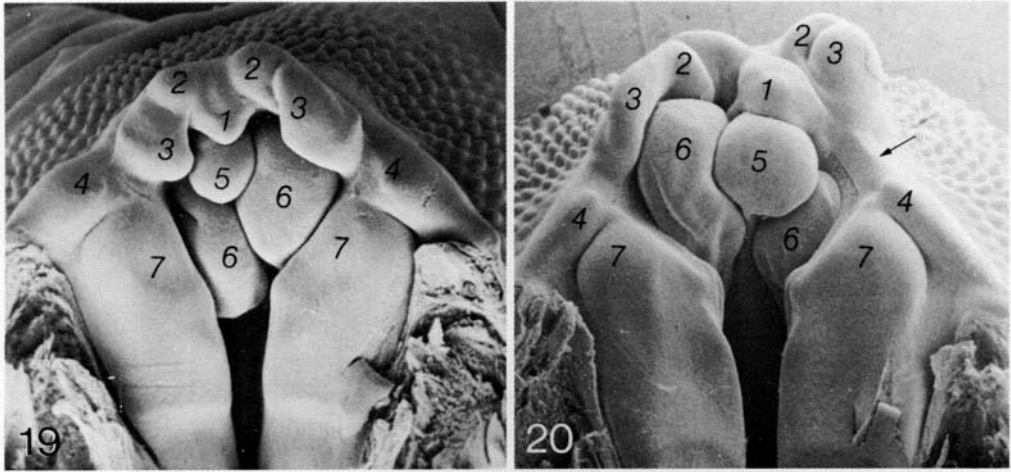


Fig. 19. Experimental specimen stage 35. This intrastomodeal projection demonstrates a rather typical arrangement of tubercles in advanced experimental specimens. Refer to legend for Fig. 18. PW = 4.0

Fig. 20. Experimental specimen stage 35. This intrastomodeal projection shows one type of variation from the typical arrangement of tubercles. Tubercles of the left lateral nasal process (2 and 3) assume a more medial and rostral position leaving a wide space (arrow) next to the maxillary tubercle (4). The frontonasal tubercle (1) is displaced caudally and its enlarged right side is displaced laterally over the medial tubercle (2) of the right lateral nasal process. The palatal tubercle (5) of the frontonasal mass inclines toward the left conchal tubercle (6) and the right conchal tubercle is enlarged and elongated. PW = 4.0

DISCUSSION

Implanting beads that have been soaked in high concentrations of retinoic acid to anterior positions in wing buds leads to abnormal morphogenesis of the face. Typically the upper beak is completely missing and the front of the face forms a scalloped rim of tissue above the mouth. No nostrils are formed. In contrast, the lower beak appears to be essentially normal.

Our analysis of the morphogenesis of normal beaks and the beaks of experimental embryos shows that the scalloped border of the mouth is derived from the tissue masses that would normally fuse to form the upper beak. Thus, seven bulges can be distinguished at the upper margin of the mouth: these are a central tubercle derived from the frontonasal mass, flanked by two tubercles on either side derived from the lateral nasal processes, which in turn are flanked laterally, at the corners of the mouth, by tubercles derived from the

maxillary processes. The indentations, that represent the sites where the nostril slits would have formed, lie between the central mass and the tubercles immediately flanking it, i.e. tubercles derived from the upper part of the lateral nasal process. Initially, it was difficult to visualize the derivation of the pair of tubercles lying on either side of the frontonasal mass, but their origin can be understood by reference to the normal development of the lateral nasal process. Normally, this tissue mass borders the lateral edge of the developing nostril and, soon after obliteration of the oronasal fissure, assumes a sigmoid shape with two swellings, one below the other, along the rim of the nostril. In the abnormal embryos these two swellings give rise to the pair of tubercles on either side of the frontonasal mass. They occupy the space created by the lack of fusion across the oronasal fissures, and their position is radically modified since they now come to lie side by side at the upper rim of the mouth.

Associated with the failure of formation of nostrils and upper beak, the development of the palate of treated embryos is also abnormal. The roof of the abnormal oral cavity has five tissue masses – a central bulge and two lateral bulges, flanked by the flat palatal shelves. By reference to the stages of normal development, the central bulge of tissue is derived from the frontonasal mass. In normal embryos, this bulge becomes the part of the primary palate which forms much of the intraoral surface of the beak. The two inner lateral bulges at the roof of the mouth of abnormal embryos are derived from the primordia of the rostral conchæ but they are now distorted and lie level with the palatal surface. The secondary palate in the treated embryos is represented by the maxillary palatal shelves which project medially and are separated by a wide space along their length. In normal embryos, these shelves meet each other in the midline behind the primary palate and extend caudally for a short distance as a united surface until interrupted by the choanal slit. This is the, so-called, 'schitzognathic' condition characteristic of galliform birds. (In anseriforms, the shelves of the secondary palate are essentially fused along their entire length) (Bellairs & Jenkin, 1960)).

From the foregoing, the primary facial defect in abnormal embryos can be classified as severe bilateral clefting of the primary palate. The accompanying complete separation of the secondary palate appears to be the consequence of the abnormal development of the primary palate.

Clefting of the primary palate could, in principle, arise because of defective morphogenesis at a number of different steps during development of the face (Trasler & Fraser, 1977). Thus, for example, it has been suggested that clefting could result from failure of the fusion process itself – involving adhesion of the facial primordia and dissolution of the adjacent epithelia – or from only a weak seam being established with subsequent rupture (Poswillo, 1974). However, the severe clefting of the primary palate in our experiments does not result from interference with this step in face development. In our embryos, the origin of the beak defect can be traced back to the failure of the frontonasal

mass to approximate to the maxillary processes. The gap between the facial processes appears to arise because the frontonasal mass does not enlarge whereas the maxillary processes appear to be less affected. Indeed, this effect is permanent and results in the later inhibited extension of the beak.

Failure of the facial primordia to enlarge has been recognized as a possible cause of cleft palate (Trasler & Fraser, 1977) and has been ascribed to a reduction in the number of cells within the developing facial processes. Since cells that populate the facial processes are derived from cranial neural crest, it has been suggested that interference with cell migration could lead to fewer cells reaching these primordia. This hypothesis is consistent with the effects of retinoic acid on the facial development of mammals. Thus rat embryos treated at the time of neural crest emigration have reduced cell numbers in the facial primordia and go on to develop cleft palate (Morris, 1973). Embryonic chicks treated with the Vitamin A derivative, retinol, during neural crest cell emigration into the face have a reduced number of cells in the face primordia (Hassell, Greenberg & Johnston, 1977; Keith, 1977). Tissue culture tests of the effect of retinol on the motility of quail cranial neural crest cells show that cell motility is lessened because their adhesiveness to the substratum is greatly reduced (Thorogood *et al.*, 1982). However, the face primordia are already populated by crest cells at the time of treatment. Indeed, in the experiments in which chicks were cultured in the presence of retinol (Hassell *et al.*, 1977), the end point for assessing the effect is the stage at which we begin the application of retinoic acid.

In our experiments, retinoic acid appears to act on cells that have already migrated into the face primordia, by killing them and/or affecting their proliferation. This would diminish the number of cells and inhibit the enlargement of the frontonasal mass. This interpretation is consistent with experiments in which chick embryos treated with a vitamin A analog, 13 *cis* retinoic acid, that is known in other systems to be less cytotoxic (reviewed, Pawson, Ehmann, Itri & Sherman, 1982) did not have facial abnormalities (our unpublished observations).

There are other examples of experimental production of cleft palate in which the primary effect appears to be on cells that have already migrated into the facial primordia. Administration of the drug 'hadacidin' to pregnant hamsters causes the embryos to develop severe clefts of the primary palate (Shah, 1977). In these experiments, the greatest production of defects was noted when treatment occurred at an analogous stage of development to that of the chick embryos in our experiments. Furthermore in another example, in which tunicamycin was locally applied to the developing primary palate of cultured rat embryos (Eto, Figueroa, Tamura & Pratt, 1981), enlargement of the frontonasal mass is specifically affected and this could result from inhibition of the proliferation of its constituent cells.

It should be noted that the effect of retinoic acid on the growth of the

frontonasal process in our embryos is permanent. Another factor, in addition to the proposed diminution of cell number by killing and/or reduction in proliferation, may be involved. This concerns the contribution of mesenchyme to the frontonasal mass from the maxillary processes as a part of the normal fusion sequence, i.e. after the epithelial partitions are perforated, mesenchyme from the maxillary processes is thought to migrate medially (Patten, 1961).

Thus the failure of the upper facial primordia to fuse could be an important factor leading to abnormal morphogenesis of the frontonasal mass. Such an explanation could also shed light on why the lower beak in these treated embryos, in contrast to the upper beak, appears to be unaffected. Morphogenesis of the lower beak does not involve the contribution of cells from adjacent primordia since the paired mandibular processes are essentially in continuity throughout development. Recent experiments on the developmental potential of the frontonasal mass, however, show that small fragments can undergo morphogenesis in isolation (Wedden: work in progress). Thus the lack of contribution of cells from adjacent primordia does not appear to be relevant to the abnormal morphogenesis of the frontonasal mass. Furthermore, this leaves the puzzling question of the apparent immunity of the lower beak still open.

It is of interest to compare our analysis of the morphogenesis of the face of retinoic acid treated embryos with that of the mutant *cyp*. The origin of the defect in this mutant (as in our study), can be traced back to the failure of the frontonasal mass to enlarge (Yee & Abbott, 1978). However it is not clear whether the basis of abnormal morphogenesis in the mutant involves defective migration of neural crest cells into the face or whether there is a later effect on the proliferation of cells in the established primordia. A further interesting feature of the *cyp* embryos relevant to experiments with retinoic acid, is that the mutation affects the limbs as well as the face. Indeed the effect of retinoic acid on facial morphogenesis reported here, emerged from a series of experiments in which we were primarily interested in the effects on pattern formation in developing wing buds. At the concentrations of retinoic acid that result in abnormal facial morphogenesis, the treated wings are truncated or have incomplete sets of symmetrical digits. In the *cyp* mutant, the legs only are defective and have reduced numbers of digits. This mutant is thus reminiscent of embryos treated with the highest concentrations of retinoic acid. At the lower retinoic acid concentrations effective in producing severe clefting of the primary palate, the associated limbs have symmetrical digit patterns. In this context, it is of interest that mutant chickens, such as *diplopodia*, that have additional digits also have short upper beaks (reviewed, Romanoff, 1972).

An association between limb and facial defects following experimental treatments of embryos has been noted for other chemicals in addition to retinoic acid. Thus, in the example of hadacidin treatment of hamster embryos already mentioned (Shah, 1977), the morphogenesis of the limbs as well as that

of the face was affected (the limb defects ranged from amelia to polydactyly but were not described in any detail). In another example, the peak of incidence of limb defects following treatment with pilocarpine coincides with that for facial defects in chicken embryos (Landauer, 1954), although in this case the limbs were truncated. One can speculate that the cellular mechanisms by which these teratogens bring about defects are the same for both the limbs and the upper beaks. However, at present an explanation in these terms is not possible because the mechanisms involved in the development of these two structures are incompletely understood. Nevertheless, the view that beak and limb defects result from interference with the same cellular mechanisms can provide new insights. For example, the limb truncations produced by high concentrations of retinoic acid could be interpreted as resulting from damage to the apical ectodermal ridge that is necessary for limb outgrowth (reviewed, Wolpert, 1976). One could then speculate that outgrowth of the upper beak may similarly require a special region of ectoderm, and failure of beak development results from damage to this epithelium.

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