MANDIBLE GROWTH IN PALATE CLOSURE IN MICE

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ABSTRACT

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It has been postulated that a small mandible causes cleft palate because the conditions are frequently associated in humans and mice. Vitamin A-induced cleft palate in mice is accompanied by micrognathia. Therefore, the effect of excess vitamin A, cortisone and 6-aminonicotinamide on growth of the mandible during palate closure and their relations to production of cleft palate were investigated in the A/J (susceptible) and C57BL (cortisone and 6-aminonicotinamide resistant) inbred strains of mice. Body weight, mandible length and mandible growth rate in A/J and C57BL control embryos were identical during palate closure. The strains differed when mandible lengths were adjusted for chronological age and morphological rating but were similar when they were adjusted for palate stages and body weight. The mandibles were not shortened during palate closure by vitamin A or the other treatments. Cortisone-treated mandibles adjusted for chronological age were longer than controls. Forward tongue movement started earlier in C57BL than in A/J. 6-aminonicotinamide inhibited the displacement of the tongue in A/J and not C57BL; vitamin A inhibited C57BL tongues. Therefore differences in tongue displacement during palate closure may contribute to the strain difference in susceptibility to drug-induced cleft palate.

MANDIBLE GROWTH DURING PALATE CLOSURE IN NORMAL AND INDUCED CLEFT PALATE IN MICE

BY

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I INTRODUCTION

With recent advances in treatment of many acute and chronic diseases, the disorders of genetic origin have assumed increasing importance in Pediatrics. Among hereditary disorders, congenital malformations are by far the most frequent, but the least understood. Studies of these structural anomalies provide a better understanding of the normal developmental mechanisms and will also lead to preventive measures for these disorders.

There are two approaches to the study of congenital malformation:

(a) the clinical approach, involving accurate diagnosis of specific syndromes, analysis of pedigrees, and the search for a specific causative defect; and (b) the experimental induction of malformations using animal models to detect genetic and environmental factors and their interaction. Studies of the genetics of human malformation have shown that some malformations are determined by gene mutation or chromosomal anomalies. Other malformations are caused mainly by environmental teratogens.. The majority of common malformations are produced by complicated interactions between polygenic predisposition and a variety of ill-defined environmental factors.

Cleft lip and cleft palate are the most representative examples of a large heterogeneous group of developmental malformations. Many of these malformations have been known since

ancient times and today many can be reproduced experimentally either by the administration of teratogens, or by mechanical means. Like many other common congenital malformations, they probably result from a failure of a developmental process to meet a certain critical threshold (Fraser, 1969; Fraser, 1971). These traits are classified as being due to quasi-continuous variation (Gruneberg, 1952). Thus in normal secondary palate closure, the shelves of the palate must change their orientation from vertical to horizontal, and move from beside to above the tongue in order to meet each other in midline. This fusion is the critical threshold that must be achieved for normality. Treatment with the right dose of cortisone at the appropriate gestational stage causes cleft palate in 100% of A/J embryos, but only in 18% of C57BL/6 embryos (Kalter, 1954). The strain difference in cortisone-induced cleft palate frequency is considered to be dependent, among other factors, on a difference in the normal time of shelf movement. The later shelf movement begins, the closer to the critical threshold and the more susceptible is the embryo to cortisone-induced cleft palate (Trasler, 1965). Thus among the various proposed factors involved in palatal closure it was suggested that the intrinsic shelf force is the most likely to be affected by cortisone treatment.

Little work has been done concerning the role of the mandible in production of cleft palate, although there are plenty of human examples in which micrognathia is associated with

cleft palate (see below). The tongue is also known to play a role in aiding shelf closure in the rabbit in conjunction with extension of the neck (Walker, 1971a). Mouth opening reflexes in response to stimulation in human embryos is reported to be a significant factor in tongue withdrawal from between the vertical palatine shelves due to traction resulting from mandibular depression (Humphrey, 1969). In the human fetus, a relative "overgrowth" in the mandible at the onset of palate closure may be a contributing factor to palate closure. This profile reversal and re-orientation of the palatal shelves occurs earlier in the male than the female, and the embryological sexual dimorphism has been suggested as an explanation for the greater incidence and severity of palatal clefting in females (Burdi and Silvey, 1969).

The present study is designed to answer the following questions: (1) Is there a strain difference in lower jaw growth during palate closure which can account for the difference in susceptibility of C57BL/6 and A/J mice to cleft production? (2) Do any of the three teratogens under consideration (cortisone, 6-aminonicotinamide and excess vitamin A) induce cleft palate by affecting mandible growth? (3) Is there a relationship between tongue and lower jaw growth?

II LITERATURE REVIEW

A. Micrognathia associated with cleft palate in humans.

Mandibular deformities are found associated with many congenital malformation syndromes in humans. A small or hypoplastic mandible (micrognathia) is quite common among newborn babies. The frequency of a mild form of micrognathia was found to be 3.2 per 1000 in newborns while the severe form of micrognathia was found with an incidence of 0.2 to 1.1 per 1000 (Marden, Smith and McDonald, 1964).

Micrognathia is often associated with the following syndromes: Treacher Collins, Hallermann-Streiff (Steel and Bars, 1970), Bloom's, Silver's, Seckel's, Progeria (Smith, 1970), Cornelia de Lange, (Pashayan et al., 1969), idiopathic infantile hypercalcemia (Rubin, 1967), and renal agenesis (Potter, 1965). This anomaly is frequently found associated with cleft palate in first arch syndrome (McKenzie, 1958), trisomy 13, trisomy 18, Cri-du-Chat syndrome, No. 4 short arm deletion, No. 21 long arm deletion, Smith-Lemli-Opitz syndrome, Goldenhar's syndrome, and aminopterin-induced syndrome (Smith, 1970).

The first recorded description of cleft palate with micrognathia to appear in the medical literature was by Fairbairn (1846). Following this Lannelongue and Menard (1891) and Shukowsky (1911) also described these associated defects in humans. In 1923 Pierre Robin described a series of children

with adenoid faces without adenoid enlargement, glossoptosis, dyspnea, and micrognathia. Although Robin did not describe cleft palate as the main feature of the syndrome, he deserves credit for calling attention to this particular syndrome and for pointing out some of the grave dangers that may accompany it (Robin, 1934). Today the Pierre Robin syndrome refers to a combination of micrognathia, glossoptosis and cleft palate in the newborn infant. The exact cause of this condition is not known and in all likelihood there is no single etiology. The possibility of intrauterine growth retardation of the mandible before 9 weeks of gestation (Lenstrup, 1925) and of simple arrest of development involving the first branchial arch and possible hereditary factors (Eley and Farber, 1930) had been proposed as the cause of this syndrome. Robin stated, however, that mandible hypotrophy is never idiopathic and as a rule is caused by tuberculosis or congenital syphilis, by a hereditary dystrophia from alcoholism or by some other infection (Robin, 1934). Hypoplasia of the mandible due to pressure exerted by the fetal body on the vertex was suggested by Davis and Dunn (1933). A review of 39 cases of Pierre Robin syndrome by Smith and Stowe in 1961 revealed that in one fourth of the 39 cases there was a history of intrauterine infection in early pregnancy. A positive family history was noticed in two instances (Smith and Stowe, 1961). Their

investigation of associated defects revealed that an ocular lesion was involved in 13 cases and limb defects in 8 cases. In the survey reported by Conway and Wagner (1966) covering 467 cases, 27% of the total examined had additional malformations. An X-linked recessive family history having affected individuals with cleft palate, micrognathia, club foot and congenital heart defects has been reported (Gorlin et al., 1970). A clinically identical syndrome has also been described in siblings but the mode of inheritance is thought to be due to dominant genes with variable penetrance (McKusick, 1968; Singh et al., 1970). Micrognathia is generally considered to be the cause of cleft palate in this syndrome. Because of the short mandible there is a failure to maintain the tongue in a forward position and the closure of the posterior shelves is disturbed due to the immobility of and obstruction by the tongue. Infants with bilateral renal agenesis possess a peculiar face with a receding chin (Potter, 1965). Relative lack of amniotic fluid due to a kidney anomaly usually leads to fetal compression, micrognathia and glossoptosis. Three babies with cleft palate and harelip were noticed among the 50 cases reported by Potter (1965). In Klippel-Feil syndrome (autosomal dominant inheritance) there is fusion of the cervical vertebrae resulting in a short neck and limited movement of the head (Sommerfeld and Schweiger, 1969). The whole head seems to sit directly on

the thorax and there is a disturbance in tongue movement. A cleft palate or a high palatal vault is a quite consistent finding (Gorlin and Pindborg, 1964).

The folic acid antagonist, 4-aminopteroylglutamic acid (aminopterin) and its methyl derivative (methotrexate) are both known to induce abnormal morphogenesis of the fetus when taken by mothers in the first trimester of pregnancy. Micrognathia and cleft palate were noted in three out of four of these babies (Thiersch, 1952; Warkany, Beaudry and Hornstein, 1959; Milunsky, Graef and Gaynor, 1968).

B. Pathogenesis of cortisone-induced cleft palate.

Maternal treatment with cortisone during pregnancy induces a higher frequency of cleft palate in the A/Jax strain than in the C57BL/6 strain of mice (Fraser and Fainstat, 1951). The steroid hormone was found to produce consistent teratogenic effects in appropriate species. Since then, the glucocorticoids have been used as convenient teratogens in the study of normal and pathological palate closure in various species of animals.

There is some evidence that adrenal steroid therapy during pregnancy may cause cleft palate and other fetal abnormalities in man. In a survey of world literature up to 1960, 4 out of 260 mothers receiving glucocorticoid during early pregnancy produced babies with cleft palate (Bongiovanni and McPadden, 1960). These workers concluded that the human fetus is rarely injured by maternal treatment with corticoid. Other

investigators also felt that there was no evidence of teratogenicity related to steroid therapy (Popert, 1962; Kenny et al., 1966; Rolf, 1966; Yackel et al., 1966), nevertheless the opinion that there is a high incidence of fetal wastage and prematurity has persisted (Werrell and Taylor, 1968; MacMillan, 1970; McGee and Makowski, 1970; Smithells and Morgan, 1970). One report does exist in the literature of an infant who was born to a mother receiving high doses of corticosterone for sarcoidosis in which the infant died shortly after birth of adrenal failure secondary to adrenal atrophy (Oppenheimer, 1964). Considering the incidence of cleft palate in the general population and the incidence in a cortisone treated population, Fraser felt there is a fairly low risk of harming the fetus yet there may very well be a risk of inducing a cleft in the babies of mothers treated with glucocorticoid during pregnancy (Fraser, 1962).

How does cortisone induce abnormalities? It appears most likely that independent adrenal function and homeostasis exist both in the mother and the fetus. Maternal cortisol is known to cross the placenta and is found in the cord blood. The ratio of the steroid between the mother and the fetus is from 2: 1 to 5: 1 (Migeon et al., 1957). A specific globulin-transcortin exists in serum which is known to bind cortisol. The capacity of maternal serum to bind cortisol is greater

than that of the fetus and this capacity is known to increase during pregnancy. This greater capacity may favour the higher concentration of hormone in maternal blood and limit cortisol transfer across the placenta (Sandberg and Slaunwhite, 1959). It is also possible that a small increase in total maternal cortisol could result in proportionally higher unbound cortisol in the fetus. In mice, hydrocortisone when injected directly into the amniotic sac, is known to induce cleft palate (Dostal, 1970). Thus, it is likely that corticosteroids are affecting the embryos directly. The effect of cortisone varies in different species; this steroid induces cleft palate in mice and rabbits but not in rats. (Nanda, 1970a; Walker, 1971b). Investigation of the genetics of strain difference in mice in response to the effect of cortisone has disclosed that the frequency of cleft palate depends on fetal genotype, maternal genotype and environmental factors such as dose of cortisone injected, maternal weight and parity (Kalter and Fraser, 1953; Kalter, 1954; Kalter, 1956; Kalter, 1957). The difference in susceptibility to cleft palate production between the two strains depends on the difference in normal developmental patterns in embryos of the two strains. During embryogenesis, the palatine shelves, which initially hang vertically on either side of the tongue, move into a horizontal plane extending across the tongue dorsally, where they meet in the midline and fuse (Walker and Fraser, 1956). The C57BL palates begin to close 10 to 12 hours

earlier than the A/J palate. In the FI hybrids from reciprocal crosses, the C57BL X A/J embryo palates close earlier and are less susceptible to cortisone induced cleft palate than those from the A/J x C57BL embryos (Trasler and Fraser, 1958). Cortisone interferes with the process underlying movement of the shelves to the horizontal (Walker and Fraser, 1957). Thus it was clearly shown that the embryos in which palate closure normally occurs earlier have the lower frequency of cleft palate following cortisone treatment. Closure of the secondary palate is therefore regarded as a threshold character and many factors in the fetal and maternal genotypes and environments interact with each other and contribute to either the success or failure of palate closure. The latest time in development at which the shelves become horizontal and still fuse can be considered the threshold (Fraser, 1961). Since there are several factors which influence palate closure, cortisone could affect any one or more than one of these factors in causing the malformation. Despite these and other investigations into cortisone-induced cleft palate, its pathogenesis remains unclear. Each of the following possibilities could lead to an abnormal palate:

1) An abnormally narrow palatine shelf with normal width of the floor of the nasal cavity, as in the urogenital mutant mice (Fitch, 1957) or in palatine shelves subjected to x-irradiation (Fraser, 1969).

2) A mechanical obstruction to the shelf movement. In phocomelic mutant mice two bars of misplaced cartilage are located in, or just above, the area which is involved in movement of palatine shelves, and interfere with shelf movement (Fitch, 1957).

3) A reduced shelf force delaying shelf movement. Cortisone and other teratogens probably act on the intrinsic shelf force.

4) An abnormally wide head, which would make it more difficult for the shelves to meet.

5) Failure of epithelial fusion when the shelves have met.

6) An increase in tongue resistance. Anything that interferes with the forward and downward displacement of the tongue from between the shelves could cause cleft palate by preventing shelf movement, as in spontaneous cleft lip embryos (Trasler and Fraser, 1963).

Walker and Fraser (1957) showed that cortisone delays initiation of shelf movement considerably, and suggested that cortisone might inhibit the synthesis of ground substance in the palate. Cortisone did not affect head growth nor retard palatine shelf growth during normal palatine closure time. There is marked ^{35}S-sulphate incorporation during closure of the palate (Larsson, Boström and Carlsöö, 1959; Larsson, 1960) and a close parallel between this incorporation and histochemical staining representing

active acid mucopolysaccharide metabolism during closure (Walker, 1960). It was therefore concluded that the ground substance of the palatine shelves contained acid mucopolysaccharides. Autoradiographic study in day 14 embryos of two strains of mice differing in susceptibility to cortisone showed less ^{35}S-sulphate incorporation in both treated groups than in the control, but no difference in labelled ^{35}S-sulphate uptake between the two strains (Larsson, 1962b). Distribution of acid mucopolysaccharides in normal and cleft palates was studied using various histochemical methods in day 13 to day 17 embryos; a 12 hour time difference between control and cleft palate in staining intensity of acid mucopolysaccharide was found (Jacobs, 1964). The results suggested that some correlation exists between the concentration of acid mucopolysaccharides in the palate shelves and the competence of embryonic palatine shelves to effect closure. Loevy (1962) did not find a difference in stainability of the ground substance by histochemical methods between control and treated embryos. Based on the finding of autoradiographic study that ^{35}S-sulphate incorporation in palatal shelves of control fetuses and those from rats treated with cortisone and vitamin A was similar, Nanda (1970a) concluded that there is no relation between disturbed sulphated mucopolysaccharide metabolism and production of cleft palate. An alternative explanation regarding strain differences in the

pathogenesis of cleft palate was offered by Harris (1964), who observed a reduction in the weight of amniotic fluid associated with cortisone-treated embryos before and during the period of palate closure. The strain difference might thus be traced in part to greater reduction of amniotic fluid volume in A/J than in C57BL. Although Walker (1965) reported reduced amniotic fluid volume in cortisone-treated animals, his data included observations of some cleft embryos with larger volume of amniotic fluid than the control animals. In an experiment designed to produce 50% incidence of cortisone-induced cleft animals, the volume of amniotic fluid reduction was exactly the same in cleft animals as in their normal litter mates (Fraser et al., 1967). Thus, the role of reduction in amniotic fluid was ruled out as the causative factor in cleft palate formation.

There is also a difference in degree of flexion in the cranial base of the embryo in the region of the craniopharyngeal canal which divides the cranial base into anterior and posterior portions. The amount of this flexion was found to be much greater in rats than in mice. In mice, the angle is greater in the C57BL strain than the A/J (Harris, 1964). Reduction in amniotic fluid and a less flexed cranial base in the A/J strain at the time of palate closure compared to the C57BL embryo could account for the strain difference.

Rapid growth and lengthening of the cranial base could provide internal shelf force and play a role in palate

closure (Verrusio, 1969). Verrusio (1970) confirmed the observation of Harris that flattening of the cranial base takes place during palate closure, and proposed the hypothesis, illustrated with a mechanical model, that straightening of the cranial base could provide the intrinsic shelf force. Whether this model would account for the strain difference in intrinsic shelf force responsible for the difference in incidence of cortisone induced cleft palate remains to be shown. Other possibilities by which cortisone could reduce shelf force include mitotic inhibition (cited by Fraser, 1969), change in hydration of palatine shelves (Jacobs, 1966), and change in the level of lysosomal enzyme (cited by Fraser, 1969).

Cortisone might be responsible for the failure of cellular fusion at the edge of the two palatal shelves. The palate shelves can either fail to fuse due to a change in potentiality of the epithelium of the palatine shelves or due to a change in the state of differentiation of the underlying mesenchyme. In vitro cultures of rat-palatal process did not respond very strongly to added cortisone; palatal closure was not seriously affected (Thompson and Schweisthal, 1969).

Interference in the downward and forward displacement of the tongue due to disturbance in mandibular growth might cause cleft palate. The relation between the mandibular growth, tongue and palate closure will be discussed in this thesis in detail.

C. Cleft palate induced by 6-aminonicotinamide.

It has been stated that any drug administered at the proper dosage and at the proper stage of development to embryos of the appropriate species will be effective in causing disturbance in embryonic development (Karnofsky, 1965).

The antimetabolite 6 AN is a good illustration of the above statement. 6 AN is a structural analogue of nicotinamide which is capable of causing niacin deficiencies in the living organism (Woolley, 1963). It has been used as an antagonist of the vitamin in attempts at chemotherapy of certain viral diseases and of cancers (Shapiro, Dietrich and Shils, 1957). This drug has extreme toxic effects both in rats and rabbits involving muscular paralysis. Johnson and McColl (1956) showed that simultaneous administration of nicotinamide in mice produced an eight fold increase in the LD_{50} of 6 AN. Nicotinic acid also gave significant protection and tryptophan appeared to give some protection. This proved that the drug was a potent nicotinamide antagonist, (Johnson and McColl, 1955, 1956). Nicotinamide is found in the tissue as a constituent of the two important coenzymes, nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP). These two coenzymes are involved in many biochemical reactions in the body (Goldsmith and Miller, 1967). The toxicity produced by 6 AN is due to production of inactive NAD analogue (Kaplan et al., 1954; Johnson and McColl, 1955).

The 6AN analogue of NAD has been detected in liver and kidney tissue of mice treated with the drug (Johnson and McColl, 1956). Administration of 6AN to mice causes a marked reduction in the activities of the NAD-dependent mitochondrial enzymes, B-hydroxybutyrate and α-ketoglutarate dehydrogenase and also marked lowering of ATP and ADP and increase in AMP concentration in the rapidly growing 755 adenocarcinoma. (Dietrich et al., 1958).

6AN acts as a teratogen in the chick embryo inducing dwarfism, micromelia and parrot beak (Landauer, 1957). In rat embryos, Murphy (Murphy et al., 1957) produced different types of malformations depending on the day of gestation treated. Another study showed that the teratogenic effect of 6AN in rat embryos was prevented when a tryptophan deficient diet was supplemented with nicotinamide (Pike, 1951).

A single injection of 6AN without protection in the first week of gestation resulted in embryonic death or prevented implantation in rats. A progressive decline in deaths was noticed when treatments were given at later stages of pregnancy. (Chamberlain and Nelson, 1962; Chamberlain, 1963).

Major skeletal malformations, growth retardation, and cleft palate were observed in offspring of 6AN treated mice by Pinsky and Fraser (1959). These authors (1960) gave 6AN followed 2 hours later by nicotinamide providing a precisely timed exposure of the embryo to the inhibitory effect of 6AN. A variety of malformations were produced, depending on the day the treatment was given (Goldstein, 1964). In four

genetically different groups of mice the peak of incidence of cleft palate was day 13 1/2 (Goldstein et al., 1963). Treatment on day 13 1/2 produced a higher frequency of cleft palate in the A/J strain than in the C57BL. Strain differences were also observed in the incidence of induced vertebral fusion. A patroclinous reciprocal cross difference was found in the FI hybrids in the frequency of vertebral fusions, while a matroclinous reciprocal cross difference was observed in the frequency of induced cleft palate between the FI offspring of A/Jax and C57BL/6J inbred strains. That is, the offspring of A/J females mated to C57BL males and treated on day 13 1/2 of gestation had more cleft palate than those of C57BL/6J females mated to A/J males. The cross difference persisted when the two types of FI offspring were backcrossed to tha A/J strain. The frequency of 6 AN induced cleft palate was higher in the offspring of AC (A/JxC57BL) females than of CA (C57BL/6xA/J) females. This suggests that embryonic genotype, the maternal genotype, or cytoplasmic factor may play an important role in cleft palate production induced by 6 AN. Another complexity was added to the analysis of this interaction by the discovery that the cytoplasmic strain difference was diet dependent (Verrusio, 1966). The frequency of cleft palate was lower in the crosses CxC or CAxA when the mother was maintained on a high niacin diet (Purina lab chow) than on a low niacin diet (Breeder chow). (Verrusio et al., 1968). Further studies on this reciprocal cross difference showed the cytoplasmic factor responsible for the strain difference is not transmitted through the male parent. The reciprocal

cross difference seen in the first backcross disappeared on the second backcross (Pollard, 1968; Pollard and Fraser, 1968). The basis for the strain difference in response to 6 AN could be interpreted as a difference in the metabolism of 6 AN between genetically different animals. Studies conducted by Verrusio showed that the C57BL strain required more 6 AN than the A/J strain to produce a comparable frequency of cleft embryos (Verrusio, 1966). Also there is evidence that genetically determined differences exist in the rate of metabolism of 6 AN and nicotinamide turnover between the two. The strain difference could also be due to the variation found between the strains in the mitochondria which would allow the C57BL strain to utilize niacin more efficiently when exposed to 6 AN (Verrusio and Watkins, 1969). Analogous observations on the importance of cytoplasmic factors taking part in biochemical changes which are responsible for malformations have been reported for the chick (Landauer and Wakasugi, 1968). What remains to be settled, however, is the way in which cleft palate is produced by 6 AN. Turbow and Chamberlain (1968) have shown that 6 AN affects rat embryos directly. It reduces somite numbers and retards growth greatly in day 10 embryos in culture. When injected into the amniotic sac directly, 6 AN causes progressively higher frequency of cleft palate with increasing concentration. Landauer and Sopher (1970) have postulated that 6 AN exert its teratogenic effects by interference with mitochondrial energy production in the affected tissues.

Inhibition of hydrogen transport and phosphorylation by 6 AN can interfere with cellular energy production in the palatine shelf and can lead to clefting of the palate. 6 AN is known to inhibit chondrification of mesenchyme and ossification of cartilage (Pinsky and Fraser, 1959). Chromosomal fragmentation and polyploidy in embryos treated with 6 AN has been reported (Ingall, Ingenito and Carley, 1964). Micrognathia or shortening of cranial base may result from 6 AN treatment and each can contribute to the production of cleft palate. A clear picture of the mechanism of cleft palate production will only be seen after further biochemical and morphological studies be carried out.

D. Vitamin A excess and cleft palate.

It is well established that both hypovitaminosis A and hypervitaminosis A in the mother may result in the birth of young with various anomalies. Administration of massive doses of vitamin A to pregnant rats can produce exencephaly, gross eye defects, skeletal changes, and cleft palate in their offspring (Cohlan, 1953; 1954; Giroud and Martinet, 1955; Millen and Woolam, 1957; Woolam and Millen, 1957). Similarly, when pregnant mice are treated with vitamin A in excess, various malformations are produced in the offspring; the typical defects commonly seen are exencephaly, microphthalmia, cleft palate, and limb defects (Mauer, 1959; Kalter, 1960).

Shortening of the mandible and maxilla with macroglossia were first described in rat embryos treated with vitamin A (Cohlan, 1954). The micrognathia led to protrusion of the tongue, which was actually normal in size, giving the impression of an enlarged tongue (Inaba, 1958). The mandible defect was primarily a shortening of the distal portion of the jaws. The ramus was rarely affected by the drug. Studies conducted by Deuschle, however, showed shortening of the more proximal portion of the jaw (Deuschle, Geiger and Warkany, 1959).

A syndrome of dentofacial malformations which included abnormalities such as dermal appendages, microstomia, and micrognathia was produced when pregnant mice were treated on day 9 1/3 (Kalter, 1960). The micrognathia involved both shortening of the mandibular corpus and reduction in the proximal end of the mandible. No strain differences were observed in three strains of mice regarding the type of malformation or the frequency of induced defect (Kalter, 1960). Walker and Crain (1960), however, noticed a strain difference in the frequency of cleft palate in the A/J and C57BL strains.

Several different theories have been advanced to explain how maternal hypervitaminosis A leads to cleft palate in the offspring after an excessive dose of vitamin A to the mother. Delay in the movement of the palatal processes from a vertical to a horizontal position was attri-

buted to a disturbance in the metabolism of sulfated mucopolysaccharides in the palatal shelf (Walker and Crain, 1960; Ross and Walker, 1967; Walker, 1961).

Blood concentration of thyroid hormone was significantly reduced in vitamin A-treated animals, and thyroid weight and thyroidal uptake of I^{131} was significantly increased in these animals (Takekoshi, 1964). From these observations, it was suggested that altered thyroid gland function was closely connected with the induction of cleft palate in vitamin A-treated embryos.

Woollam and Millen (1960) proposed that vitamin A exerts its teratogenic power by interfering at an unspecified level in the carbohydrate metabolism of the mother, placenta and/or fetus. The direct action of a toxic dose of vitamin A on the embryos had been proposed by others (Cohlan and Stone, 1961; Giroud, Gounelle and Martinet, 1957). A distinct cellular necrosis in the somites of young hamster embryos was found after vitamin A treatment.. A mesodermal derangement and mesodermal collapse is more likely to contribute to the malformation (Marin-Padilla, 1966). The palatine shelves are very small and grossly malformed; there is inefficient outfolding or a deficiency of mesenchymal tissue. In spite of this distortion, movement of the palate shelves from the vertical to the horizontal position is initiated on time (Kochhar and Johnson, 1965). There is also replacement of maxillary bone by cartilage. Lack of ossification of the maxilla interferes with normal pressures

that developing bone exerts to force the horizontal palatal processes toward the midline. Based on these findings, Kochhar and Johnson, (1965), suggested that cleft palate in rat embryos in response to maternal vitamin A treatment is not due to failure of shelf movement. Altered ^{35}S-sulphated mucopolysaccharides metabolism of mesenchymal tissues and cartilage has been shown in the malformed embryos (Kochhar and Johnson, 1965; Kochhar, Larsson and Boström, 1968). Observations made by Nanda (1970b) on vitamin A-treated embryos differ from those of Kochhar and Johnson. The palatal processes were horizontal only in the anterior region and not posteriorly. Heterotopic cartilage formation induced maxillo-mandibular ankylosis in the affected embryos and this led to partial or complete immobility of the mandible. This immobility contributed to disturbed development of mandible which showed up after birth as micrognathia and retrognathia. As a consequence of this maxillo-mandibular ankylosis and condylar agenesis, normal mouth-opening reflexes are delayed or prevented in these embryos. This in turn impairs tongue mobility and descent. Abnormal epithelial proliferation which was observed in the oral cavity could result in disruption of synchronized development and spatial organization of the surrounding mesoderm (Kochhar, 1968). Radioactive thymidine uptake studies revealed inhibition of cell proliferation in loose mesenchymal tissue in the posterior region of the palate shelves.

Ross and Walker (1967) have shown that the embryonic tongue plays a significant role in palate closure. Whether Vitamin A treatment affects the active role of the tongue during palate closure is unknown, however, Fujino et al., (1964), reported defective tongue musculature as one result of this teratogen. Vitamin A in excess can also influence the competency of the palatal shelves to fuse along their medial margin. Addition of Vitamin A to day 14 and day 15 rat embryonic palate in vitro inhibits palate fusion, but when the median edges of the sliced palatine shelves are placed together, they retain the ability to fuse even after Vitamin A treatment. This indicates that excess Vitamin A does not alter the shelf surface (Myers, Petrakis and Lee, 1967).

E. Cleft palate induced by amniotic sac puncture

It was found that cleft palate could be induced in high frequency in mouse embryos by inserting a hypodermic needle through the uterine wall and into the amniotic sac on day 13 1/2 of gestation (Trasler, Walker and Fraser, 1956; Trasler, 1958). These cleft palates resulted from a mechanical compression of the fetus by the fetal membrane (Walker, 1959) because of leakage of amniotic fluid from the puncture. The affected animals were deprived of normal extension of the cephalic flexure and were unable to raise their chins from their chests

prior to mandible descent at the crucial time of palate closure (Poswillo and Roy, 1965). In these clefted animals, acute flexion of the head had pressed the lower jaws firmly against the chest and forced the tongue up between the palatine shelves. As a result the palatine shelves were prevented from fusing.

Whether the resistance of the tongue caused by the amniotic sac puncture is produced by arrest of lower jaw growth locally by direct pressure from the embryonic membrane or results merely from immobilization of the embryos with inhibition of normal spontaneous mouth-opening movements by the apposed elastic membrane remains to be answered.

The association of CP* with amniotic sac puncture in mice leads a clinician to wonder whether there is any evidence of a similar association of CP* with amniocentesis and oligohydramnios in human patients. Poswillo (1968) has called attention to the striking similarity between the Pierre Robin syndrome and the characteristic morphology of embryos recovered from punctured amniotic sacs. Oligohydramnios as well as hydramnios occurring during pregnancy are known to be associated with malformed babies (Stevenson, 1960). Bilateral renal agenesis is always associated with scanty or absent amniotic fluid. The fluid deficiency may be partly due to a physiologically and morphologically

* CP: cleft palate.

abnormal placenta and umbilical cord and partly due to absence of the kidney (Abramovich, 1970). In 50 reported cases of renal agenesis, 3 cases of cleft palate and hare lip were found (Potter, 1965).

Can cortisone or 6 AN treatment induce clefts in embryos through a reduction in the volume of amniotic fluid? Harris (1964) has shown that a definite reduction in the weight of amniotic fluid was associated with cortisone-treated embryos in C57BL, A/J, and CBA strains of mice. This decrease in fluid was proposed as a factor in the etiology of the cleft palate produced by maternal treatment with cortisone in mice. Walker (1965) confirmed the finding by Harris, but concluded that reduction in the volume of amniotic fluid is not primarily responsible for the induced cleft palates. Verrusio (1966) has shown that in A/J mice treated with 1/2 dose of 6 AN on day 13 1/2 there is no significant difference in the reduction in amniotic fluid between embryos with cleft palates and their normal littermates.

Thus amniotic fluid reduction cannot be the sole factor that determines which embryos develop cleft palate after 6 AN or cortisone treatments.

F. Mandible deformity and tongue resistance in experimental production of cleft palate.

According to Asling et al., (1960) an increase in the resistance of the tongue which will induce cleft palate

can be produced in micrognathic rats. During normal development of the palate in rats, the lateral palatine processes maintain a ventromedial orientation under the lateral margins of the tongue until the 16th day of gestation. Thereafter the tongue drops in the mouth and the palatine shelves move into a horizontal position and subsequently there is complete fusion. Under maternal folic acid deficiency the tongue is high in the oral cavity until the 18th day with the lateral palatine processes maintaining their position below the tongue. By the time the tongue descends the head has grown so wide that fusion is impossible. Micrognathia and agnathia caused by inadequate growth and arching of the mandible contribute directly to increase tongue resistance because of the reduced space available for the tongue.

Examples of small size and abnormal shape of the lower jaws (responsible for the failure of the tongue to descend and permit the normal positioning and fusion of the palatine shelves) are the reduction in forward growth of the mandible in chondrodystropic mutants (Seegmiller, Fraser and Sheldon, 1971). Micrognathia in mutant musculodysgenesis is probably secondary to skeletal myopathies and the presence of an abnormally small tongue lodged in the cleft palate, impeding fusion of the palatine shelves (Pai, 1965).

The cleft palate seen in A/J embryos with spontaneous cleft lip and cleft palate is due to delay of the normal tongue

descent by the large abnormal median process (Trasler and Fraser, 1963). Whether the tongue drops in the mouth before the palatine shelves move to their horizontal position (Burston, 1959; Asling et al., 1960) or, conversely, that descent of the tongue is caused by movement of the palatine shelves (Trasler and Fraser, 1963) remains to be settled. Poswillo and Roy (1965) concluded from their studies on rats that fusion of the posterior palate involved the simultaneous action of tongue descent and palate shelf movement. The forward and downward displacement of the tongue is aided by a change in the contour of the pharyngeal wall, by outgrowth of the mandible and the inclined plane of the primary palate, and in rabbits by a sudden change in the degree of flexion of the head (Walker, 1967, 1968).

The need for further evaluation of the role of the tongue in palate closure was raised by Ross and Walker in 1967 (Ross and Walker, 1967). In 1969 Walker observed spontaneous embryonic movement coinciding in its onset with the time of onset of palate closure. He also found that swallowing movements involving both forward and backward movement of the tongue also flatten the palatal shelves and thus, help them meet. (Walker, 1969).

Humphrey (1969) has shown a correlation between mouth-opening reflexes that depress the mandible of human fetuses before and during the period of palatal shelf closure in the study of the movements of 18 fetuses recorded cinemato-

graphically. From the correlation of palatal closure with the change in mandible size in her study Humphrey concluded that increase in growth of the mandible follows palatal closure. This accelerated growth of the mandible was attributed in part to the stimulating effect of reflex activity, to the pressure of the tongue, and to increase blood circulation in the mandibular region resulting from movement of the mucosal surface. When the evidence is considered for an active role of the tongue in closure of the palate, for a small tongue associated with cleft palate seen in the mutant "shorthead" (Fitch, 1961), and for a weakness in the tongue as in the case of Muscular Dysgenesis (Pai, 1965), it is quite possible that a teratogen such as 6 AN or vitamin A excess could cause cleft palate via a direct effect on the tongue or affect the tongue indirectly via an effect on the mandible.

III METHODS AND MATERIALS

This investigation was carried out between June 1970 and June 1971.

1. Mice.

The inbred mice used were of the A/J and C57BL/6J strains mainly obtained from the Jackson Memorial Laboratory, Bar Harbor Maine. Small numbers of mice of either strain also came from the maintanance colony (substrains) of Dr. D. G. Trasler at the McGill University.

Male and female mice were kept separately in plastic cages in an air-conditioned (72°F) room with a light-dark cycle such that there was 8 hours darkness from 11 pm to 7 am and artificial light for 16 hours.

All adult mice were fed on a diet of Purina Laboratory Chow and water ad libitum which was supplemented once a week with whole wheat bread, milk and lettuce.

2. Timing and collection of embryos.

When an experimental animal was required, a male of the appropriate genotype was placed in a breeding cage with 4 or 5 mature females in the early evening and removed the following morning. The females were examined for vaginal plugs indicating successful copulation, in the morning. If the vaginal plug was present, the female was weighed and kept in its home pen until the day of appropriate treatment. For the purpose of estimating

chronological age of the embryos, fertilization was assumed to occur at 2 am of the day the plug was found, (Runner and Ladman, 1950; Snell et al., 1940) which was considered to be day 0 of gestation. For the control series pregnant C57BL females were killed from day 14/14 hours to day 14/20 hours. For the A/J control the females were killed between day 14/20 hours and day 15/2 hours of gestation. The treated groups of each strain of mice were killed 6 hours later than the control animals in order to obtain embryos at palate closure time. The whole uterus with a right ovary attached (for identification) was removed and fixed in Bouin's solution for a week and then stored in 70% ethanol for later examination.

3. Cortisone treatment.

A standard method was used for all cortisone treatments. Intramuscular injection in the flank with 2.5mg of cortisone acetate[1] was given daily for 3 consecutive days starting on day 11/12 hours of gestation.

4. 6 AN treatment.

The 6-aminonicotinamide (6 AN[2]) and nicotinamide (N[3]) used in these experiments were dissolved in sterile distilled

1. Cortisone acetate. (Cortone 50mg/ml, Merck, Sharp and Dohme) was kindly supplied by Dr. W.D. Dorian of Merck, Sharp and Dohme Co.
2. 6-aminonicotinamide was supplied by Dr. J.D. McCall of Frank W. Horner Ltd.
3. Nicotinamide also was supplied by Dr. J.D. McCall. Both 6 AN and N are dissolved in distilled water for appropriate concentration.

water in concentrations of 225 mg and 85 mg per 100 c.c. respectively. Dosages were calculated according to the weight of the female at 1/3 day of gestation. A full dose of 6 AN was the equivalent of 19mg/kg body weight while a full dose of N was 7.3mg/kg body weight. For C57BL/6 one full dose of 6 AN was administered intraperitoneally followed 2 hr afterward with a full dose of nicotinamide while for A/J a three fourth (3/4) dose of 6 AN followed 2 hours later by a 3/4 dose of N was given. The pregnant females were all treated with 6 AN at day 13/12 hr of gestation. These treatments were found to give approximately 70% cleft palate (CP) in C57BL/6 and 91% CP in A/Jax.

5. Vitamin A treatment

10,000 I.U. of Vitamin A[4] were given once to pregnant females on day 10 1/2 of gestation by administration of 0.1 ml of a 2: 1 dilution of Arovit in distilled water (freshly made each time) by intraperitoneal injection.

6. Amniotic sac puncture

Pregnant females on day 13 1/2 were etherized, and the uterus was exposed through a midline abdominal incision. A number 26 gauge needle was then inserted into the amnion of each embryo in one uterine horn but nothing was injected.

4. Roche Arovit. Water-soluble 150,000 I.U. per c.c. (approximately 82 mg Vitamin A palmitate), 7.5 ml/bottle.

The embryos in the other horn were untreated controls. The mother was then sewn up and killed later at various times.

7. Examination of embryos.

The fixed embryos were dissected out of the uterus and examined under a dissecting microscope for morphological features. Individual embryos were then weighed on a Mettler balance in the following manner. The embryos were removed from the storage petri dish and placed on Kimwipes momentarily for 30 seconds to remove excess moisture. They were then placed on the balance and the final weight was recorded immediately. This was done to minimize any errors resulting from evaporation.

8. Free-hand razor blade sections.

After morphological evaluation, the head was separated from the body by slicing through the neck with a double edge razor blade. The head was then cut sagittally through the midline on a paraffin block under the dissecting microscope. The head with cut surface up was then placed in a depression on the paraffin block. The distance between the tip of the lower jaw and opening of thyroid duct was measured with the aid of an ocular micrometer. The distance between the tip of the tongue to thyroid duct opening was taken as length of the tongue. The position of the tip of tongue relative to the primary palate was observed in some embryos. After the measurements were taken, the tongue and lower jaw were removed gently from the head and the stages of palate shelf closure were recorded. (Figure 1).

9. Frequency of cleft palate in the two strains after various treatment

The frequency of cortisone, 6 AN or Vitamin A induced cleft palate was evaluated after killing a group of pregnant females on day 17 1/2. Embryos were examined for gross abnormalities and the resorptions were counted.

10. Examination of cranial base

Control embryos from the two strains were checked for the angle of cranial base at the time of palate closure. After decolorizing with lithium chloride in 70% alcohol for 24 hours, the head was stained with 0.1% Alcian Blue overnight and counter-stained with 1% Neutral red for 2-4 hours. The heads were then washed with 50%, 60% and 70% alcohol.

11. Morphological rating of the embryos

Using the method of Walker (1954) a standard series of observations were made on the following morphological features, degree of webbing of the digits, extent of hair follicle development, appearance of the eyelid and the pinnae. Each feature was assigned a numerical value and the morphological rating was taken to be the sum of these figures. (See Appendix Table 1).

12. Evaluation of palate stages (Walker and Fraser, 1956; Trasler and Fraser, 1963)

Stage 0: The palate shelves are vertical, hanging down in the oral cavity, and lie parallel to each other.

Stage 1: The shelves begin to move into their final position above the tongue. The tongue is still cupped between the shelves but it has moved forward and under the primary palate. The posterior ends of the shelves, which are still vertical, have begun to move medially.

Stage 2: The posterior 2/3 of the shelves on both sides have become horizontal and lie above the tongue.

Stage 3: One shelf is in a horizontal position above the tongue along its entire length, and the other shelf still remains vertical anteriorly.

Stage 4: Both shelves have assumed a horizontal position and lie dorsal to the tongue.

Stage 5: Fusion begins between the two shelves near the midpoint along their length.

Stage 6: About 1/3 to 2/3 of the palatal shelves are fused, fusion having proceeded from the midpoint in a antero-posterior direction.

Stage 7: Epithelial fusion has spread along the entire length of the palatine shelves.

Figure I: Sagittal section of embryonic head showing the measurement taken for lower jaw and tongue length.

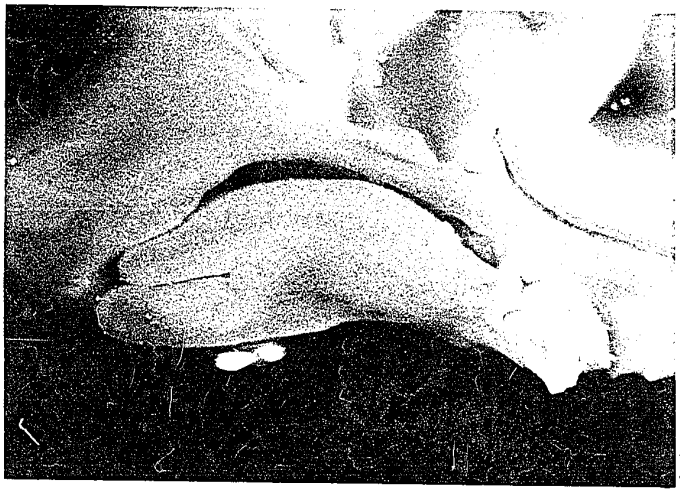


Figure I: Sagittal section of embryonic head showing the measurement taken for lower jaw and tongue length.

IV RESULTS

Table 1 represents: 1) Number of litters; 2) total implantation number; 3) resorption rate; 4) frequency of spontaneous cleft lip and palate, and 5) the total number of embryos used for measurement of the lower jaw length in control and treated groups in the A/J and C57BL/6 strains. A slightly lower resorption rate is found in the 6AN-treated group in both strains, but statistical analysis shows that there is no significant difference between control (17%), cortisone (26%), 6AN (9%) and vitamin A (16%) treated resorption rate in C57BL. However, there is a significantly lower resorption rate in A/J 6AN (13%) treated compared to control A/J (23%) (p: .05-.025). No spontaneous cleft lip and palate was found in the control and treated groups in C57BL, but occasional microphthalmia and anophthalmia (1%) and aglossia (1%) were found.

The incidence of cleft palate (CP) induced by the teratogens under consideration is shown in Table 2. Although the sample numbers are small, the strain difference is clear. Cortisone induced 100% CP in A/J while causing only 35% CP in C57BL. 6AN produced 91% and 68% CP in A/J and C57BL respectively. The incidence of CP due to excess vitamin A is the same in both strains.

Chronological age of the control and treated embryos from the two strains of mice is shown in Table 3. In order to collect embryos of appropriate palate stages, embryos had to be

Table 1

Control and treated groups of A/J and C57BL/6 examined on day 14 to 15 of gestation.

Strain	Treatment	Number Litter	Number Implantation	Resorptions (%)	Total Embryos	Embryos Examined*	CLP (%)
A/J							
	Control	25	218	51 (23.4)	167	144	12.0
	6 AN	16	145	19 (13.1)	126	105	7.0
	Cortisone	14	127	32 (25.2)	95	78	18.0
	Vitamin A	10	97	19 (19.5)	78	71	9.0
C57BL/6							
	Control	41	296	49 (16.6)	247	222	0
	6 AN	17	128	12 (9.4)	116	111	0
	Cortisone	19	138	36 (26.1)	102	195	0
	Vitamin A	15	127	20 (15.7)	107	101	0
	Amn. Punct.	7	56	12 (21.4)	44	20 (24)**	0

* The number of embryos used for the measurement of lower jaw length.

** 20 embryos were punctured and 24 were control.

Table 2

Incidence of cleft palate (CP) after treatment in embryos examined on day 17 of gestation

Strain	Treatment	Number Litters	Number Implantations	Resorptions (%)	Number Embryos	CP Embryos (%)	CLP Embryos (%)
A/J	6 A N	4	30	6 (20%)	23	21 (91%)	1 (4%)
	Cortisone	4	31	11 (35%)	18	18 (100%)	2 (10%)
	Vitamin A	2	16	1 (6%)	13	12 (92%)	2 (13%)
C57BL/6J	6 A N	3	24	5 (21%)	19	13 (68%)	
	Cortisone	3	24	1 (4%)	23	8 (35%)	
	Vitamin A	2	17	2 (12%)	15	14 (93%)	

Table 3

Number of embryos collected at each chronological age in A/J and C57BL strains in control and treated groups

Strain	Treatment	D14/8h	D14/12h	D14/16h	D14/20h	15/2h	15/6h	15/10h	15/14h	15/18h
A/J	Control			14	78	21	8	7		4
	6 A N			–	18	48	19	16		4
	Cortisone			4	9	39	12	14		
	Vitamin A			8	26	30	7	–		
C57BL	Control	22	112	44	37	–	7	–		
	6 A N	–	–	6	63	13	21	8		
	Cortisone	–	16	19	39	13	8	–		
	Vitamin A	–	19	35	25	–	22	–		

collected at somewhat different chronological ages in control and experimental groups in both strains.

A. Observation on the growth of lower jaw during palate closure in control and treated groups

1. Palate stage in relation to morphological rating.

Tables 4 and 5 show the pattern of palate closure plotted against morphological rating in control and treated groups in the two strains. All three teratogens cause a delay in the process of palate closure when morphological rating is used as the criterion of embryonic development. A diagrammatic representation of the relation between morphological rating and palate stage and the effect of teratogens on palate closure is shown in Figure 2, in which, mean morphological rating is plotted against palate stage. The finding in the controls of both strains confirm the previous findings by Walker (1954) and Trasler (1958). That is, there is a difference in time of palate closure in relation to developmental features in A/J and C57BL which parallels the susceptibility to teratogen induced cleft palate. The number of embryos, mean morphological rating (MR), standard error and the range of MR at each palate stage (PS) are shown in Appendix Tables 2a and 2b. In Table 6, the mean MR of control and treated groups of both strains at each palate stage is shown. The mean MR at each palate stage in the treated group is far more advanced than that of the control group in both strains.

Table 4

Palate stages (PS) vs morphological rating (MR) A/J Embryos

MR	Control PS								6 AN PS								Cortisone PS								Vitamin A PS							
	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
-2																									1							
-1																																
0	1								3								2								1							
1	1								4																							
2	2								2								2								8							
3	9	1							2								2								5							
4	13	1							5								3								3	1						
5	9	1							7								1								2							
6	8	1							9								4								1	3						
7	4	4							8								4	1							3							
8	5	6		1					10	1							3									1			1			
9	3	14							7	4							2	1								2			1			
10		12							4	3							1	1								3		2				
11		8	2	1			2		2	8		1		1			1	4	1						1	2	1	1	1	1	1	
12		2	1	2		1	2		2	4	1														5	3	1	1	1	1		
13				2			1	1		4		3	1			1	1	2	1						2	2		3		1	1	
14							1	7		1	2	5	1			1		3	1	4					3							1
15								12			1	2	1			1			3	1					2							
16						1		1						1				2	1	1	1											
17													1					2	2	2												
18														1						1												
19																					1											
20																		1														
21																			2	1												
22																			1													
23																			2													
24																				2												
25																			3													
26																																

Table 5

Palage stages (PS vs morphological rating (MR) C57BL embryos

MR	Control PS							6 AN PS							Cortisone PS								Vitamin A PS							
	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7	0	1	2	3	4	5	6	7
-1	1																								1							
0	5								3																							
1	2								1								1								6							
2	4								1								2								2							
3	5								5								2								2							
4	29	10							11								7								3	1		1				
5	6	8	1						2								4								9			1				
6	4	17	1						7	3							4	1							9							
7		11	8	3	1				3	3							1	2							9	1						
8		4	6	3	2	1	1		3	6								4	1						3	4						
9			3	5	15	5			1	5		3						1	2	2					4	4	1	1				
10				2	4	5	6	2		4		4	2						3							1	2					
11						3	11	4				5	4		2	1			1	5						1		1	1	1		1
12						1	6	12		1		4	11		2	1				1	3	1	3				1			1	3
13								6		1					3	1			1	1	1	1							3	2	3
14							1	9					1		2	1						2							1	2	5
15								3								3				2	1	2	6						1	3	3
16																					3		1	1						2	2
17								1													1		3	1						2	2
18																								1						1	2
19																						1	4	3						2	2
20								3													1			4					1		
21																								2							
22								2																1							
23																															
24																															
25								1																							

Figure: 2

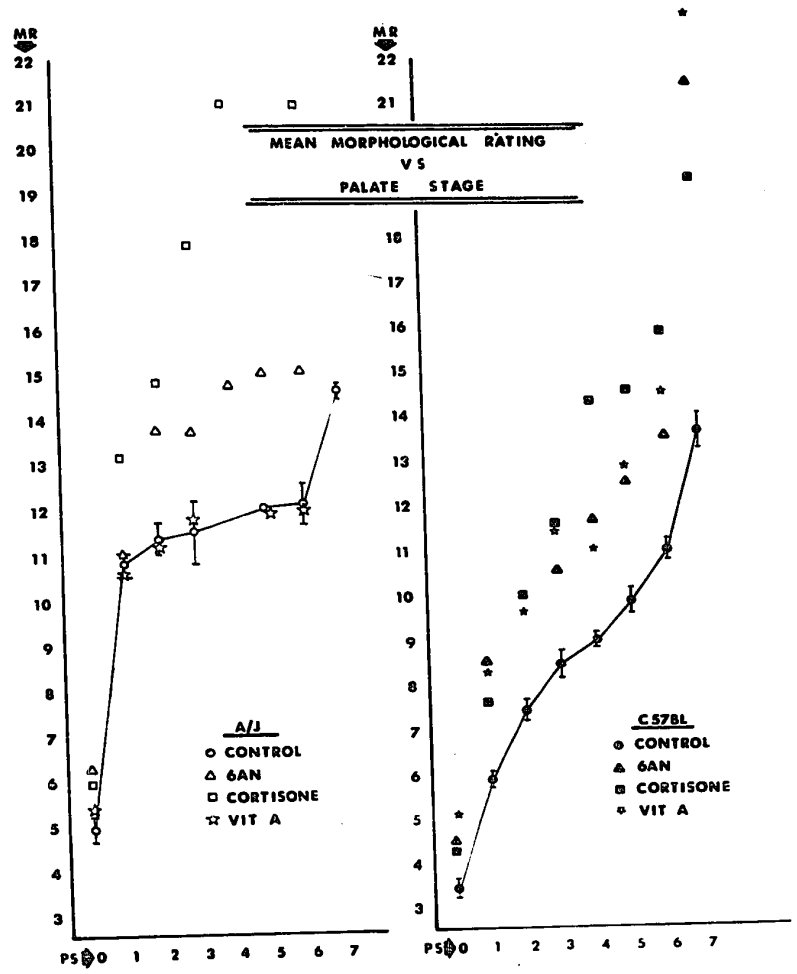

MEAN MORPHOLOGICAL RATING
VS
PALATE STAGE
MR
PS
A/J
CONTROL
6AN
CORTISONE
VIT A
C57BL
CONTROL
6AN
CORTISONE
VIT A

Table 6

Mean morphological rating of embryos from A/J and C57BL strain, control and treated at each palate stage

		palate stages							
Treatment	Strain	0	1	2	3	4	5	6	7
Control	A/J	4.95	9.04	11.33	11.50		14.00	12.17	14.62
	C57BL	3.46	5.82	7.47	8.46	9.00	9.87	11.00	13.65
6 AN	A/J	6.28	11.04	13.75	13.64	14.75	15.00	14.50	14.50
	C57BL	4.54	8.57		10.63	11.67	12.56	13.57	21.50
Cortisone	A/J	5.96	13.18	14.78	17.79	21.50			
	C57BL	4.29	7.63	10.00	11.64	14.33	14.57	15.82	19.38
Vitamin A	A/J	5.47	10.64	11.50	11.70	10.00	12.0	12.00	14.00
	C57BL	5.19	8.33	9.67	11.57	11.0	12.80	14.57	15.13

2. Lower jaw length and palate stage.

The number of embryos used for measuring the lower jaw length at each palate stage, the mean lower jaw length (in micrometer units: 1 u.=0.06mm), standard error and the range of jaw length are presented in the Appendix Table 3. In Table 7 and Table 8, the mean jaw length at each palate stage of control and treated groups in each strain are shown. In the control groups at earlier palate stages (stage 0 to stage 3), the A/J embryos seem to have longer lower jaws compared to C57BL; by the end of palate closure (stage 7), their lower jaws are smaller than in C57BL. This finding may be due to the small sample size of the A/J embryos. At stages 4 and 5, no A/J embryos were suitable for measurement while in the C57BL control there were enough samples at each stage to get satisfactory measurements. Figure 3 illustrates that in spite of strain differences shown previously when morphological rating was used as the criterion of development, in palate closure, no difference was found in the length of the mandible or in the rate of its growth throughout the critical period of normal palate closure in the untreated control embryos of both strains. The mean lower jaw length at each palatal stage in the treated groups is also shown in Figure 3. None of the teratogens acted by slowing the growth of the lower jaw relative to palate stage. This finding does not, however, indicate that the teratogens do not affect the growth of the

Table 7

Mean and standard error of lower jaw length during palate closure in A/J embryo of control and treated group

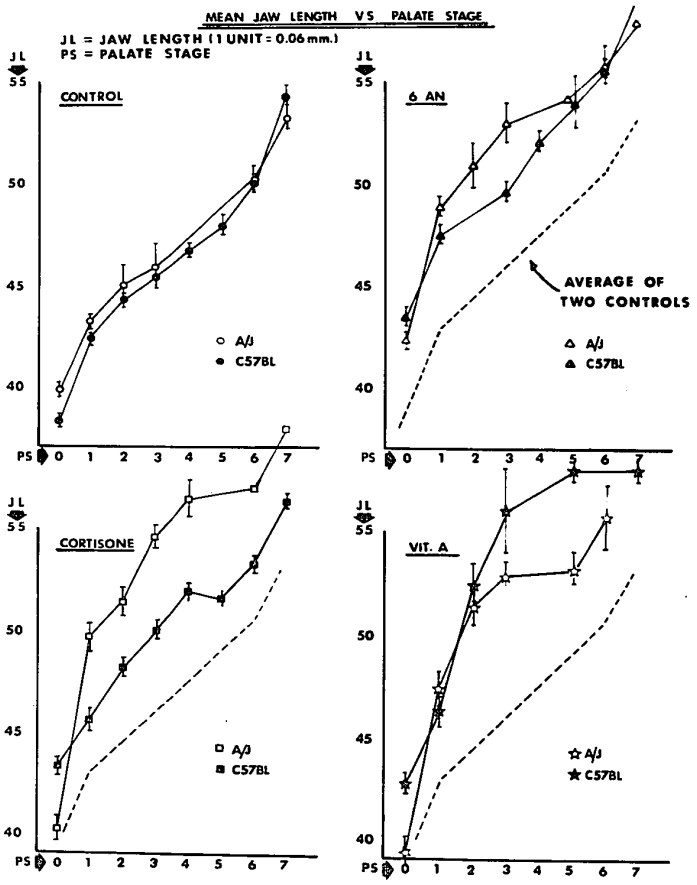
	Control	6 A N	Cortisone	Vitamin A
Stage 0	39.86 ± 0.33	42.35 ± 0.39	40.22 ± 0.73	39.27 ± 0.95
Stage 1	43.62 ± 0.31	48.90 ± 0.45	49.76 ± 0.87	47.40 ± 0.90
Stage 2	46.75 ± 1.11	50.88 ± 1.33	51.39 ± 0.84	51.33 ± 1.01
Stage 3	45.90 ± 1.13	52.90 ± 0.67	54.78 ± 0.65	52.96 ± 0.57
Stage 4		56.83 ± 0.17	56.50 ± 1.04	46.25 ± 0.63
Stage 5		54.00 ± 5.00		53.33 ± 0.88
Stage 6	50.25 ± 0.68	55.75 ± 1.11		55.75 ± 1.75
Stage 7	53.21 ± 0.66			

Table 8

Mean and standard error of lower jaw length during palate closure in C57BL embryo of control and treated group

	Control	6 A N	Cortisone	Vitamin A
Stage 0	38.87 ± 0.35	43.49 ± 0.51	43.31 ± 0.52	42.76 ± 0.52
Stage 1	42.74 ± 0.23	47.38 ± 0.50	45.64 ± 0.58	46.19 ± 0.85
Stage 2	44.26 ± 0.26		48.14 ± 0.66	52.50 ± 1.04
Stage 3	45.46 ± 0.50	49.47 ± 0.55	50.00 ± 0.58	56.00 ± 3.91
Stage 4	46.73 ± 0.37	52.03 ± 0.50	51.90 ± 0.66	60.25 ± 7.25
Stage 5	47.81 ± 0.55	53.92 ± 1.36	51.57 ± 0.38	58.10 ± 0.68
Stage 6	49.93 ± 0.48	55.64 ± 0.54	53.21 ± 0.49	56.60 ± 0.93
Stage 7	54.22 ± 0.51	67.00 ± 2.38	56.32 ± 0.42	58.03 ± 0.72

Figure: 3


MEAN JAW LENGTH VS PALATE STAGE
JL = JAW LENGTH (1 UNIT = 0.06 mm.)
PS = PALATE STAGE
CONTROL
A/J
C57BL
6 AN
AVERAGE OF TWO CONTROLS
CORTISONE
VIT. A
JL
PS
55
50
45
40
0 1 2 3 4 5 6 7

mandible at all.

3. Lower jaw length in relation to morphological rating.

Since the embryos in each treated palate stage consist of a heterogeneous group differing in chronological age, morphological developmental features, and other factors due to treatment, a better evaluation of possible interactions of lower jaw length and palate closure can be made when jaw length is compared to morphological rating. In Tables 9 and 10, the means and standard errors of lower jaw length of embryos with various morphological ratings in control and treated groups of both strains are shown. As the morphological rating advances, the jaw also grows in both strains. Figure 4 compares the growth trends in the control groups of A/J and C57BL based on morphological rating. At any particular morphological rating, the lower jaw length of C57BL is longer than that of A/J. Statistical evaluation of the differences between means using t-tests and analysis of variance shows that there is a significant difference in mean lower jaw length between C57BL control and A/J control from MR 7 to MR13 which corresponds to the period of active palate closure in both strains (see Tables 4 and 5) and Appendix Table 6). The values for mean jaw length of the treated groups of both strains are plotted against MR in Figure 5. These figures also suggest that the jaw length is not affected by each teratogen when morphological rating is used as the developmental measure.

Table 9

Mean lower jaw length vs morphological rating A/J embryos

MR	Control	6 A N	Cortisone	Vitamin A
0		36.33 ± 0.33	37.00 ± 1.00	
1		36.63 ± 0.38		
2	37.25 ± 1.25	37.00 ± 1.00	38.00 ± 2.00	37.31 ± 0.47
3	38.31 ± 0.82	40.00 ± 1.00	38.25 ± 0.25	34.80 ± 1.00
4.	39.54 ± 0.50	40.20 ± 0.73	38.00 ± 0.91	39.87 ± 0.52
5	40.65 ± 0.52	41.43 ± 0.34		37.00 ± 5.00
6	40.94 ± 0.70	42.81 ± 0.50	41.63 ± 0.75	41.88 ± 0.77
7	39.58 ± 1.56	43.83 ± 0.50	41.50 ± 0.71	43.00 ± 0.76
8	42.00 ± 0.83	44.09 ± 0.52	42.33 ± 1.33	45.00 ± 1.00
9	43.03 ± 0.33	45.95 ± 0.82	44.50 ± 1.76	46.25 ± 0.25
10	44.39 ± 0.60	46.67 ± 0.75	45.00 ± 2.00	49.20 ± 1.44

To follow...

Table 9

(suite)

Mean lower jaw length vs morphological rating A/J embryos

MR	Control	6 A N	Cortisone	Vitamin A
11	45.70 ± 0.96	48.27 ± 0.64	47.00 ± 0.68	50.00 ± 1.64
12	46.81 ± 0.98	48.07 ± 0.61		49.92 ± 1.30
13	47.50 ± 0.50	52.29 ± 1.03	49.80 ± 1.08	51.00 ± 1.30
14	51.93 ± 0.74	53.91 ± 0.70	52.14 ± 0.66	52.33 ± 0.87
15	54.11 ± 0.98	54.67 ± 1.12	51.50 ± 1.04	
16	51.75 ± 2.25		53.20 ± 0.86	
17			52.92 ± 0.47	
21			56.88 ± 0.66	

Table 10

Mean lower jaw length vs morphological rating C57BL embryos

MR	Control	6 A N	Cortisone	Vitamin A
0	34.30 ± 0.80	38.50 ± 2.47		
1	38.50 ± 0.50			37.50 0.88
2	38.50 ± 0.50		39.75 ±0.50	
3	37.50 ± 0.52	42.30 ±1.28	41.50 ±0.50	
4	40.04 ± 0.32	43.23 ±0.35	43.57 ±0.52	41.58 ± 0.84
5	41.80 ± 0.43	43.50 ±1.50	44.13 ±0.24	42.20 ± 0.73
6	42.50 ± 0.31	45.56 ±0.75	44.60 ±0.51	42.06 ± 0.49
7	44.17 ± 0.35	46.25 ±0.62	46.00 ±1.53	45.80 ± 0.48
8	45.00 ± 0.32	46.11 ±0.52	46.00 ±0.54	46.67 ± 0.25
9	46.63 ± 0.37	47.33 ±0.68	47.75 ±0.32	47.35 ± 0.89
10	47.89 ± 0.49	49.10 ±0.49	47.33 ± 0.88	51.83 ± 1.59

To follow...

Table 10

(suite)

Mean lower jaw length vs morphological rating C57BL embryos

MR	Control	6 A N	Cortisone	Vitamin A
11	51.21 ± 0.67	51.41 ±0.63	49.75 ± 0.40	52.60 ± 1.33
12	52.28 ± 0.49	52.09 ±0.59	50.00 ± 0.50	56.20 ± 1.11
13	53.50 ±0.41	54.13 ±0.97	52.25 ± 0.48	56.44 ± 0.66
14	55.00 ± 0.36	55.38 ±0.75	52.20 ± 1.00	59.25 ± 0.85
15	57.50 ± 2.50	56.83 ±0.60	52.45 ± 0.45	58.50 ± 1.66
16			53.60 ±1.02	61.00 ± 2.00
17			54.63 ± 1.21	61.50 ± 0.50
18				
19	61.83 ± 0.73		55.00 ± 0.58	
20		63.67 ±4.04	55.25 ± 0.72	61.5 ± 0.50

Figure: 4

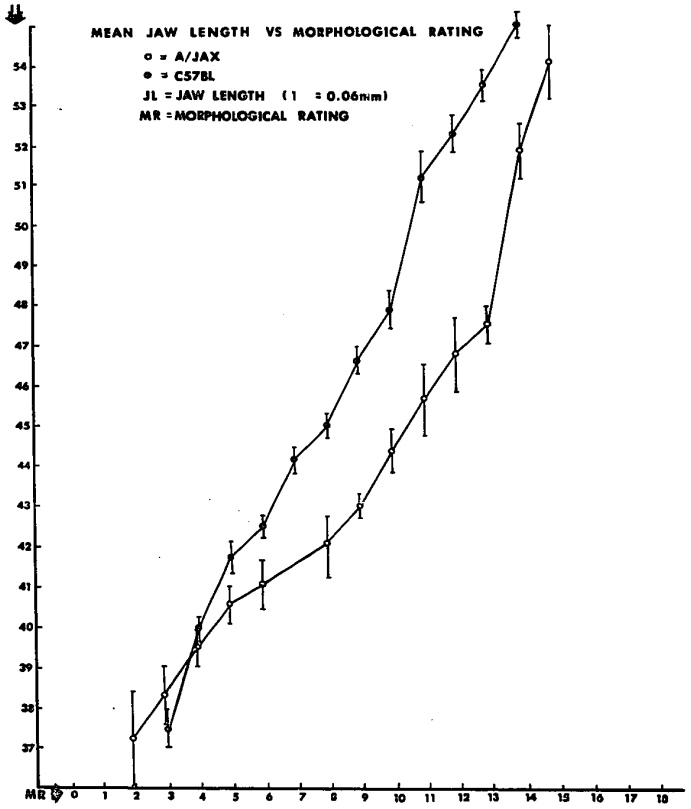
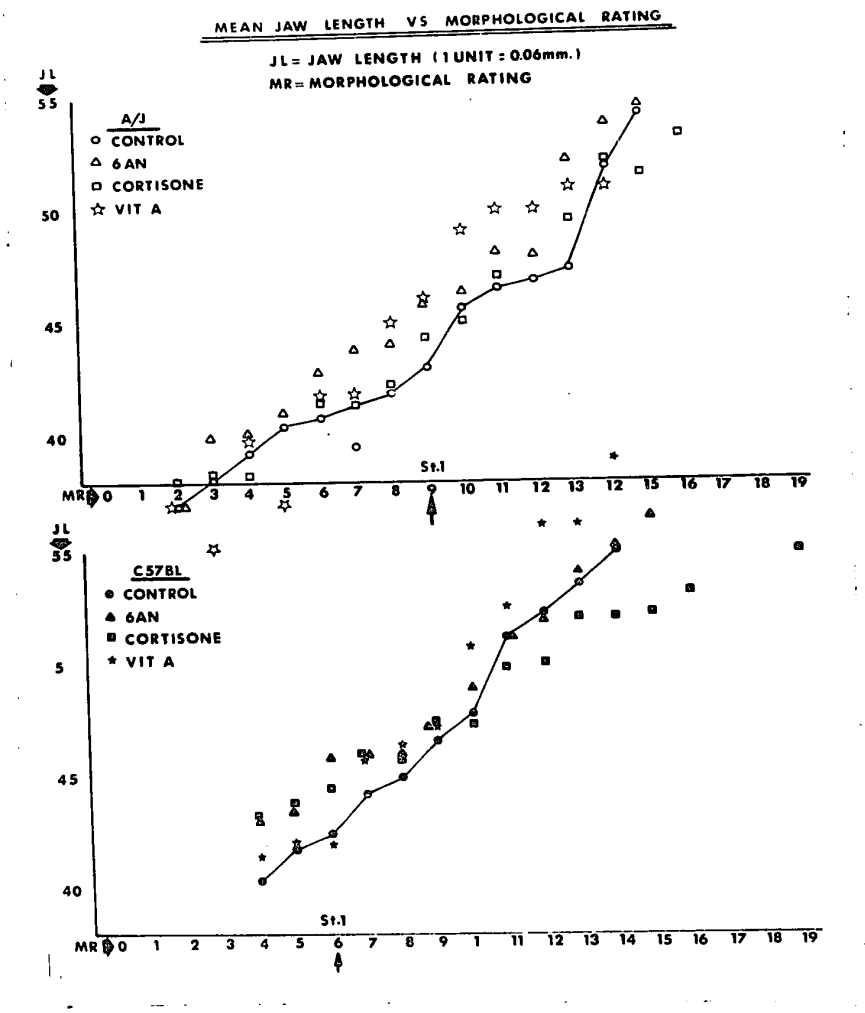

MEAN JAW LENGTH VS MORPHOLOGICAL RATING
o = A/JAX
• = C57BL
JL = JAW LENGTH (1 = 0.06mm)
MR = MORPHOLOGICAL RATING
JL
MR
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54

Figure: 5



Note: Arrows indicate Stage 1 of palate closure.

The lower jaw length in the cortisone-treated group for the A/J strain is not significantly different from the control for MR2 to MR16. A significantly larger lower jaw was found in the 6 AN treated group between MR6 and MR11 (P: .05–.025) and for the vitamin A treated group between MR8 and MR12 (P: .05–.025) (Appendix Table 6c). The t-test for difference in the means of lower jaw length between cortisone and 6 AN-treated groups and C57BL control is significant (P: $<$ .001) only at the beginning of palate closure, when the treated lower jaws are longer (between MR4 and MR6). However, none of the treated C57BL embryos are significantly different from the control embryos during palate closure (between MR7 and MR11). At higher MR the vitamin A treated embryos have longer jaws (P: 0.005-0.001) and cortisone-treated embryos have shorter lower jaws (P: 0.025-0.01) compared to the control. This could be due either to the small sample size (See Appendix, Table 3, Table 6d) or to the enhancement of morphological features due to cortisone treatment, or to difficulty and inaccuracy in rating as a result of vitamin A treatment.

4. Palate stage in relation to body weight of the embryos.

In Tables 11a and 11b, the mean body weight of the embryos in control and treated groups in the A/J and C57BL strains at each palatal stage is presented. The number of embryos between

Table 11a

Mean body weight during palate closure in control and treated A/J embryos

PS	Treatment	Number Embryos	Mean (g)	Standard error	Range
Stage 0	Control	56	.142	.0017	.111 - .169
	6 A N	65	.164	.0024	.120 - .199
	Cortisone	27	.144	.0039	.096 - .189
	Vitamin A	32	.153	.0056	.097 - .224
Stage 1	Control	45	.169	.0025	.133 - .199
	6 A N	25	.205	.0031	.177 - .251
	Cortisone	17	.194	.0043	.167 - .225
	Vitamin A	22	.211	.0067	.161 - .276
Stage 2	Control	3	.177	.0133	.150 - .190
	6 A N	4	.229	.0132	.208 - .263
	Cortisone	9	.211	.0055	.180 - .232
	Vitamin A	2	.229	.0070	.222 - .236
Stage 3	Control	5	.193	.0060	.177 - .208
	6 A N	11	.234	.0038	.216 - .251
	Cortisone	18	.235	.0073	.202 - .298
	Vitamin A	7	.233	.0050	.218 - .251

To follow...

Table 11a

(suite)

Mean body weight during palate closure in control and treated A/J embryos

PS	Treatment	Number Embryos	Mean (g)	Standard error	Range
Stage 4	Control				
	6 A N	4	.255	.0027	
	Cortisone	5	.255	.0149	.203 - .292
	Vitamin A	5	.190	.0071	.171 - .207
Stage 5	Control	1			.178
	6 A N	3	.262	.0221	.218 - .289
	Cortisone				
	Vitamin A	3	.245	.0153	.214 - .261
Stage 6	Control	7	.214	.0020	.209 - .224
	6 A N	4	.254	.0036	.244 - .261
	Cortisone	1			.245
	Vitamin A	2	.243	.0160	.227 - .259
Stage 7	Control	21	.242	.0047	.213 - .282
	6 A N	2	.268	.0010	.267 - .268
	Cortisone	1			.283
	Vitamin A	1			.216

Table 11b

Mean body weight during palate closure in control and treated C57BL embryos

PS	Treatment	Number Embryos	Mean (g)	Standard error	Range
Stage 0	Control	57	.146	.0020	.118 - .172
	6 A N	36	.176	.0039	.126 - .253
	Cortisone	21	.151	.0027	.122 - .168
	Vitamin A	48	.184	.0048	.110 - .266
Stage 1	Control	49	.167	.0016	.146 - .194
	6 A N	22	.214	.0070	.184 - .341
	Cortisone	8	.170	.0026	.159 - .184
	Vitamin A	12	.217	.0107	.167 - .308
Stage 2	Control	18	.177	.0024	.164 - .203
	6 An				
	Cortisone	8	.179	.0034	.165 - .198
	Vitamin A	3	.220	.0075	.208 - .234
Stage 3	Control	14	.188	.0027	.162 - .205
	6 A N	16	.225	.0032	.212 - .256
	Cortisone	11	.197	.0055	.175 - .243
	Vitamin A	6	.232	.0239	.170 - .317

To follow...

Table 11b

(suite)

Mean body weight during palate closure in control and treated C57BL embryos

PS	Treatment	Number Embryos	Mean (g)	Standard error	Range
Stage 4	Control	22	.192	.0033	.157 - .213
	6 A N	18	.235	.0033	.210 - .260
	Cortisone	10	.210	.0047	.195 - .239
	Vitamin A	1			.239
Stage 5	Control	15	.196	.0030	.174 - .215
	6 A N	9	.251	.0060	.216 - .278
	Cortisone	8	.211	.0093	.189 - .273
	Vitamin A	6	.294	.0092	.271 - .327
Stage 6	Control	25	.218	.0041	.184 - .276
	6 A N	7	.263	.0049	.243 - .286
	Cortisone	17	.214	.0028	.190 - .226
	Vitamin A	6	.305	.0175	.272 - .389
Stage 7	Control	36	.251	.0061	.165 - .357
	6 A N	6	.382	.0158	.369 - .439
	Cortisone	14	.243	.0072	.198 - .272
	Vitamin A	24	.306	.0061	.232 - .357

stage 2 and stage 5 is very small, particularly in control groups for the A/J strain; there are also few embryos of stage 4 to stage 7 for the treated A/J groups. In Figure 6 the body weights of the two control groups are plotted against their palate stages. Except for the missing stages 4 and 5 in A/J, the mean body weights at the beginning of palate closure and those by the end of palate closure in the A/J and C57BL strains are similar. The t and F values are shown for these two control groups in Appendix, Table 7. The mean body weights of the embryos at each palatal stage do not differ significantly, although the F test showed a significantly different variance between the two strains in stages 1, 2, 6 and 7. This difference could be due to the small sample size in the A/J group stage 2, 3, and 6. In Figure 7, mean body weights of the treated groups in the two strains for each palatal stage are shown. The mean weight of the treated group appears much larger than the control, perhaps because of the 4 to 6 hour delay in collection of the samples of the treated embryos or due to edema of the affected embryos. However, the body weight of the cortisone-treated C57BL embryos, is similar to that of control C57BL embryos.

5. Lower jaw growth versus body weight.

In Figure 8, the jaw length is plotted against body weight of individual embryos in control A/J and C57BL. The rate of growth of the lower jaw when body weight is used as abscissa is identical in both strains. Figure 9 shows the

Figure: 6

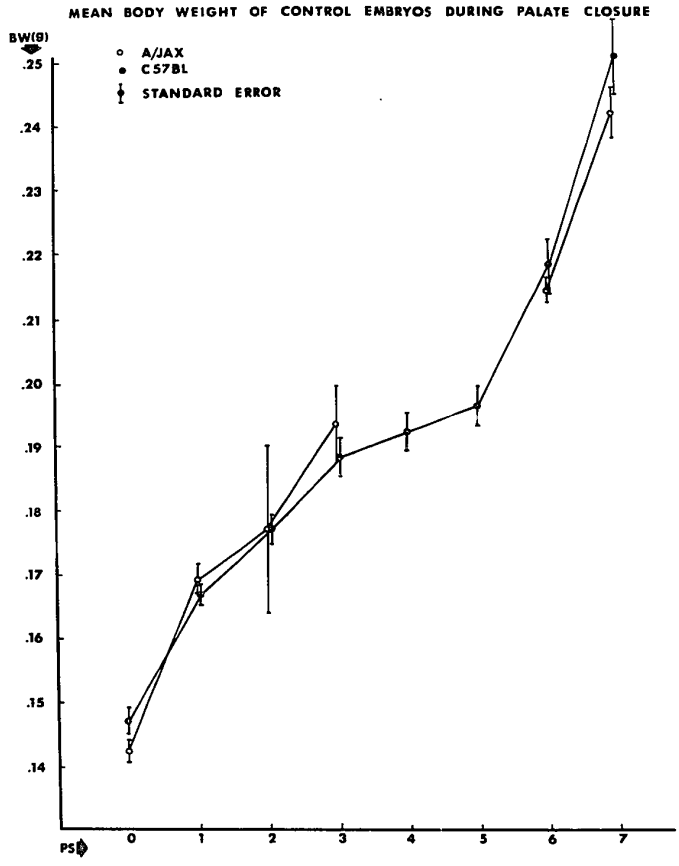

MEAN BODY WEIGHT OF CONTROL EMBRYOS DURING PALATE CLOSURE
BW(G)
A/JAX
C57BL
STANDARD ERROR
.25
.24
.23
.22
.21
.20
.19
.18
.17
.16
.15
.14
PSD
0
1
2
3
4
5
6
7

Figure: 7

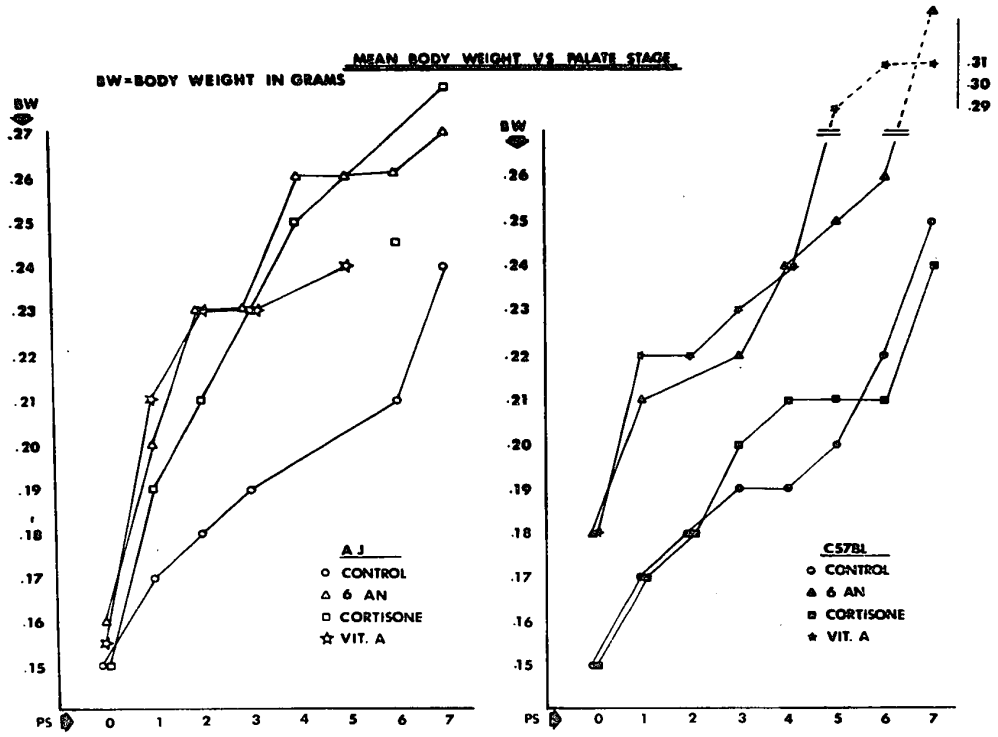
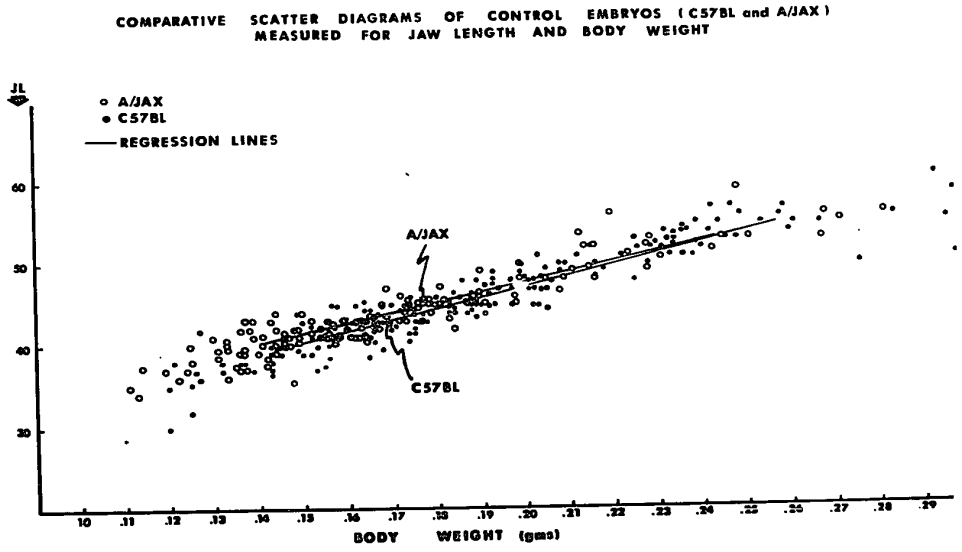

MEAN BODY WEIGHT VS PALATE STAGE
BW=BODY WEIGHT IN GRAMS
BW
.27
.26
.25
.24
.23
.22
.21
.20
.19
.18
.17
.16
.15
PS
0 1 2 3 4 5 6 7
A J
CONTROL
6 AN
CORTISONE
VIT. A
BW
.26
.25
.24
.23
.22
.21
.20
.19
.18
.17
.16
.15
.31
.30
.29
PS
0 1 2 3 4 5 6 7
C57BL
CONTROL
6 AN
CORTISONE
VIT. A

Figure: 8


COMPARATIVE SCATTER DIAGRAMS OF CONTROL EMBRYOS (C57BL and A/JAX)
MEASURED FOR JAW LENGTH AND BODY WEIGHT
JL
A/JAX
C57BL
REGRESSION LINES
60
50
40
30
A/JAX
C57BL
10
.11
.12
.13
.14
.15
.16
.17
.18
.19
.20
.21
.22
.23
.24
.25
.27
.28
.29
BODY WEIGHT (gms)

mean lower jaw length of the three treatments and control which are plotted against body weight of the embryos. The lower jaw in 6AN-treated A/J and C57BL does not differ from that of the control groups. Slight retardation in lower jaw growth in the vitamin A-treated A/J and accelarated growth of the lower jaw in cortisone-treated A/J and C57BL can be seen. Although mean jaw lengths of 6 AN-and Vitamin-treated A/J embryos were significantly below that of control embryos, and the variance differed significantly (6 AN $t = 4.38$ $p < 0.001$ $F= 3.88$ $p=0.1-0.05$, Vitamin A $t = 2.69$ $p= 0.02-0.01$, $F= 1.36$ $p= 0.5-0.25$) in the body weight range between 0.130-0.139 gm, this difference subsequently disappeared as body weight increased. After treatment with cortisone, no differences in mean jaw length between treated and control embryos were seen until body weight was in the range of 0.180-0.220 gm, from which point on, mean jaw lengths of these treated embryos were significantly higher than these of the controls (See Appendix, Table 8a). In the C57BL strain, 6AN-and Vitamin A-treated embryos do not differ significantly from the control when body weight of the embryos is used as the parameter of comparison (Appendix, Table 8b). However, the jaw length is significantly longer in embryos treated with cortisone ($p.<.001$) at all body weight ranges compared (Appendix, Table 8c). Mean lower jaw length of the treated groups is compared to that of control for both strains in Tables 12a and 12b.

Table 12a

Mean jaw length vs body weight

A/J

BW	Control	6 AN	Cortisone	Vitamin A
.090-.099			32.00	31.0
.100-.109				35.0
.110-.119	35.81	37.0		34.0
.120-.129	39.17	36.69		35.50
.130-.139	39.50	36.70	38.44	37.56
.140-.149	40.55	40.25	40.56	
.150-.159	41.85	41.25	42.69	
.160-.169	42.73	43.25	42.83	41.71
.170-.179	44.79	44.03	45.25	43.92
.180-.189	44.88	45.72	47.50	42.25
.190-.199	47.00	48.08	51.50	47.67
.200-.209	47.50	47.60	52.04	48.00
.210-.219	50.67	49.67	54.08	50.28
.220-.229	52.20	53.00	52.50	52.31
.230-.239	51.00	52.10	53.50	51.17
.240-.249	54.67	54.50	56.83	52.42
.250-.259		56.10		54.75
.260-.269		55.50	57.25	
.270-.279				
.280-.289			59.67	
.290-.299			59.50	

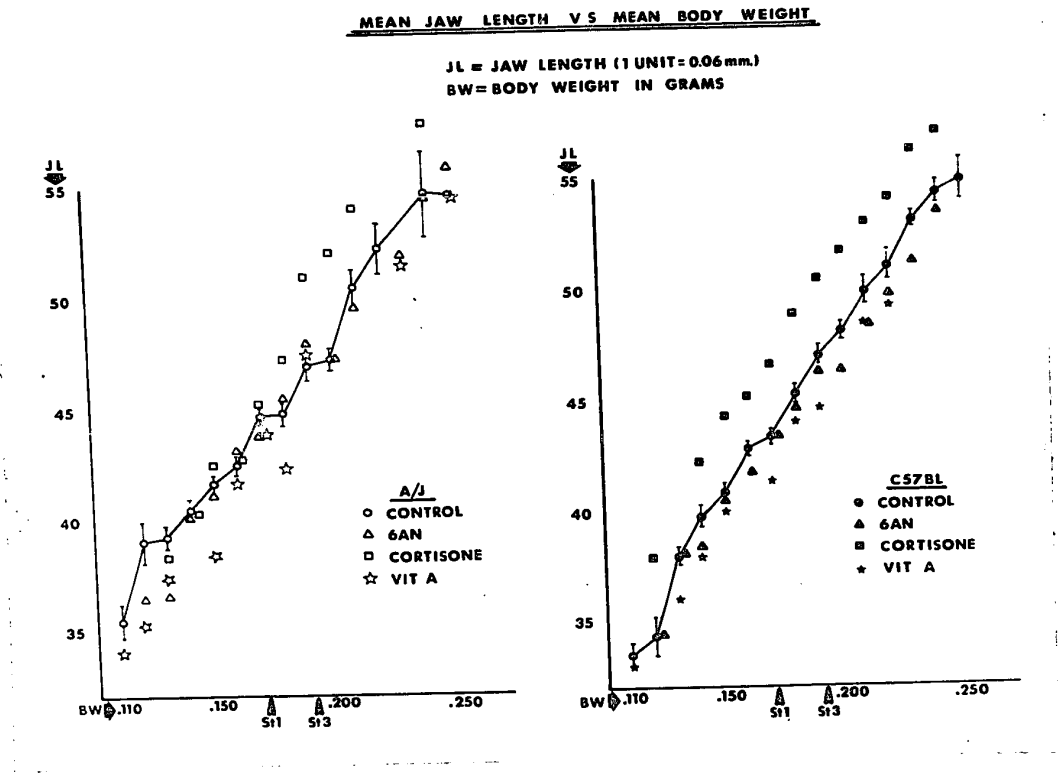
Table 12b

Mean jaw length vs body weight

C57BL

BW	Control	6 AN	Cortisone	Vitamin A
.100-.109				
.110-.119	33.50			
.120-.129	36.21		38.00	
.130-.139	37.92	40.50		36.17
.140-.149	39.79	38.75	42.58	
.150-.159	40.93	40.75	44.50	40.33
.160-.169	42.82	41.90	45.22	41.91
.170-.179	43.39	43.77	46.79	41.50
.180-.189	45.29	44.75	49.00	44.25
.190-.199	46.97	46.50	50.62	44.85
.200-.209	48.10	46.50	51.83	49.33
.210-.219	49.83	48.73	53.13	48.92
.220-.229	51.00	49.95	54.18	48.50
.230-.239	53.10	51.63	56.33	49.36
.240-.249	54.23	53.65	55.67	45.00
.250-.259	54.88	52.44		50.00
.260-.269	54.67	54.38		47.67
.270-.279		56.50	60.00	56.00
.280-.289				56.75
.290-.299	56.50			57.33
.300-.309	61.17			
.310-.319			64.67	61.00
.320-.329				60.00
.330-.339				61.33
.340-.349				63.00
.350-.359	62.00			
.360-.369				

Figure: 9



Note: Arrows indicate Stage 1 and Stage 3 of palate closure.

In Figure 9 mean jaw length of embryos of treated groups are plotted against body weight in both strains.

6. Chronological age and jaw length in A/J control and treated groups.

Since there is a 4 to 6 hour difference between the time of collection of embryos in control and treated, the above observation of effect of treatment could be partly due to the age difference of the embryos in the control and treated group. A simple regression analysis and an analysis of covariance was carried out comparing jaw length and chronological age in the treated and control groups of the A/J strain. Chronological age was taken as the independent variable and the length of lower jaw as the dependent variable. The results of the regression analysis are presented in Table 13. Mean lower jaw length of the treated embryos are significantly different from that of control embryos ($P: < 0.001$ in all three treatments). There is significant difference in the slope of vitamin A treated embryos ($P: < 0.001$), but the 6 AN and cortisone-treated slopes are not different from the control slope. The difference between adjusted means of jaw length of the control and treated groups was tested by simple analysis of covariance, adjusted for chronological age. The results of this test are shown in Table 14. After correction, mean jaw length of the A/J cortisone-treated group is significantly longer than that of the control group ($P: < 0.001$). The mean jaw length of 6 AN- ($P: > 0.75$) and vitamin A- ($P: = 0.25-0.1$) treated embryos do not differ significantly from that of controls.

Table 13

Regression analysis in A/J control and treated groups

chronological age vs lower jaw length

	Control	6 A N	Cortisone	Vitamin A
Number	80	114	76	68
Mean chron. age	15.15	22.67	22.14	21.98
Mean jaw length	42.52	46.26	48.50	45.48
b	0.306	0.537	0.201	1.37
t	2.42	4.7	1.175	5.07
P	0.025-001	<0.001	0.4-0.2	<0.001
Fittness	5.86	23.34	1.38	25.78
P	0.025-0.01	<0.001	0.25-0.1	<0.001
Joint regression		31.5918	4.401	27.124
P		<0.001	0.05-0.025	<0.001
Comparison of slopes		1.4673	0.190	14.8866
P		0.25-0.1	0.75-0.50	<0.001
Comparison of means		31.9534	44.205	14.995
P		<0.001	<0.001	<0.001
Comparison of residual variance		2.99	3.90	2.192
P		<0.001	<0.001	<0.001

Table 14

Analysis of covariance in A/J control and treated groups

chronological age vs lower jaw length

	6 A N	Cortisone	Vitamin A
Mean adjusted age	19.57	18.12	18.29
Mean adjusted jaw length treated	44.79	47.77	42.49
control	44.61	43.21	44.69
b	0.473	0.234	0.693
t	7.7	2.63	7.7
P	<0.001	0.01-0.001	<0.001
Difference between the two corrected jaw length			
F	0.046	16.472	1.999
P	>0.75	<0.001	0.25-0.1
Fitness of points			
F	31.52	4.42	24.75
P	< 0.001	0.05-0.025	<0.001

7. Comparison of lower jaw growth in two control groups.

Regression analysis and analysis of covariance was also carried out comparing the jaw length and chronological age, and jaw length and body weight in A/J and C57BL control embryos. Table 15 contains the results of regression analysis on both chronological age and body weight to jaw length in A/J control and C57BL control embryos. When analysis is based on jaw length versus chronological age, the two strains differ significantly in slopes (P: 0.01-0.005) and mean jaw length (P: $<$ 0.001). When analysis is based on jaw length versus body weight, there is still a significant difference between the two mean jaw lengths (P: $<$ 0.001), but the slope difference disappears. This implies that the rate of jaw growth does not differ in the two strains under consideration when body weight is used as parameter. Furthermore, after adjustment of body weight, the lower jaw of A/J strain is significantly longer than the control C57BL embryos (P: $<$ 0.001). The difference in the jaw length at adjusted body weight is, however, very small (0.589m.u.) and it might not have any biological meaning (See Table 16).

Table 17 and Table 18 show the results of an analysis of covariance and a simple regression analysis of the two control groups between morphological rating (MR) and jaw length (JL); and palate stage (PS) and jaw length (JL). A highly significant difference in slope and mean were observed when MR was used as the abscissa. The results show that with adjustment for the MR, the C57BL embryo has a significantly longer jaw than the A/J

Table 15

Regression analysis between A/J control and C57BL control embryos

	chronological age vs jaw length		body weight vs jaw length	
	A/J	C57BL	A/J	C57BL
Number	80	94	129	94
Mean age	15.15	8.62	Mean B.W. 0.170	0.187
Mean jaw length	42.52	45.28	43.80	45.28
b	0.306	0.814	126.3768	134.382
t	2.42	7.10	29.77	29.49
P	0.025-0.01	<0.001	<0.001	<0.001
F	5.86	50.45	883.73	864.6
P	0.025-0.01	<0.001	<0.001	<0.001
Joint regression F	60.09		F 1752.165	
P	<0.001		P <0.001	

To follow...

Table 15

(suite)

Comparison of slopes				
	F	7.671	F	1.5578
	P	0.01-0.005	P	0.25-0.1
Comparison of means				
	F	18.16	F	6202.41
	P	<0.001	P	<0.001
Comparison of residual variance				
	F	2.15	F	1.327
	P	0.005-0.001	P	0.1-0.05

Table 16

Analysis of covariance between A/J and C57BL controls

	chronological age vs jaw length			body weight vs jaw length	
	A/J	C57BL		A/J	C57BL
Mean adjusted age	11.62	11.62	mean adjusted body weight	0.177	0.177
Mean adjusted jaw length	40.198	47.254		44.709	44.12
b	0.657	0.657		129.883	129.883
t	3.2	3.2		2.805	
P	0.001	0.001		0.01-0.001	
Difference between the two corrected mean jaw length	F	65.92		F	11.25
	P	0.001		P	0.001
Goodness of fit	F	9.39		F	1746
	P	0.005~0.00		P	<0.001

Table 17

Regression analysis between A/J control and C57BL control embryos

morphological rating vs jaw length	A/J	C57BL
Number	80	94
Mean MR	7.90	7.73
Mean jaw length	42.518	45.28
b	0.822	1.453
t	10.673	29.88
P	<0.001	<0.001
F	111.967	893.155
P	<0.001	<0.001

palate stage vs jaw length	A/J	C57BL
Number	59	128
Mean PS	1.457	2.117
Mean jaw length	44.81	45.563
b	2.052	2.546
t	6.8	16.55
P	<0.001	<0.001
F	46.24	274.176
P	<0.001	<0.001

To follow...

Table 17

(suite)				
Joint regression	F P	775.69 <0.001	F P	297.405 <0.001
Comparison of slopes	F P	50.937 <0.001	F P	2.463 0.25-0.1
Comparison of means	F P	76.144 <0.001	F P	55.29 <0.001
Comparison of residual variance	F P	1.414 0.05	F P	1.539 0.05-0.025

Table 18

Analysis of covariance between A/J and C57BL control embryos

		morphological rating vs jaw length	palate stage vs jaw length
Mean MR		7.807	1.909
Mean jaw length		44.011	45.326
b		1.2034	2.4117
t		25.129	17.939
P		<0.001	<0.001
Difference between the corrected jaw length	F	68.017	6.864
	P	<0.001	0.01-0.005
Fitness of points	F	239.021	295.057
	P	<0.001	<0.001
Mean adjusted jaw length	C57BL	45.375	45.061
	A/J	42.405	45.448

embryos ($P: < 0.001$). Tables 9 and 10 and Figure 4 also show that C57BL has the longer jaw although its palate closure occurs at lower MR than that of the A/J. The rate of growth of the jaw is also different in the two strains. There is no significant difference between the slopes of the regression lines of JL on PS. That is, the jaw grows at the same rate in both strains during palate closure but the mean JL of A/J is significantly longer than C57BL after correction for palate stage. Thus, the differences seen graphically in Figure 3 indicate that the A/J jaw is indeed longer at least during early palate closure stages 0 to 3.

8. Chronological age and lower jaw length in C57BL control and treated groups.

A regression and a covariance of C57BL between jaw length and chronological age, are summarized in Tables 19 and 20. There is no difference in rate of growth of the lower jaw when the treated groups are compared to the control, but a significant difference between the means of control and treated. When the adjustment for chronological age between the treated and control groups is made, no difference is found in length of lower jaw in the vitamin A treated and cortisone treated groups, but the 6 AN treated embryos lower jaws remain, as before adjustment, longer than the control. Again as with the A/J strain, it is clear that lower jaw length is not reduced by any of the three treatment during the period of palate closure.

The above findings point to the fact that there is no retardation of lower jaw growth when susceptible A/J strain is compared to resistant C57BL strain at the time of palate closure.

Table 19

Regression analysis in C57BL control and treated group

chronological age vs lower jaw length

		Control	6 A N	Cortisone	Vitamin A
Number		94	110	72	99
Mean chron. age		8.617	17.71	14.208	14.277
Mean jaw length		45.28	49.03	50.576	49.24
a		0.814	0.713	0.729	0.735
b		7.10	6.646	5.84	7.41
P		<0.001	<0.001	<0.001	<0.001
Fitness of points		50.45	44.048	34.12	55.00
P		<0.001	<0.001	<0.001	<0.001
Joint regression					
F			143.84	84.097	85.252
Comparison of slopes	F value		0.622	0.255	0.199
	P		0.5-0.25	0.75-0.5	0.75-0.5
Comparison of means	F		41.59	47.86	19.90
	P		<0.001	<0.001	<0.001
Comparison of residual variance	F		1.53	1.19	2.40
	P		0.05-0.025	0.25-0.1	<0.001

Table 20

Analysis of covariance in C57BL control and treated groups

chronological age vs lower jaw length

		6 A N	Cortisone	Vitamin A
Mean adjusted age		13.52	11.04	11.52
Mean adjusted jaw length				
	treated	45.878	48.127	47.147
	control	41.587	47.156	47.485
b		0.753	0.774	0.759
t		13.46	10.796	10.449
P		<0.001	<0.001	<0.001
Difference between the two corrected jaw length				
F		20.58	1.168	0.116
P		<0.001	0.25-0.1	0.75-0.5
Fitness of points				
F		221.774	164.169	105.483
P		<0.001	<0.001	<0.001

They also indicate that treatment with the three teratogens under consideration did not affect the lower jaw length during palate closure even after time adjustment was made. It is highly unlikely that cleft palate induced in these instances is a direct result of growth retardation of the lower jaws.

B. The relation of tongue, lower jaw growth and palate closure.

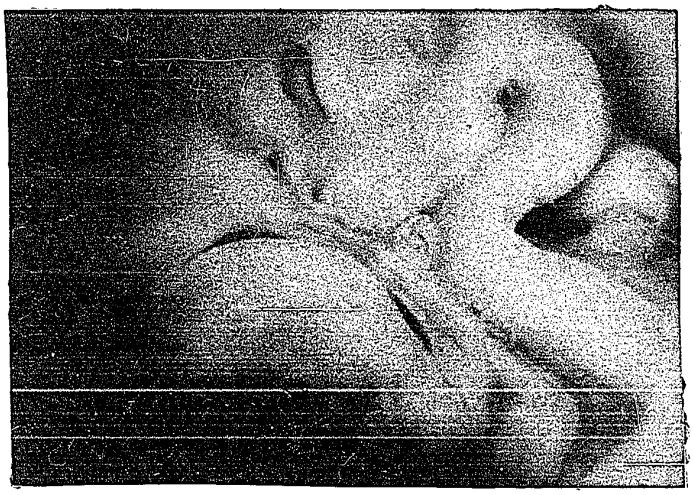
The length, width and thickness of the tongue in embryos of control and treated groups were measured according to the illustration in Figure 1. Tongue growth was analyzed using the relation of its position relative to the primary palate and to lower jaw length (See Figures 10a, b, c). Figures 11a and b compare the position of the tongue relative to the primary palate at different palate stages and lower jaw lengths in A/J and C57BL control and cortisone-treated embryos. The horizontal axis represents mandible growth and the vertical axis indicates state of the palate. In the jaw length during palate closure (38 m.u. to 48 m.u.) in the control groups, more C57BL embryos (16) than A/J embryos (8) have a forwardly displaced tongue, whereas only 4 C57BL embryos have their tongues behind and under the primary palate, in contrast to A/J embryos (18). Chi square tests either by Fisher's exact method or with Yates correction have shown the difference to be highly significant ($p < 0.001$). There is a tendency for the A/J tongue to stay behind or just under primary palate in stage 0 and stage 1, whereas the C57BL tongue seems to be able to move forward beyond primary palate even before the start of palate closure at stage 1. The same

Figure 10

Tongue position relative to primary palate during palate closure.

A) tongue behind primary palate.

B) tongue under primary palate.



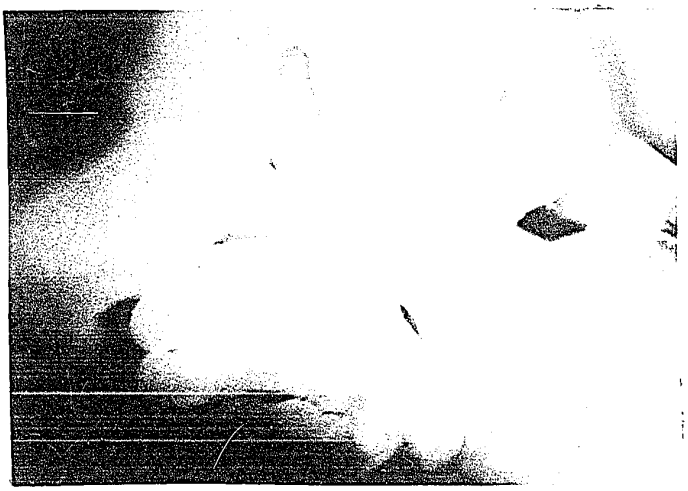
C) tongue over primary palate. (Stage 0).

Figure 10

Tongue position relative to primary palate during palate closure.

A) tongue behind primary palate.

B) tongue under primary palate.



C) tongue over primary palate. (Stage 0).

Figure: 11a

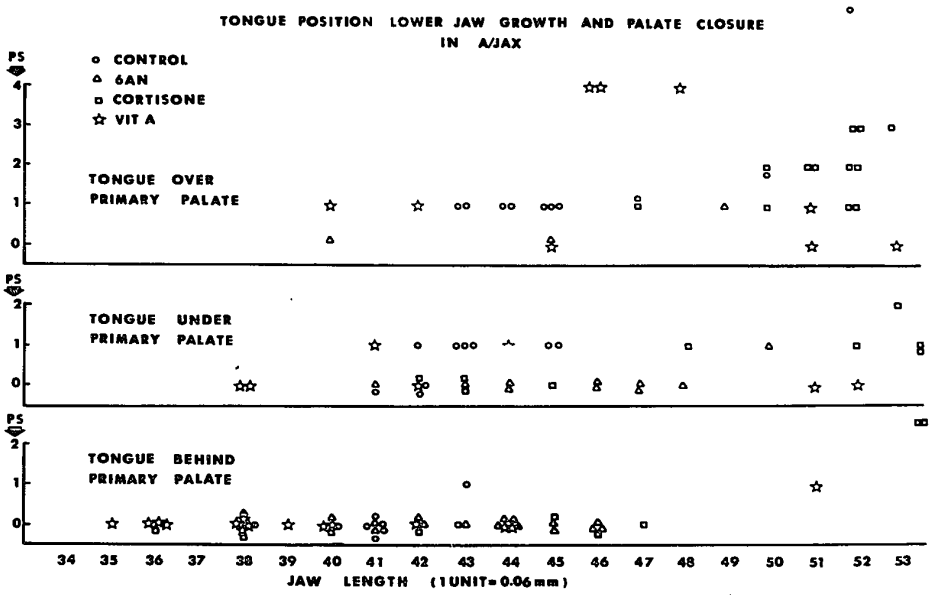
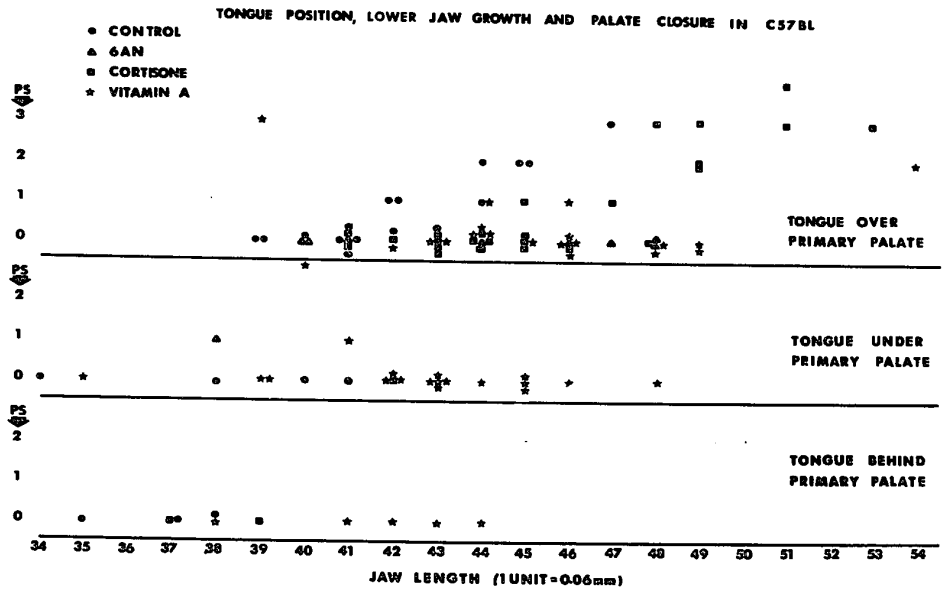

TONGUE POSITION LOWER JAW GROWTH AND PALATE CLOSURE
IN A/JAX
PS
CONTROL
6AN
CORTISONE
VIT A
TONGUE OVER
PRIMARY PALATE
TONGUE UNDER
PRIMARY PALATE
TONGUE BEHIND
PRIMARY PALATE
34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
JAW LENGTH (1 UNIT = 0.06 mm)

Figure: 11b


TONGUE POSITION, LOWER JAW GROWTH AND PALATE CLOSURE IN C57BL
CONTROL
6AN
CORTISONE
VITAMIN A
PS
TONGUE OVER PRIMARY PALATE
TONGUE UNDER PRIMARY PALATE
TONGUE BEHIND PRIMARY PALATE
34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
JAW LENGTH (1 UNIT=0.06mm)

trend is observed in these two control embryo groups when position of the tongue under consideration is compared with palate stage and morphological rating (Figures 11c and d). Treatment with cortisone does not affect forward displacement of the tongue in C57BL embryos (p: = 0.001). 6 AN and vitamin A treatments also influence movement of the tongue beyond primary palate in both strains.

In Table 21, the numbers of embryos of jaw length 41-50mμ in control and treated groups are shown according to the position of tongue relative to primary palate. Chi-square test have been done between control and treated groups for both strains to test for any effect of teratogen on tongue position. There is a significant difference (P = .05-.025) between A/J control and A/J 6 AN-treated and a significant difference (P = .01-.005) between C57BL control and vitamin A-treated C57BL embryos.

Table 22 compares the tongue positions of control embryos and embryos treated with amniotic sac puncture at each palate stage. There is a tendency for embryos with open palate to have their tongue behind the primary palate, whereas embryos with forward, lowered tongue have their palate closed.

C. The effect of teratogens on developmental features.

The strain difference in palate closure of A/J and C57BL embryos is based on chronological age and morphological rating. Treatment delays the time of palate closure relative to these two criteria. The effect of the teratogens on indi-

Figure: 11c

Tongue position, morphological rating and palage stage in A/J and C57BL control embryos.

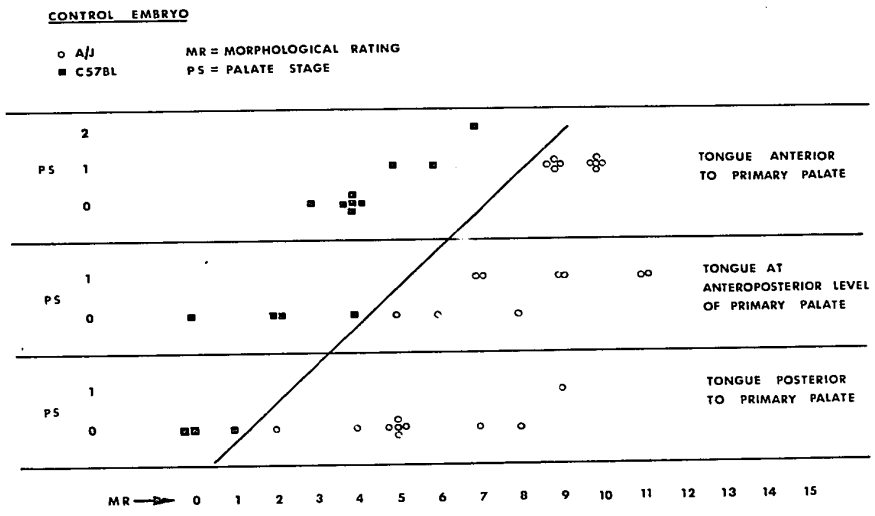


Figure: 11d

Tongue position, morphological rating and palate stage in A/J and C57BL embryos treated with cortisone.

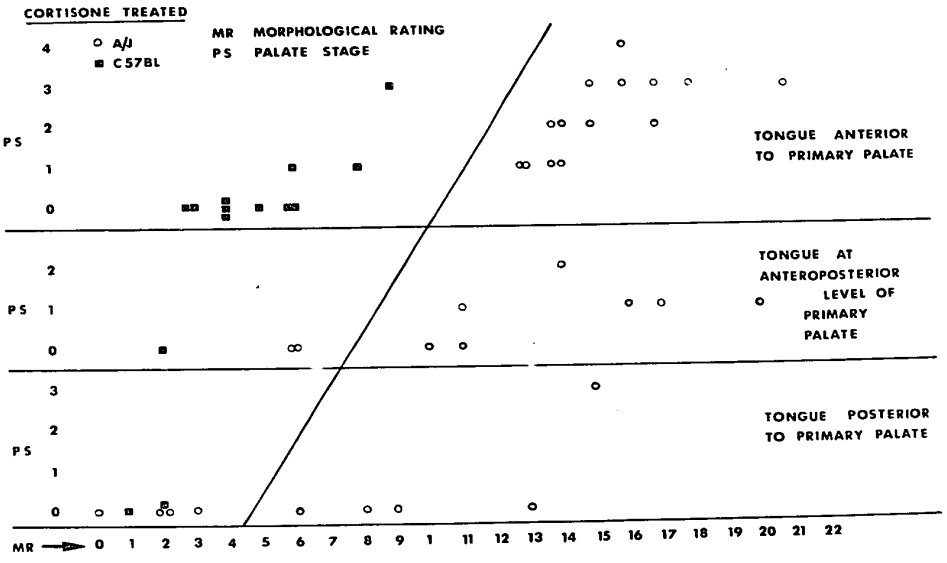


Table 21

Effect of teratogens on tongue position relative to primary palate within the range of jaw length 41-50mu.

A/J embryos		T* behind P.P.**	T. over P.P.	Total
	Control	16	9	25
	6 AN	24	2	26
	Cortisone	9	3	12
	Vitamin A	7	5	12
C57BL embryos				
	Control	1	13	14
	6 AN	0	6	6
	Cortisone	1	22	23
	Vitamin A	19	17	36

*T: tongue
**P.P. : primary palate

	X^2 and P. of control and treated			
	A/J		C57BL	
	X^2	P.	X^2	P.
6 AN	4.48	0.05-0.025	3.21	0.1-0.05
Cortisone	0.086	>0.9	0.15	0.9-0.5
Vitamin A	0.48	0.5-0.1	6.95	0.01-0.005

Note: The jaw length 41 is the first measurement at which A/J control embryo with the tongue under primary palate is found.

Table 22

Comparison of status of tongue in embryos of control and amniotic sac punctured embryos in C57BL

	Control embryos		Amniotic sac control horn		Punctured embryos	
	T*over P.P.**	T behind P.P.	T over P.P.	T behind P.P.	T over P.P.	T behind P.P.
PS						
0	3/7	4/7	1/1	0/1		2/2
1	6/6	0/6	1/1	0/1	1/3	2/3
2	5/5	0/5				
3	6/6	0/6	1/1	0/1		
4	8/8	0/8				3/3
5	2/2	0/2				1/1
6	1/1	0/1	3/3	0/3		
7	13/13	0/13	16/16	0/16	10/10	

*T: tongue

**P.P.: primary palate

vidual morphological features was looked at in relation to chronological age and the data are summarized in Tables 23a and 23b. The average scores of four features (hair-follicles, ears, forefeet and hind feet) in control and treated embryos are presented here. The scores of eyes development are not included here, for eye lid fusion takes place after palate closure. 6 AN tends to reduce morphological scores in every feature of all age groups in both A/J and C57BL embryos. The effect of Vitamin A on limbs is somewhat confused due to deformities induced by the teratogen. Inhibition of morphological development is more obvious in the limbs of A/J embryos but the hair follicles do not seem to be affected. A similar trend is also noticed in Vitamin A-treated C57BL embryos. Cortisone generally produces an increase in morphological scoring in each of the features from D15/2hr on in A/J, and from D14/12hr on in C57BL. However, in A/J embryos at the earlier time of D14/16hr to D14/20hr, the opposite phenomenon is observed with cortisone treatment. This difference may be due partly to small sample size, and partly to the inaccuracy and undependability of using chronological age as criteria for embryonic development. The data can only be considered as suggestive as to how each teratogen affects differentiation in the two strains under observation. Figure 12 presents graphically the effect of cortisone on morphological rating in the two strains.

Table 23a

Developmental features and chronological age: A/J embryos

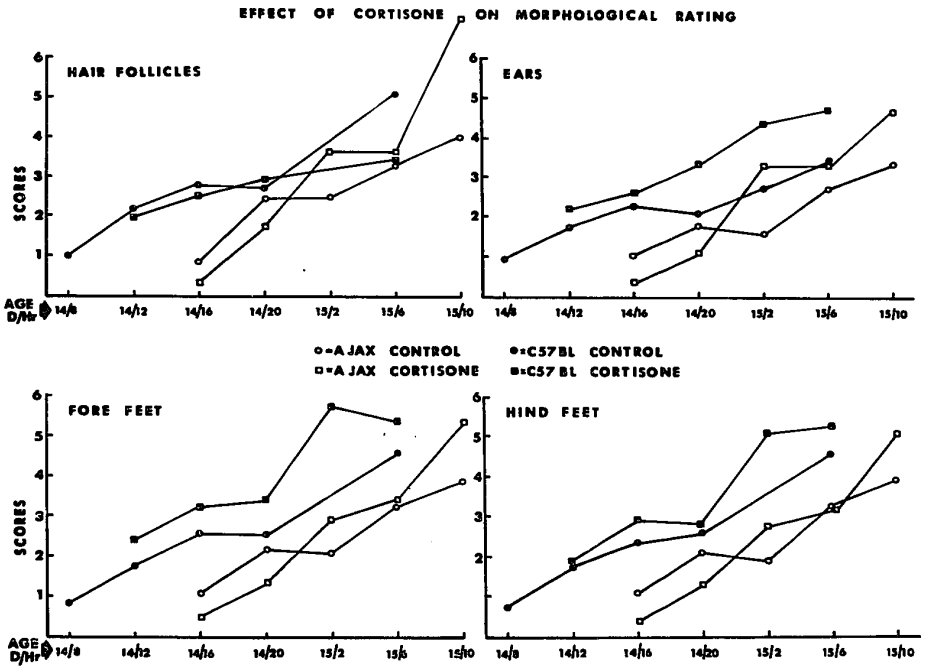
Developmental features	Treatment	chronological age D14/16h	D14/20h	D15/2h	D15/6h	D15/10h
Hair follicles	Control	0.91	2.25	2.50	3.35	4.0
	6 AN		1.78	2.22	2.72	2.82
	Cortisone	0.38	1.78	3.65	3.63	7.0
	Vitamin A		0.56	3.07	2.66	3.71
Ears	Control	1.03	1.87	1.64	2.70	3.31
	6 AN		1.75	2.14	2.22	2.00
	Cortisone	0.38	1.17	3.37	3.38	4.71
	Vitamin A		0.81	2.25	1.80	2.57
Forefeet	Control	1.06	2.14	2.05	3.20	3.75
	6 AN		2.08	2.38	2.81	2.56
	Cortisone	0.50	1.33	2.99	3.46	5.36
	Vitamin A		0.44	2.39	1.95	2.71
Hind feet	Control	1.09	2.07	1.91	3.20	3.88
	6 AN		1.92	2.29	2.81	2.59
	Cortisone	0.38	1.28	2.71	3.04	5.00
	Vitamin A		0.44	2.32	1.98	2.79

Table 23b

Developmental features and chronological age: C57BL embryos

Developmental features	Treatment	chronological age D14/8h	D14/12h	D14/16h	D14/20h	D15/2h	D15/6h
Hair follicles	Control	1.0	2.21	2.85	2.72		5.13
	6 AN			2.08	1.82	3.17	3.18
	Cortisone		1.91	2.52	2.88	5.20	3.40
	Vitamin A		2.93	2.97	2.44		5.07
Ears	Control	0.94	1.76	2.34	2.12		3.44
	6 AN			2.08	1.78	2.80	2.93
	Cortisone		2.21	2.60	3.35	4.47	4.70
	Vitamin A		2.48	2.67	1.69		3.59
Forefeet	Control	0.80	1.76	2.56	2.55		4.50
	6 AN			2.17	1.82	2.97	3.73
	Cortisone		2.47	3.26	3.43	5.77	5.30
	Vitamin A		1.88	2.08	1.50		4.48
Hind feet	Control	0.8	1.69	2.35	2.44		4.50
	6 AN			2.08	1.73	2.90	3.68
	Cortisone		1.85	2.90	2.76	5.07	5.10
	Vitamin A		1.88	2.08	1.50		4.28

Figure: 12


EFFECT OF CORTISONE ON MORPHOLOGICAL RATING
HAIR FOLLICLES
EARS
FORE FEET
HIND FEET
SCORES
AGE D/Hr
14/8
14/12
14/16
14/20
15/2
15/6
15/10
o=AJAX CONTROL
□=AJAX CORTISONE
●=C57BL CONTROL
■=C57BL CORTISONE

D. Prevention of cleft palate induced by 6 AN with nicotinamide-adenine dinucleotide (NAD).

Four C57BL females and three A/J females were given NAD after 6 AN injection either simultaneously or 2 hours after treatment. The amount of NAD injected was 5 to 10 times greater per kilogram body weight in comparison to 6 AN. Females were allowed to give birth over wire and the newborns were examined for cleft palate (Table 24). Fisher's exact X^2 test on the 0/29 CP incidence in the C57BL strain compared with expected 68% CP (13/19) showed that the result is highly significant. ($P: < 0.001$). This test was carried out to support the hypothesis that 6 AN affects normal palatal development in the embryos by interfering with utilization of NAD.

E. Measurement of intercartilage distance.

The measurement of the lower jaw in the present study involves the microscopic observations of the distance between the front tip of the lower jaw to the opening of the thyroid duct (Figure 1). This does not in fact measure the length of the mandible cartilage body, and it is possible (though unlikely) that the length of the mandible cartilage might be affected by any of the teratogens without affecting the entire mandible length, since the mandible included considerable soft tissue. Twenty-seven C57BL embryos which were investigated for the effect of amniotic sac puncture were stained with Alcian Blue and the distance between the anterior tip of Meckel's cartilage and the hyoid cartilage was measured in each embryo.

Table 24

Prevention of CP by NAD

Strain A/J	female	6 AN	NAD	Nicotinamide	Number offspring	Number CP	Number CLP
	59	3/4 dose	2mg	+	2	0	0
	900	3/4 dose	4mg	-	1	0	1
	901	3/4 dose	4mg	+	9	0	2
Total	3				12	0	3
C57BL	896	1 dose	4mg	-	9	0	0
	897	1 dose	2mg	+	9	0	0
	910	1 dose	4mg*	-	7	0	0
	914	1 dose	2mg	+	4	0	0
Total	4				29	0	0

*NAD given 2 hours after 6 AN injection.

The intercartilage distance was then compared to the usual measurement. A linear relation exists between the two modes of lower jaw measurement. The result is shown in Table 25. It is probably quite satisfactory to use the present method of measuring distance involving soft tissue for a relatively precise reflection of the growth of the lower jaws. The data in Table 25 seems to indicate that amniotic sac punctured embryos show little evidence of retarded jaw growth.

F. Cranial base angles.

Nine A/J and 19 C57BL control embryos were used in this preliminary survey. Measurement of the cranial base angle were made on photographs taken of the horizontally placed cut surface of Alcian Blue stained embryos. The angle between the anterior and posterior portion of the cranial base at the point of the craniopharyngeal canal is measured as indicated in the accompanying Figure 13.

Since the measurement was done on embryos after removal of tongue and the lower jaws for the purpose of examining palate stage, a possibility of distortion of the cranial base angle exists. However, the data supports the idea that the angle increases and the cranial base straightens during palate closure in both strains. The cranial base angles of A/J tend to be larger than C57BL at stage 0 and stage 1 of palate closure. Further experiments are in progress using this method to analyze the effect of the teratogens on cranial flexure.

Table 25

Intercartilage distance vs soft tissue jaw length in C57BL embryos treated with amniotic sac puncture

Control embryos		Punctured embryos	
Cartilage distance	soft tissue distance	Cartilage distance	soft tissue distance
50	79.5	44	69
48	77.5	44	71
44	73	34	57
37	58	32	55
35	59	34	58
35	58.5	37	62
36	60.5	30	55
31	49	38	63
34	55.5	36	64
30	55	34	55
37	60	33	58
37	62	32	52
38	62		
34	57		

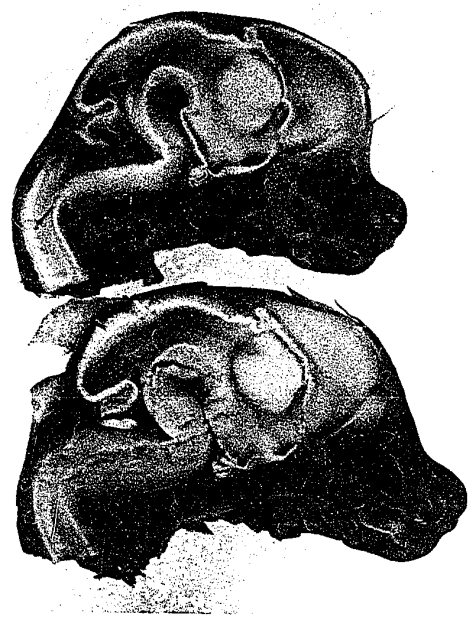
Table 26

Cranial base angle in A/J and C57BL control embryos during palate closure

	Stage 0	Stage 1	Stage 4	Stage 5	Stage 6	Stage 7
A/J	170	175				177
	167	174				
	164	172				
		170				
		163				
C57BL	161	158	169	171	163	170
	161	161	170	168	168	180
	161		174		168	
	164				171	
	164				173	

Figure: 13

Cranial base angle of A/J and C57BL embryos during palate closure.



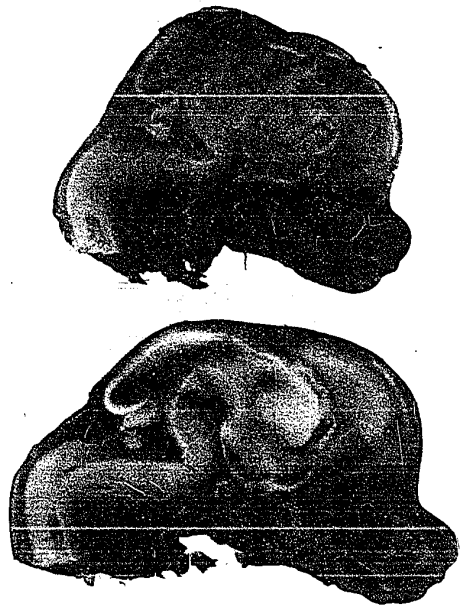
A/J

C57

Figure: 13

Cranial base angle of A/J and C57BL embryos during palate closure.

A/J



C57

V. DISCUSSION

A. The growth of the lower jaw during palate closure in A/J and C57BL control embryos.

In this investigation the normal growth pattern of the lower jaw in the two strains was studied during palate closure time using several growth parameters (chronological age, morphological age, and also body weight).

There is a steady increase in the length of the lower jaw at each progressive palate stage. The average increase in the length of the jaws in the two strains is quite similar, from stage 1 to stage 6 where in A/J it is 6.63 mu and in C57BL, 7.19 mu. At initiation of palate closure (stage 0 to stage 1), the A/J lower jaw is longer than C57BL but by stage 7 with completion of palate closure the C57BL lower jaws seem to be longer. The question of whether this represents a true higher growth rate of the lower jaw in C57BL which could account for a strain difference in cleft palate production was considered. Chronological age, morphological rating and body weight were used as growth parameters to look for evidence for the presence of a growth spurt in C57BL.

When analyzed by adjusting the chronological age of the two strains, the length of the lower jaw in the C57BL strain appeared significantly longer than the A/J strain. Since chronological age, due to high variability in the short time span, does not really reflect the true growth parameter, the length of the jaws of the two strains can be compared at each

morphological rating. When this comparison was made it turned out that at the MR in each strain corresponding to palate closure C57BL had tended to have significantly longer jaws (Table 9 and Table 10, Figure 4) than A/J embryos. The morphological rating adjusted mean jaw length in C57BL embryos is significantly larger than that of the A/J embryos and also the rate of growth of the lower jaw in relation to morphological rating is significantly greater in C57BL than A/J embryos. In relation to the body weight of the embryos, there is no significant difference in the rate of growth of the lower jaw between the two strains but the difference in mean length of the two were significant. After adjustment of body weight the A/J lower jaw is significantly longer than the C57BL lower jaw. Covariance analysis on palate stage and jaw length between the two control embryo groups also showed that after adjusting for palate stage, the jaw in A/J is significantly longer than in the control C57BL embryos. When the body weight of A/J and C57BL control embryos are compared at corresponding palate stages (Table 11a, 11b,Figure 6), they are found to be the same. This can be interpreted to mean that for palate closure to come about it is essential that the embryo attain a particular range of body weight; that is jaw growth and weight gain of the embryo are closely related processes. The strain difference which is observed in MR then has little meaning in relation to palate closure. Thus, A/J embryos

close their palates at a greater chronological age with higher morphological rating than C57BL but with the same range of body weight. There is little evidence for strain difference when body weight is considered but differences are noticed when age and MR are considered. This means that body weight can be a better parameter for judging growth of the embryo. The conclusion reached regarding normal palate closure is that there is neither difference in the rate of lower jaw growth between the two strains, nor is there a significant difference in jaw length which could account for a strain difference in the susceptibility of A/J to cortisone induced teratogenesis. The finding that there is at palate closure time, the same range of body weight and similar growth rate and length of the lower jaws favours the idea that lower jaw growth is not a factor in the strain differences in susceptibility to cleft palate.

B. Effect of teratogens on growth of lower jaw.

The growth of the lower jaw is not retarded at the time of palate closure in treated embryos of either strain. Statistical analysis has shown that when samples of control and treated embryos from each strain are corrected for age difference, no significant difference is observed between 6 AN- and cortisone-treated A/J and control A/J, or between 6 AN-, cortisone- and vitamin A-treated C57BL and control C57BL in the rate of growth of the lower jaws. Furthermore, none of the mean jaw lengths of the treated groups was shorter

than that of control embryos after age adjustment.

A trend toward decreased jaw length in embryos of higher MR in cortisone-treated C57BL embryos is probably due to an enhancing of MR by cortisone (Walker, 1954; Trasler, 1958); this trend toward enhanced MR is not as marked for the A/J strain. At the MR corresponding to palate closure there is no shortening of the jaw length in all three teratogen-treated embryos for both strains.

In the A/J strain, except at a lower weight range, the jaw length of treated embryos at body weights corresponding to palate closure (0.170-0.220 gm.), does not differ significantly from that of A/J control embryos, but cortisone-treated C57BL embryos have significantly longer jaws than those of control embryos at corresponding body weight. A slight trend toward increased jaw length is also observed in A/J embryos at a higher body weight range. The reduction of embryonic weight by cortisone treatment (Fraser, Chew and Verrusio, 1967) is probably responsible for the slight increase in jaw length in cortisone-treated embryos. The effect of cortisone on MR and body weight is more obvious in C57BL than A/J strain.

Several types of analysis used in the present investigation all indicate that there is no evidence of mandibular shortening which could be implicated as the cause for induced cleft palate by 6 AN, cortisone and vitamin A. All three teratogens are known to affect fetal development (body weight, MR, etc.,). When mandible length is analyzed on these growth

parameters, it is difficult to assess the interaction between the parameters and their interaction with the lower jaw growth in the treated animals. A multiple regression analysis may provide a general solution to this problem.

C. Effects of cortisone treatment on lower jaw growth.

Deuschle and Kalter (1962) reported that mandibles of cortisone treated A/J newborns with cleft palate were relatively longer than the control mandibles; this size difference was due solely to a relative increase in the length of the premolar corpus. There was no difference in mandible length between control mice and A/J spontaneous cleft lip animals. They also reported that in man, cleft lip and palate is accompanied by shortening of the mandible, contrary to the findings in mice. The observations in mice were based on a very limited sample, and were not related to the critical period of palate closure itself; thus, little information was provided on to the role of the mandible in palate embryogenesis. A different result was presented by Schwartz (1967), who found significant shortening in the mean mandible length of cortisone-treated A/J newborns. He also noticed shortening of the posterior and anterior heights of the mandible in these animals compared with untreated controls, but not significantly different from a vehicle-treated group. He stated that a close relationship exists in cortisone-induced cleft palate and micrognathia. Neither of these studies examined the cause and effect relationship between micrognathia and induced cleft palate during the time of palate closure, and thus, they contribute little

regarding the role of the mandible in palate closure.

Cortisone exerts little or no site-specific teratogenic action. Although cleft palate is the predominant lesion, other embryopathic features are also observed in the offspring of cortisone treated mice. Fraser and Fainstat (1951) reported shortening of the head, spina bifida and shortening of the mandible in cortisone-treated newborn mice. Observations of embryos treated with cortisone during the time of normal palate closure, however, did not show evidence for growth retardation of the head and palatine shelves (Walker and Fraser 1957). A relative reduction in shelf width was observed by the same authors at times beyond normal palatine closure. The present investigation has demonstrated that there is no evidence of growth retardation in the lower jaw in the experimental group which could suggest this as a causal factor of the cleft palate induced by cortisone treatment. This does not mean there is no untoward effect of cortisone on the embryos.

Cortisone exerts its effect on the metabolism of water electrolytes, proteins, fat and carbohydrates. The hormone influences basic physiologic processes including cell permeability, nucleic acid and protein synthesis, and is known to induce cellular enzymes. Understanding of the mechanism of action of glucocorticoids is complicated by their opposing manifest actions, causing hyperplasia in some tissues, cytolysis in others. What is the primary mechanism of glucocorticoid

action? There is evidence for the existence of a common basic mechanism to account for the response of different tissues to the same hormone (Tomkins and Martin 1970). A stimulation of the synthesis specific proteins may be responsible for all the adrenal corticosteroid action. Studies on the growth of mouse lymphosarcoma cells indicated that action of the steroid involves the role of the specific receptor molecules in the mechanism of hormone action. Formation of a complex between a given steroid hormone and its specific receptor system is regarded as an obligatory first step in the action of hormones. Steroid hormones are assumed to act as inducers by antagonizing specific gene repressors, thereby increasing the rate of synthesis of specific messenger RNA (Catt 1970). The resultant accumulation of specific RNA accounts for enzyme induction by the steroid. An alternate model for this induction is that steroids act to stabilize messenger RNA rather than increase its synthesis. That is, the steroid hormone functions by regulating post-transcriptional events in gene expression.

Effects of corticosteroids on mammalian tissues have been extensively studied. Since our interest concerns its effect on palatal closure, only the effect of steroids on connective tissue and bone will be discussed here. The glucocorticoids are known to exert their influence on connective tissue primarily via mesenchymal cells. In non-physiological concentrations, glucocorticoids are known to inhibit fibroblast proliferation; morphological change and cell destruction often

accompany corticosteroid treatment. In contrast, corticosteroids in physiological concentration stimulate cell growth. In bone, the action of corticosteroids involves similar complex responses. The effect of cortisone upon bone is dose-dependent and time dependent; high doses suppress bone resorption, but low doses stimulate bone resorption. At low steroid concentration an increase in the number of osteoclasts and decrease in the number of osteoblasts is observed, whereas at high concentration, periodontal ligament fibroblast progenitor cell proliferation and bone formation are depressed. Cortisone also indirectly stimulates parathyroid hormone secretion which results in increased bone resorption (Jee et al., 1970).

The most important intercellular substances in connective tissue are the mucopolysaccharides. These compounds occupy key positions in physiological and pathological processes of the connective tissue. In addition to forming the structural basis for the amorphous intercellular substance, they also determine the distribution of water and electrolytes in connective tissue. High concentrations of glucocorticoids inhibit the synthesis of mucopolysaccharides. These steroids interfere with synthesis of chondroitin sulphate in cartilage, and inhibit proliferation of chondrocytes in cartilage. Thus, profound disturbances of endochondral bone formation are caused by cortisone in the rabbit (McClusky and Thomas, 1959). Boström and Odeblad (1953) reported that uptake of ^{35}S-sulphate by carti-

lage slices was diminished after incubation with cortisone. Glucocorticoids also inhibit synthesis of collagen by acting on the fibroblasts.

Corticosteroids exert a profound influence on carbohydrate and protein metabolism (Williams, 1968). Among the effects of corticosteroids on protein metabolism are an increase in protein breakdown and mobilization of amino acids from skin, muscle and bone, that is, there is a catabolic action of the steroids on protein. A simultaneous depression in synthesis of collagen, mucopolysaccharides and protein in general indicates the anti-anabolic influence of corticosteroids and reveals the complex actions of this hormone on proteins (Ebert and Prockop, 1963, 1967). Houck and Patel (1965) postulated that the corticosteroids may potentiate the release of a cellular protease into the extracellular compartment which would account for the induction of collagenolytic activity in the dermis.

The investigations so far discussed mostly concerned the study of corticosteroid action on adult connective tissues. Studies of glucocorticoid action on embryonic chick connective tissues have also demonstrated a catabolic action of corticosteroid on early extracellular embryonic connective tissue fibrils (Carlson and Low, 1971). There was a reduction in perinotochordal microfibrils 1 hour after treatment with corticosteroid with subsequent recovery by 24 hours. Inhibition of ^{35}S-sulphate incorporation into mucopolysaccharide, of ^{3}H

proline incorporation into collagen, and of ^{3}H proline into acid insoluble protein after corticosteroid treatment, has been shown in chick embryos (Ebert and Pyrockop, 1963; Ebert and Pyrockop, 1967). The growth of embryonic chick bone in organ culture was also inhibited by hydrocortisone (Fell and Thomas, 1961). At very low concentration, corticosteroids did not show significant inhibitory effect of the growth of embryonic chick tibiotarsi in organ culture and had no significant inhibitory effect on the synthesis of chondroitin sulphate by the bone (Schryver, 1965). Studies on radioactive ^{35}S-sulphate uptake by mesenchymal connective tissue of the palatine shelves in 11 to 17 days old mouse embryos have shown marked ^{35}S-sulphate incorporation in the connective tissue of the palatine shelves (Larsson, Boström and Carlsöö, 1959; Larsson, 1962a). Larsson, (1962b) studied the effect of cortisone on mucopolysaccharide incorporation in the A/J (100% CP) and CBA (12-20% CP) strains of mice, which differed in induced cleft palate frequency. Cortisone was shown to inhibit or reduce incorporation of ^{35}S-sulphate into palatine tissue mucopolysaccharides, but no difference could be demonstrated between the two strains by either the autoradiographic or the histochemical method (Larsson, 1962b). Loevy (1962) showed no difference in histological stainability of mucopolysaccharides between control and cortisone-treated Strong a mice. A 12-hour time difference in both movement of the shelves to the horizontal and staining intensity of

acid mucopolysaccharide existed between strain CD control and cortisone-treated embryos (Jacobs, 1964). In rats, although cortisone did not produce cleft palate in these embryos, significant reduction of ^{35}S-sulphate incorporation was observed in the palatine shelves (Nanda, 1970a). Based on this observation, Nanda concluded that cleft palate is not positively correlated with disturbance in mucopolysaccharide metabolism.

Abnormalities such as dilatation of the marginal venous sinus in both foreand hind-limb buds, sub-epithelial hemorrhage blisters in head (temporal region), neck, and both were noticed in both A/J and C57BL embryos in the present investigation. These abnormalities are presumably due to primary changes taking place in endothelial cells of the marginal venous sinuses (Jurand, 1968). An increase in the number of Golgi groups with primary and secondary lysosomes in their vicinity was noted on electron microscopic examination and overproduction of lysosomes and increased synthesis of hydrolytic enzymes were suggested (Jurand, 1968). This view is contrary to that of many people who consider corticosteroids lysosomal stabilizers (De Duve, Wattiaux and Wibo, 1961; Jacobson, 1964; Lloyd and Beck, 1969).

Umansky (1968) using an <u>in vitro</u> model with free mesenchymal cells from embryonic mouse limb bud studied the effect of cortisone on myogenesis and chondrogenesis. No effect of cortisone was found with low concentrations, but cell death was noticed with high concentrations. The abnormalities observed

include inhibition of compact cell aggregation. Chondrogenenis was inhibited with high concentration of cortisone. Disturbance of chondrogenesis by cortisone is related to stage of exposure and concentrations of the hormone. Cortisol was also found to interfere with embryonic myogenesis and the severity of the effect varied with duration of exposure and concentration.

A model was proposed to explain the cause of cleft palate induced by cortisone treatment, by Zimmerman, Andrew and Kalter (1970) who suggested inhibition of mRNA synthesis by glucocorticoid in the fetal palate which led to later inhibition of protein synthesis and to cleft palate.

Profound inhibition of RNA synthesis and protein synthesis was observed in whole C3H/AN embryonic homogenate after triamcinolone. A simultaneous inhibition of RNA synthesis (by triamcinolone) and protein (by cyclohexamide) synthesis reduced the incidence of cleft palate in the embryos (Zimmerman, Andrew and Kalter, 1970). The authors indicated that the growth inhibitory effect of steroid through inhibition of mRNA and protein synthesis is a possible mechanism for cleft palate in these embryos (Andrew and Zimmerman, 1971).

Inhibition of thymidine kinase by cortisone has been reported in Erlich cells (Kaneko and Lepage, 1970). Marked inhibition of DNA synthesis is a possible cause of cleft palate (Mott et al., 1969).

Based on the finding of cytosine arabinoside, a potent DNA synthesis inhibitor, which produces a large range of malformations with cleft palate, and specificity of glucocorticoids in producing cleft palate in mice, Zimmerman, Andrew and Kalter, (1970) did not consider inhibition of DNA synthesis as a cause of cleft formation. An autoradiographic study of ^{3}H proline uptake in the palate of normal mice showed continuous protein synthesis in palatine shelf before and during palate closure. In cortisone-treated group, normal protein synthesis is observed up to the time of normal palate closure where there is an abrupt decrease in protein synthetic activity. It is suggested that cortisone acts as teratogen by causing local alteration of fetal protein metabolism (Shapiro, 1969).

Inhibition of mouse fibroblasts in culture by glucocorticoid is probably associated with decreased synthesis of DNA and RNA, and inhibition of hexose uptake and amino acid transport. The hexoses inhibited include galactose, glucose, deoxy-glucose and fructose. It was suggested that an alteration in the phosphorylation and also the energy production or utilization in these cells is probably responsible for the disturbance in carbohydrate metabolism (Gray, Pratt and Aronow, 1971). This disturbance in carbohydrate is rather an attractive hypothesis for teratogenicity of corticosteroids.

The present study did not show alteration of mandible growth at the time of investigation. In view of the profound inhibitory effect of cortisone on growth of the embryos affecting

connective tissues, protein, carbohydrate and water metabolism, the possibility of micrognathia after birth is not excluded.

D. Effect of 6-aminonicotinamide on the skeletal system.

Murphy et al., (1957) have reported that 6 AN will produce gross skeletal abnormalities in chicks and produce a variety of skeletal malformations, including syndactyly, club feet, hare-lip, cleft palate and rib anomalies in rat embryos. Similar skeletal malformations of the tibia and foot such as oligodactyly, deformity of the ankle joint, fusion of neural arches, absence of vertebral bodies and defects of the supra occipital bone were noted in mice treated with 6 AN. The basic mechanism of 6 AN-induced teratogenicity therefore brings about a disturbance of chondrification of the mesenchyme and ossification of cartilage (Pinsky and Fraser, 1959).

Despite this marked tendency of 6 AN to induce skeletal changes, the present results indicate no obvious retardation during palate closure of lower jaw growth in either strain. An explanation of why 6 AN does not affect jaw growth is therefore necessary. Examination of newborn mice of the two strains treated with 6 AN showed an incidence of 9.7% in A/J and 3·7% in C57BL of newborn mice with small lower jaws. (The incidence of cleft palate, however, was 91% and 68% respectively.) According to Asling (1960), mandible development begins to accelerate in rats only from the eightee-th day on. Thus, it is difficult to make a diagnosis of micrognathia before this acceleration

takes place. In mice the relative growth rates of the maxillary and mandibular region indicate a differential increase in mandibular length from days 13-16 with no growth spurt during this period (Smiley, 1967). Smiley's results indicate that the mandible out-grows the maxilla in mouse embryos; the relative mandibular increase is evident through the 13-16 day period, but not so obvious at the beginning of palate closure which occurs on day 14 in the C57BL strain and on day 15 in the A/J strain. Since treatment with 6 AN generally retards embryonic growth for half a day it is probable that the method employed here for lower jaw measurement would not be able to detect a possible change introduced by 6 AN. Sicher (1915) has reported in the rat that there is a marked growth spurt of the mandible simultaneously with transposition of the palatine shelves. The present study has been mainly concerned with antero-posterior dimension of the lower jaw which can influence tongue descent.

The relative width of the mandible and its height both anteriorly and posteriorly are all involved in determining the capacity of the oral floor, however. The importance of the anterior vertical dimension, measured by a perpendicular from the midpoint of the anterior convexity of the nasal septum to an anterior extension of the mandible measurement which increases prior to and during the critical time for palate closure, has been mentioned by Hart, Smiley and Dixon (1969).

These authors consider that an increase of anterior-posterior vertical dimension was the most significant finding for the changes in mandibular position and tongue position prerequisites for shelf movement. The effect of 6 AN on the height and width of the mandible must be studied to construct a complete picture of the effect of the drug on lower jaw growth.

6 AN can also interfere with the growth of the chondrocranium, thereby interfering with the straightening of the cranial base angle in treated embryos at the crucial period of palate closure. 6 An has been shown to delay straightening of the cranial base in the rat embryo (Verrusio, 1969).

As already discussed in the literature review, the evidence indicates that 6 AN acts initially by forming abnormal NAD in mammalian tissues and interferes with oxidative phosphorylation in liver mitochondria. Excessive amounts of nicotinamide adenine dinucleotide injection into the pregnant female prevent cleft palate in the offspring. Although, the data are few, they give direct proof that the action of 6 AN is competitive with the natural cellular coenzyme. Chamberlain and Goldyne (1970) injected 6 AN into rat embryos intra-amniotically together with NAD and NADP and prevented malformations as well as fetal and placenta weight loss. Injection of 6 AN with ATP did not correct the malformations induced by 6 AN, but both oxidized and reduced coenzymes NAD, NADP were capable of reducing malformation in the rat.

Contrary to the previous prediction that certain nucleotides can not cross the cell membrane or are unable to enter mitochondria (Verrusio, 1966) NAD was able to penetrate and counteract the teratogenic action of 6 AN, even 2 hours afterwards. Teratogenesis of 6 AN thus, is probably due to the inability of the palatine shelf to carry out normal metabolic steps after the replacement of NAD with the 6 AN analogue. The fact that ATP did not reduce teratogenicity serves as proof for Coper and Neubert's (1964) conclusion that 6 AN interferes with the reaction leading to the formation or release of NADH-P from mitochondria but not with the transphosphorylating reaction.

Johnson and Kanics (1969) have shown that nicotinic acid and its derivatives (particularly 6 AN) are potent stimulators of adrenacortical secretion (corticosterone) in rats through increased production and secretion of ACTH. It is possible that 6 AN exerts its teratogenicity through excess production of corticosterone. The model is attractive in that increased corticosterone impairs glucose metabolism and thus, contributes to teratogenicity. Nevertheless, this hypothesis is unlikely to be true, however, since nicotinamide, which stimulates secretion of corticosteroid, actually protects against the effects of 6 AN on the embryo. Furthermore, the rat is resistant to cleft palate induction by cortisone, and finally, there is evidence that nicotinic acid derivatives

do not exert their effects on adrenal corticosterone synthesis through effects on pyridine nucleotide synthesis.

Ingall, Ingenito and Curley (1964) claimed that chromosomal anomalies were induced in animals with cleft palates. Since no mention of the strain of mice, dosage, or protection with nicotinamide was made their results are difficult to assess.

Electron microscopic change induced by 6 AN in limb cartilage of 6 AN treated chick embryos included reduction in rough endoplasmic reticulum and Golgi, reduction of metachromasia and number of collagen fibrils, reduction in acid mucopolysaccharide matrix, and impaired chondrogenic activity.

Although the lower jaw length of 6 AN-treated embryos do not differ from that of control when the comparison was based on chronological age, morphological rating or body weight and palate closure both in A/J and in C57BL embryos are in general depressed. It is clear that 6 AN depresses the morphological features and also is known to affect fetal and placenta weight greatly (Turbow and Chamberlain, 1968). The effect is probably a combined alteration in both fetal and maternal tissue which leads to the production of abnormalities.

E. Vitamin A induced cleft palate.

Treatment with excess vitamin A (10^4IU) on day 10 1/2 of gestation caused 93% CP in C57BL embryos and 92% CP in A/J embryos. No strain difference in response to vitamin A can be

shown in the two strains from the frequency of CP. Vitamin A has been chosen as the third teratogen for its production of micrognathia in rats and mice (Deuschle and Kalter, 1962). Administration of 3,000 IU vitamin A daily intraperitoneally from day 7 to day 9 caused 10% micrognathia, 5% CP and 15% CLP in C57BL, 8.7% micrognathia in DBA and 41% micrognathia with 52% CP in the C3H strain of mice in previous investigations.

Malformations encountered in this study other than CP are exophthalmos, syndactyly, oligodactyly and shortened fore- and hind limbs. Fetal resorption is not increased. Treated embryos of both strains are at slightly later developmental stages when compared to control groups at all palate stages, and the inhibition of shelf movement by the teratogen is more marked in C57BL embryos than A/J embryos. The jaw length in vitamin A treated embryos of both strains is not shortened by the treatment at all. When various growth parameters are taken into account, the differences in jaw length between control and treated groups in both strains are not statistically significant. There are, however, significantly more embryos with their tongue behind the primary palate in the vitamin A-treated group than in the control group in C57BL (p: .01 - .005)., and the effect of vitamin A on tongue position may contribute to cleft palate. No significant difference (p. .5-.1) is seen between treated A/J and control A/J embryos with respect to tongue position.

Micrognathia has consistantly been produced by various investigators using vitamin A. (Cohlan, 1954; Deuschle, Geiger and Warkany, 1959; Kochhar and Johnson, 1965; Nanda 1970b). The vitamin is reported to affect the more proximal portion of the mandible (Deuschle, Geiger and Warkany, 1959), to cause abnormal osteogenesis in maxillary region, heterotopic chondrogenesis of maxilla and maxilla-mandibular ankylosis (Kochhar and Johnson, 1965), and also to cause abnormal Meckel's cartilage, maldevelopment of the mandibular bony region and defects of salivary glands and musculature (Nanda, 1970b). Thus, vitamin A affects mandibular length as well as its height. Since reduction in the size of the orbit is responsible for various degrees of exophthalmos (Kalter and Warkany, 1961), it is not unreasonable to suspect that this teratogen can also affect the cranial base in treated embryos.

The mode of action of vitamin A on chondrogenesis and osteogenesis is still little understood. Baume (1970) has proposed three mechanisms: 1) Direct action of vitamin A on the activity of the osteoblast and osteoclast, with trabecular ossification being regulated directly by the vitamin. 2) Vitamin A acts both on the adrenals and the osteogenic tissues directly, regulating endochondral ossification, in a pattern resembling the action of hormonal regulation. 3) Bone changes in vitamin A deficiency or in hypervitaminosis A were found to be associated with changes in chondroitin sulphate. Accordingly vitamin A may directly influence the sulphate and hexosamine metabolism in

the synthesis of mucopolysaccharides.

Varying doses of vitamin A were given to young Wistar rats; their temporo-mandibular joint (for condylar cartilage), tibia (proximal epiphyseal cartilage), basicranial synchondroses and the metacarpal of foreleg (articular cartilage) were examined for their differential response to the vitamin A. The study disclosed that vitamin A has different effects on different types of cartilage (Baume, 1970). Hypervitaminosis A causes rapid atrophy of cartilage, and produces sclerotic, dense bone in the condyle. High doses of vitamin A induce acceleration of chondrogenesis; low doses of the vitamin decelerate endochondral ossification in basicranial synchondroses. Thus, excess vitamin A causes arrest of chondrogenesis, cartilage atrophy, acceleration of trabecular ossification and bone transformation in the condylar cartilage of the mandible while accelerating cartilage erosion and slowing down bone formation and bone transformation in the cranial base. The difference in response of the two types of cartilage has its basis on the embryonic origin of the cartilage center. The cranial base or synchondrotic cartilage is derived from primordial cartilage skeleton, whereas the condylar cartilage is formed secondarily, on original membrane bone. In rats, the condylar cartilage starts endochondral ossification at the seventeenth fetal day, and contributes to the growth in height and length of the mandible. In mice, the ossification center of the condyloid process starts at day 15, and the cranial base cartilage is visible after day 14. Although the present investigation

failed to show shortening of the mandible during palate closure, it is obvious that hypervitaminosis A affects both mandibular height and length and the cranial base in fetal mice. Further study of the cranial base during palate closure and after birth is necessary in order to determine the effect of the vitamin.

The mechanism of action of excess vitamin A in experimental production of cleft palate is not yet understood. Changes in acid mucopolysaccharide metabolism of embryonic tissues has been suggested by various investigators (Walker and Crain, 1960; Kochhar and Johnson, 1965; Kochhar, Larsson and Boström, 1968). Dingle, Fell and Lucy (1966) have previously demonstrated that adding excessive vitamin A to the culture medium decreases weight and amino sugar concentration in chick limb bone rudiments grown in tissue culture. This is probably due to an increased release of lysosomal enzymes in the tissue caused by high levels of retinol in the medium. The total protease content of the rudiments is increased, and release of protease from cells and subcellular particles stimulated. Total synthesis of amino sugars is probably not affected by retinol, but breakdown of the compounds containing hexosamine is increased by adding rather large doses of retinol to the growth medium. Fell and Thomas (1961) have shown that hydrocortisone alone severely inhibits the growth of the cartilage-

nous limb bone rudiments from 7- day chick embryos, but a combination of hydrocortisone or cortisone with vitamin A in the culture inhibits the effect of the excess vitamin A. Studies by these investigators has led to the hypothesis that the vitamin acts directly on the lysosomes in vivo and in vitro; furthermore, hydrocortisone is thought to increase the stability of lysosomes. The effect of vitamin A on lysosome is probably due to its action on the lipoproteins of membranes (Lucy and Dingle, 1964, Dingle, 1963). Thus, hypervitaminosis has been shown to cause degradation of the organic matrix of cartilage by a stimulation of synthesis and export of lysosomal enzymes; the degradation is mainly by lysosomal cathepsin D. The release of lysosomal enzyme from the cell by excess vitamin A is now believed to be due to fusion of enzyme-containing Golgi vesicles (primary lysosomes) with the plasma membrane of the cell (Dingle, 1968). The penetration of vitamin A into the plasma membrane induces a physiocochemical change that facilitates the fusion of the membrane with the primary lysosome.

Incorporation of sulphate groups into mucopolysaccharides is dependent on vitamin A; hence, the vitamin is necessary for the biosynthesis of mucopolysaccharides (Varandani, Wolf and Johnson, 1960). Studies of effects of vitamin A on mucopolysaccharides of lung tissue showed that incorporation of $^{35}SO_4$ and glucosamine-1-^{14}C into three distinct classes of mucopolysaccharide-peptide complexes (hyaluronic acid, a sulphated hyaluronic acid, and heparan sulphate) is higher in vitamin A

deficient tissues. The investigators concluded that vitamin A deficiency either stimulates synthesis or inhibits degradation of the mucopolysaccharides. It was also observed that there are varying effects of vitamin A deficiency on various mucopolysaccharides, from stimulation of the synthesis of the unsulphated, to inhibition of the synthesis of the sulphated. This variation is probably due to the different response of different cell types to vitamin A (De Luca and Wolf, 1968).

F. Role of tongue in palate formation.

Shifting of the tongue downward and forward from above the palatine shelves frees the margins of the palatine shelves to swing upward and toward the midline for eventual fusion. This change in relation between the tongue and the palatal shelves is crucial for the completion of the palate (Patten, 1971).

In spontaneous cleft lip embryos, there is obstruction to the forward movement of the tongue by a huge primary palate. This leads to increased resistance of the tongue to movement of the shelves from the vertical to the horizontal position and delays shelf movement.

Ross and Walker (1967) demonstrated that manual displacement of the tongue rapidly led to movement of the palatine shelves from a vertical to a horizontal plane at the time of palate closure in fetal mice. Additional effects of the tongue were also shown by complete removal of the tongue. The tongue induced greater flattening of palatine shelves in the horizontal plane than was achieved with the tongue removed in

the in vitro observation. These authors observed spontaneous embryonic movements whose onset coincides with the onset of palate closure in fetal mice. Movement of limbs, neck, trunk, lower jaw and tongue were seen (Walker, 1969).

The above mentioned observations reinforce the circumstantial evidence correlating tongue displacement and palate closure. Preliminary results have shown in this investigation that the growth of tongue parallels that of the lower jaws. Analysis based on the position of the tongue relative to the primary palate shows more C57BL embryos to have a forwardly displaced tongue even when their palate remains open. In contrast, in A/J embryos, the tongue tends to stay behind the primary palate at the beginning of palate closure. This trend is consistently shown when the observations are based on either jaw length as parameter or MR as criterion.

Is there any strain difference in response to teratogens for tongue movement which can be responsible for the strain difference observed in induced cleft palate? A marked effect of 6 AN has been seen on A/J tongue and vitamin A inhibits forward displacement of the C57BL tongue as judged from observed tongue position. Cortisone does not affect the C57BL tongue, but the A/J tongue movement is more susceptible to inhibition by the steroid.

Trasler and Fraser (1963) have shown that in A/J/Fr strain the tongue does not drop before shelf closure begins

and that shelf movement can begin even when forward and downward movement of the tongue is prevented. The present study suggests that a different type of relationship may exist between tongue descent and shelf movement in the C57BL/6J strain. The tongue tends to be forwardly and downwardly displaced even before the beginning of palate closure in many control embryos and also in cortisone-treated embryos. This early displacement of tongue may be one of the factors which contribute to the resistance to cortisone induced CP in C57BL. Whether this observation can be counted as one of the factors in the susceptibility of A/J embryos to CP needs further confirmation. The finding in embryos subjected to amniotic sac puncture that those with open palate have the tongue behind the primary palate and those with closed palate have a forwardly displaced tongue favors the above view.

Peter (1924) and Lazaro (1940) investigated closure of the human palate and concluded that palate closure involves the integral processes of palatal shelf movement and tongue withdrawal. The role of intrinsic shelf movement has been extensively investigated and has been suspected to result from a combination of differential growth of the shelves per se, plus additional impetus from an intrinsic shelf force. With concomitant growth in length, depth and width of the floor of the mouth, there is a steady forward and downward displacement of the tongue. This change in tongue position coincides with growth of the body of the mandible. The present results seem to indicate

that the mandible and tongue are not, in fact, actively involved in normal closure of the palate, in agreement with previous studies (Walker and Fraser, 1956; Trasler and Fraser, 1963). In teratogen-induced cleft palate there is no retardation of jaw growth which could account for the cause of palate closure, but some indication of increased tongue resistance has been observed after teratogen administration in both strains. The possibility of an active role of tongue, involving withdrawal from between the shelves, forward and downward movement, and application of pressure to and flattening of the palatine shelves may be part of the strain difference between A/J and C57BL embryos.

The fact that there is no difference in embryonic weight and mandible length between the two control strains,but an obvious difference for chronological age and morphological rating between the two strains during palate closure indicates the importance of embryonic weight in determining palatal development. It is possible that maternal environment may be vital in determining the strain differences of susceptibility to cleft palate. The extent of the decidual cell reaction on the 7th day of pregnancy, and placental weight, foetal weight and placental morphology on the 18th day are found to be dependent on the genotype of the conceptus and the environment provided by the mother (Hetherington, 1971). A preliminary study indicates that there is a strain difference in the time of appearance and extent of decidual cell reaction between A/J and C57BL (Berntson, 1971).

Other factors which may be involved in production of cleft palate have already been mentioned in the review. The process of palatal fusion has been studied *in vitro*. Moriarty, Weinstein and Gibson,(1963) showed that the *in vitro* process of rat palatal shelf fusion was similar to the *in vivo* process; capability to fuse *in vitro* was acquired about thirty hours before the normal *in vivo* time of fusion in rat palate (Pourtois, 1966), and probably induced by stimuli from the underlying mesoderm. Palatal shelves from cleft lip and palate A/J mice have the potential to fuse at the normal *in vivo* time of palatal fusion (Pourtois, 1967). There may be alteration in competence of the palate fusion in the teratogen-treated embryos.

Cleft palate is a threshold character. Successful palatal closure requires harmonious integration of a number of different growth and developmental factors. Whether a particular embryo develops a cleft or not depends at least partly on its normal pattern of palate closure, which pattern depends on the embryo's own genotype, maternal genotype, environmental influence and their interactions. Various teratogens probably exert their effects on both embryos and the mothers either transiently or permanently, thus shifting the threshold for palate closure.

The present thesis suggests that the mandible is not actively associated with palate closure process. Cortisone, 6 AN and vitamin A probably delay shelf movement and tongue movement by changing the subtly balanced developmental state of

the embryos.

Further investigation is necessary in the following areas: 1) Tongue role in normal palatal closure and effects of teratogens on tongue. 2) The search for the basis of intrinsic shelf force. Change of cranial base configuration in normal and treated groups. 3) Effects of teratogens on epithelial-mesodermal interactions in palate closure. 4) Effects of teratogens on maternal environment.

SUMMARY

1. Both lower jaw lengths and the rates of growth of lower jaw in the A/J and C57BL strains of mice are identical during palate closure in untreated groups.
2. Embryonic weight is more consistently related to palatal development than chronological age and morphological rating.
3. Strain differences in susceptibility to cleft palate induced by cortisone and 6 AN have been demonstrated in the A/J and C57BL strains of mice.
4. Vitamin A induces high a frequency of cleft palate in both strains.
5. All three teratogens fail to shorten the lower jaws during palate closure.
6. Cortisone stimulates the growth of the lower jaws in both strains compared to normal controls and enhances developmental features. A strain difference in response to cortisone is observed for morphological features between the two strains.
7. 6 AN does not alter the rate of jaw growth in A/J and C57BL during palate closure, although slight retardation of developmental features are observed in both strains.
8. Vitamin A does not affect the rate of lower jaw growth in C57BL, but does affect the rate of growth of the lower

jaw in A/J. The age-adjusted lower jaw length is shorter than the control in A/J, but the difference is not statistically significant. Due to syndactyly and oligodactyly produced by vitamin A, morphological rating is rather difficult to assess, and probably inaccurate in vitamin A-treated embryos.

9. Strain differences seem to exist between A/J and C57BL regarding descent of the tongue and its forward displacement: in C57BL mice, significantly more embryos have a forwardly displaced tongue compared to A/J embryos at the beginning of palate closure.
10. Cortisone does not affect the tongue in C57BL, but seems to affect it in A/J embryos.
11. 6 AN inhibits the displacement of the tongue in A/J embryos but not C57BL, whereas the effect of vitamin A on tongue position is greater in C57BL.
12. Amniotic sac puncture does not retard mandible growth of C57BL embryos as measured by the distance between hyoid and Meckel's cartilage. Cleft palate is probably due to immobility of and increased resistance from the tongue.
13. The previous finding that there is a difference in time of palate closure in relation to developmental features between the two strains has been confirmed.

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APPENDIX

Table 1

Developmental features and their arbitrarily assigned values used to calculate morphological ratings for mouse embryos

Value assigned to each feature	Developmental features				
	Forefeet	hindfeet	ears	hair follicles	eyes
-2	un.pl.				
-1	in.pl.	un.pl.	sm.ra.		
0	plate	in.pl	ra.	none	open
1	3/4 webbed	plate	1/3 closed	body	1/2 closed
2	1/2 webbed	3/4 webbed	1/2 closed	b.f.s.	closed
3	1/4 webbed	1/2 webbed	2/3 closed	b.s.	
4	free	1/4 webbed	3/4 closed	b.h.	
5	1/4 fused	free	closed		
6	1/2 fused	1/4 fused			
7	3/4 fused	1/2 fused			
8		3/4 fused		w.b.	

To follow...

APPENDIX

Table 1

(suite)

ABBREVIATIONS:

un.pl., unindented plate

in.pl., indented plate

sm.ra., small right-angled pinna

ra., right-angled pinna

b.f.s., follicles on body and a few on side of head

b.s., on body and side of head

b.h., on body and head

w.b., wrinkled body

From Trasler, D.G., 1965.
Strain differences in susceptibility to terato-genesis: Survey of spontaneously occurring malformations in mice in "Teratology, principles and techniques", 1965.

APPENDIX

Table 2a

Relation of morphological rating and palate closure (PS) in control and treated groups of the A/J strain

PS	treatment	number embryo	mean MR	standard error	range
0	Control	55	4.95	0.28	0-9
	6 AN	65	6.28	0.37	0-12
	Cortisone	26	5.96	0.63	0-13
	Vitamin A	32	5.47	0.77	2-13
1	Control	50	9.04	0.26	3-12
	6 AN	25	11.04	0.31	8-14
	Cortisone	17	13.18	0.81	7-12
	Vitamin A	22	10.64	0.68	4-15
2	Control	3	11.33	0.33	11-12
	6 AN	4	13.75	0.63	12-15
	Cortisone	9	14.78	0.64	11-17
	Vitamin A	2	11.50	0.50	11-12
3	Control	6	11.50	0.76	8-13
	6 AN	11	13.64	0.34	11-15
	Cortisone	14	17.79	1.15	14-23
	Vitamin A	7	11.70	0.52	10-13

To follow...

Table 2a

(suite)					
4	Control	--	--	--	--
	6 AN	4	14.75	0.85	13-17
	Cortisone	6	21.50	1.45	16-25
	Vitamin A	4	10.00	0.91	8-12
5	Control	2	14.00	2.0	12-16
	6 AN	3	15.00	2.08	11-18
	Cortisone				
	Vitamin A	3	12.00	0.58	11-13
6	Control	6	12.17	0.48	11-14
	6 AN	4	14.50	0.50	13-15
	Cortisone				
	Vitamin A	2	12.00	1.00	11-13
7	Control	21	14.62	0.15	13-16
	6 AN	2	14.50	0.50	14-15
	Cortisone				
	Vitamin A	1			14

APPENDIX

Table 2b

Relation of morphological rating (MR) and palate closure (PS) in control and treated embryos of the C57BL strain

PS	treatment	number embryo	mean MR	standard error	range
0	Control	56	3.46	0.23	-1-6
	6 AN	37	4.54	0.37	0-9
	Cortisone	21	4.29	0.33	1-7
	Vitamin A	48	5.19	0.36	-1-9
1	Control	50	5.82	0.17	4-8
	6 AN	23	8.57	0.37	6-13
	Cortisone	8	7.63	0.33	6-9
	Vitamin A	12	8.33	0.50	4-11
2	Control	19	7.47	0.23	5-9
	Cortisone	8	10.00	0.53	8-13
	Vitamin A	3	9.67	0.33	9-10
3	Control	13	8.46	0.29	7-10
	6 AN	16	10.63	0.27	9-12
	Cortisone	11	11.64	0.61	9-15
	Vitamin A	7	11.57	2.45	4-21

To follow...

Table 2b

(suite)					
4	Control	22	9.00	0.15	7-10
	6 AN	18	11.67	0.21	10-14
	Cortisone	9	14.33	0.69	12-17
	Vitamin A	1			11
5	Control	15	9.87	0.27	8-12
	6 AN	9	12.56	0.38	11-14
	Cortisone	7	14.57	0.84	12-19
	Vitamin A	5	12.80	0.49	11-14
6	Control	25	11.00	0.22	8-14
	6 AN	7	13.57	0.61	11-15
	Cortisone	17	15.82	0.58	12-19
	Vitamin A	7	14.57	1.13	12-21
7	Control	43	13.65	0.39	10-19
	6 AN	6	21.50	0.81	20-25
	Cortisone	13	19.38	0.46	16-22
	Vitamin A	24	15-13	0.54	11-20

APPENDIX

Table 3a Mean lower jaw length and palate stages in A/J control and treated embryos

PS	Treatment	Number Embryo	Mean	Standard Error	Range
0	Control	52	39.86	0.33	34.0-4.35
	6 AN	64	42.35	0.39	36.0-48.0
	Cortisone	27	40.22	0.73	31.0-47.5
	Vitamin A	30	39.27	0.95	31.0-53.0
1	Control	46	43.62	0.31	40.0-50.0
	6 AN	26	48.90	0.45	43.0-53.5
	Cortisone	17	49.76	0.87	42.0-54.0
	Vitamin A	20	47.40	0.90	40.5-54.0
2.	Control	4	46.75	1.11	44.0-49.0
	6 AN	4	50.88	1.33	47.5-54.0
	Cortisone	9	51.39	0.84	47.0-56.0
	Vitamin A	3	51.33	1.01	49.0-53.0
3	Control	5	45.90	1.13	42.0-48.0
	6 AN	10	52.90	0.67	49.5-55.0
	Cortisone	18	54.78	0.65	52.0-60.0
	Vitamin A	7	52.96	0.57	51.0-55.0
4	Control				
	6 AN	3	56.83	0.17	56.0-57.0
	Cortisone	5	56.50	1.04	53.0-59.0
	Vitamin A	4	46.25	0.63	45.0-48.0
5	Control	1			45.0
	6 AN	2	54.0	5.0	49.0-59.0
	Cortisone				
	Vitamin A	3	53.33	0.88	52.0-55.0
6	Control	6	50.25	0.68	48.0-52.0
	6 AN	4	55.75	1.11	53.0-58.0
	Cortisone	1			57.0
	Vitamin A	2	55.75	1.75	54.0-57.5
7	Control	17	53.21	0.66	48.0-59.0
	6 AN	1			58.0
	Cortisone	1			61.0
	Vitamin A	1			52.5

APPENDIX

Table 3 b Mean lower jaw length and palate stages in C57BL control and treated embryos

PS	Treatment	Number Embryo	Mean	Standard Error	Range
0	Control	55	38.87	0.35	32.0-43.5
	6 AN	37	43.49	0.51	34.5-50.0
	Cortisone	21	43.31	0.52	37.0-48.0
	Vitamin A	46	42.76	0.52	33.0-49.0
1	Control	46	42.74	0.23	38.0-46.5
	6 AN	21	47.38	0.50	45.0-52.0
	Cortisone	7	45.64	0.58	43.0-47.0
	Vitamin A	13	46.19	0.85	40.0-50.0
2	Control	17	44.26	0.26	42.0-46.0
	6 AN				
	Cortisone	7	48.14	0.66	46.0-51.0
	Vitamin A	3	52.50	1.04	51.0-54.0
3	Control	13	45.46	0.50	43.0-50.0
	6 AN	16	49.47	0.55	47.0-53.0
	Cortisone	11	50.00	0.58	47.5-53.0
	Vitamin A	9	56.00	3.91	39.0-69.0
4	Control	22	46.73	0.37	44.0-50.0
	6 AN	17	52.03	0.50	50.0-57.5
	Cortisone	10	51.90	0.66	49.0-56.0
	Vitamin A	2	60.25	7.25	53.0-67.5
5	Control	13	47.81	0.55	45.0-52.0
	6 AN	6	53.92	1.36	48.5-58.0
	Cortisone	7	51.57	0.38	50.5-53.0
	Vitamin A	5	58.10	0.68	57.0-60.0
6	Control	22	49.93	0.48	44.0-54.0
	6 AN	7	55.64	0.54	54.0-58.0
	Cortisone	17	53.21	0.49	49.0-57.0
	Vitamin A	5	56.60	0.93	54.0-59.0
7	Control	34	54.22	0.51	48.5-63.0
	6 AN	6	67.00	2.38	58.0-72.0
	Cortisone	11	56.32	0.42	54.0-59.0
	Vitamin A	16	58.03	0.72	54.0-62.0

APPENDIX

Table 3 c Lower jaw length and palate stages

Strain: C57BL Amniotic Sac Puncture

Palate Stages

		Number Embryo	Mean	Standard Error	Range
0	Control	1			42.0
	Punctured	2	55.00	3.00	52.0-58.0
1	Control	1			49.0
	Punctured	3	52.17	3.56	52.0-64.0
2	Control	1			49.0
	Punctured				
4	Control				
	Punctured	3	72.00	7.21	58.0-82.0
5	Control				
	Punctured	1			62.0
6	Control	3	54.83	2.33	50.5-58.5
	Punctured	1			56.0
7	Control	16	65.88	2.37	57.0-81.0
	Punctured	10	64.00		

APPENDIX

Table 4a Mean lower jaw length of A/J control embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
0	1			37.0
1	1			39.0
2	2	37.25	1.25	36.0-38.5
3	8	38.31	0.82	35.0-41.5
4	13	39.54	0.50	37.0-42.5
5	10	40.65	0.52	38.0-43.0
6	9	40.94	0.70	37.0-45.0
7	6	39.58	1.56	34.0-43.0
8	11	42.00	0.83	37.5-47.0
9	16	43.03	0.33	40.0-45.0
10	14	44.39	0.60	41.0-49.5
11	10	45.70	0.96	42.0-51.0
12	8	46.81	0.98	43.0-52.0
13	3	47.50	0.50	46.5-48.0
14	8	51.94	0.74	48.0-55.0
15	9	54.11	0.98	49.0-59.0
16	2	51.75	2.25	49.5-54.0

APPENDIX

Table 4b Mean lower jaw length of 6 AN treated A/J embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
0	3	36.33	0.33	3.60–37.0
1	4	36.63	0.38	36.0–37.5
2	2	37.00	1.00	36.0–38.0
3	2	40.00	1.00	39.0–41.0
4	5	40.20	0.73	38.0–42.0
5	7	41.43	0.34	40 –42.5
6	8	42.81	0.50	41.0–45.0
7	9	43.83	0.50	42.0–46.0
8	11	44.09	0.52	42.0–48.0
9	11	45.95	0.82	42.0–51.0
10	6	46.67	0.75	44.0–49.0
11	13	48.27	0.64	44.0–51.5
12	7	48.07	0.61	45.0–50.0
13	7	52.29	1.03	49.5–57.0
14	11	53.91	0.70	50.0–58.0
15	6	54.67	1.12	51.0–58.0

APPENDIX

Table 4 c Mean lower jaw length of cortisone treated A/J embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
0	2	37.00	1.00	31.0-33.0
1				
2	2	38.00	2.00	36.0-40.0
3	2	38.25	0.25	38.0-38.5
4	4	38.00	0.91	36.0-40.0
5	1			
6	4	41.63	0.75	39.5-43.0
7	5	41.50	0.71	39.0-43.0
8	3	42.33	1.33	41.0-45.0
9	3	44.50	1.76	41.0-46.5
10	2	45.00	2.00	42.0-47.0
11	6	47.00	0.68	45.0-49.0
12				
13	5	49.80	1.08	47.0-52.0
14	7	52.14	0.66	49.0-54.0
15	4	51.50	1.04	49.0-54.0
16	5	53.20	0.86	51.0-56.0
17	6	52.92	0.47	51.5-54.5
18	1			
19	1			
20	1			
21	4	56.88	0.66	55.0-58.0
22	1			
23	3	56.17	0.44	55.5-57.0
24				
25	3	59.17	1.01	57.5-61.0
26	3	59.33	0.33	59.0-60.0

APPENDIX

Table 4d Mean lower jaw length of Vitamin A treated A/J embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
0	1			35
2	8	37.31	0.47	35.0-39.0
3	5	34.80	1.01	33.0-38.0
4	4	39.88	0.52	38.5-41.0
5	2	37.00	5.00	32.0-42.0
6	4	41.88	0.77	40.5-44.0
7	3	43.00	0.76	42.0-44.5
8	2	45.00	1.00	44.0-46.0
9	2	46.25	0.25	46.0
10	5	49.20	1.44	44.0-52.5
11	8	50.00	1.54	40.0-54.0
12	12	49.92	1.30	40.0-55.5
13	9	51.00	1.30	44.0-57.5
14	3	52.33	0.87	51.0-54.0
15	1			

APPENDIX

Table 4e Mean lower jaw length of C57BL control embryo with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
-1	1			32.0
0	5	34.30	0.80	32.5-37.0
1	2	38.50	0.50	38.0-39.0
2	4	38.50	0.50	38.0-40.0
3	6	37.50	0.52	36.0-39.5
4	37	40.04	0.32	35.5-43.0
5	15	41.80	0.43	38.5-45.0
6	19	42.50	0.31	39.0-44.5
7	21	44.17	0.35	42.0-48.0
8	17	45.00	0.32	42.5-48.0
9	28	46.63	0.37	43.0-50.5
10	19	47.89	0.49	44.0-52.0
11	14	51.21	0.67	47.5-57.0
12	18	52.28	0.49	48.5-57.0
13	6	53.50	0.41	52.5-55.0
14	10	55.00	0.36	53.0-56.5
15	2	57.50	2.50	55.0-60.0
16	1			61.0
17	2	62.00	1.00	61.0-63.0
18	1			59.0
19	3	61.83	0.73	60.5-63.0

APPENDIX

Table 4f Mean lower jaw length of 6 AN treated C57BL embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
0	3	38.50	2.47	34.5-43.0
1	1			37.5
2	1			40.5
3	5	42.30	1.28	40.0-46.5
4	11	43.23	0.35	41.0-45.0
5	2	43.50	1.50	42.0-45.0
6	9	45.56	0.75	43.0-50.0
7	6	46.25	0.62	44.5 -48.0
8	9	46.11	0.52	45.0-50.0
9	9	47.39	0.68	45.0-51.0
10	10	49.10	0.49	47.0-52.0
11	11	51.41	0.63	49.0-54.0
12	17	52.09	0.59	48.5-58.0
13	4	54.13	0.97	51.5-56.0
14	4	55.38	0.75	54.0-57.0
15	3	56.83	0.60	56.0-58.0
16				
17				
18				
19				
20	3	63.67	4.04	58.0-71.5
21				
22	2	69.50	0.50	69.0-70.0
23				
24				
25				

APPENDIX

Table 4g Mean lower jaw length of cortisone treated C57BL embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
1	1			39.0
2	2	39.75	2.75	37.0-42.5
3	2	41.50	0.50	41.0-42.0
4	7	43.57	0.52	41.0-45.0
5	4	44.13	0.24	43.5-44.5
6	5	44.60	0.51	43.0-46.0
7	3	46.00	1.53	43.0-48.0
8	4	46.00	0.54	44.5-47.0
9	4	47.75	0.32	47.0-48.5
10	3	47.33	0.88	46.0-49.0
11	6	49.75	0.40	49.0-51.0
12	8	50.00	0.50	48.0-52.0
13	4	52.25	0.48	51.0-53.0
14	2	52.00	1.00	51.0-53.0
15	11	52.45	0.45	50.5-54.0
16	5	53.60	1.02	51.0-57.0
17	4	54.63	1.21	51.0-57.0
18	1			53.0
19	7	55.00	0.58	53.0-57.0
20	4	55.25	0.72	53.0-57.0
21	1			57.0
22	1			59.0
⋮				
28	1			54.0
29	4	66.00	0.41	65.0-67.0
30	2	61.75	0.25	61.5-62.0

APPENDIX

Table 4h Mean lower jaw length of Vitamin A treated C57BL embryos with various morphological rating

MR	Number Embryo	Mean	Standard Error	Range
1	6	37.50	0.88	35.5-41.5
2	2	43.50	0.50	43.0-44.0
3	1			42.0
4	6	41.58	0.84	39.0-44.0
5	10	42.20	0.73	38.5-45.0
6	9	42.06	0.49	39.5-44.0
7	10	45.80	0.48	44.5-49.0
8	6	46.67	0.25	46.0-47.5
9	10	47.35	0.89	41.0-51.0
10	3	51.83	1.59	49.0-54.5
11	5	52.60	1.33	49.0-57.0
12	5	56.20	1.11	54.0-60.0
13	8	56.43	0.66	54.0-59.5
14	6	59.25	0.85	57.0-62.0
15	4	58.50	1.66	56.0-63.0
16	2	61.00	2.00	59.0-63.0
17	2	61.50	0.50	61.0-62.0
18	1			61.0
19	2	63.50	1.50	62.0-65.0
20	2	61.50	0.50	61.0-62.0
⋮				
23	2	66.00	2.00	64.0-68.0
⋮				
25	1			67.0
26	1			67.5
27	2	69.00	0.00	69.0

APPENDIX

Table 4i

Mean lower jaw length of C57BL embryo with various morphological rating

Amniotic sac puncture

	AMNIOTIC PUNCTURE				CONTROL EMBRYO			
MR	number embryo	mean	standard error	range	number embryo	mean	standard error	range
5					1			42.0
9	1			55.0	2	49.00	0.00	49.0
10	1			52.0				
11	5	55.30	0.97	52.0-58.0	1			56.0
12	3	59.33	1.33	58.0-62.0	4	57.75	0.78	55.5-59.0
13	3	56.67	0.88	55.0-58.0	1			57.0
14					2	57.75	2.75	55.0-60.5
15	1			64.0	1			50.5
16					2	60.25	0.25	
17	1			63.0	2	62.0	0.00	62.0
26	2	70.00	1.00	69.0-71.0	1			73.0
29					3	78.00	0.76	77.0-79.5
30	4	79.00	1.32	76.0-82.0	2	78.50	2.50	76.0-81.0

APPENDIX

Table 5a: Mean lower jaw length and body weight

A/J: Control

BW(g)		Number Embryo	Mean	Standard Error	Range
.110	.119	4	35.81	0.80	34.0-37.5
.120	.129	6	39.17	1.08	36.0-43.0
.130	.139	17	39.50	0.47	36.0-43.0
.140	.149	20	40.55	0.45	35.5-44.0
.150	.159	13	41.85	0.31	40.0-44.0
.160	.169	15	42.73	0.44	40.5-47.0
.170	.179	14	44.79	0.36	43.0-48.0
.180	.189	8	44.88	5.73	42.0-47.0
.190	.199	7	47.00	0.76	45.0-50.0
.200	.209	3	47.50	0.50	46.5-48.0
.210	.219	6	50.67	0.87	48.0-53.5
.220	.229	5	52.20	1.16	49.0-56.0
.230	.239	2	51.00	0.50	50.5-51.5
.240	.249	3	54.67	2.19	52.0-59.0
.250	.259	1			53.0
.260	.269	3	54.33	0.88	53.0-56.0
.270	.279	1			55.0
.280	.289	1			56.0

APPENDIX

Table 5b: Mean lower jaw length and body weight

A/J : 6 A N treated

BW (g)		Number Embryo	Mean	Standard Error	Range
.100	.109				
.110	.119	1			37.0
.120	.129	4	36.69	0.51	36.0-38.0
.130	.139	5	36.70	0.44	36.0-38.0
.140	.149	4	40.25	0.48	39.0-41.0
.150	.159	8	41.25	0.40	39.0-42.5
.160	.169	16	43.25	0.38	41.0-46.0
.170	.179	16	44.03	0.38	42.0-47.0
.180	.189	9	45.72	0.62	42.0-48.0
.190	.199	13	48.08	0.71	43.0-51.5
.200	.209	10	47.60	0.87	45.0-51.0
.210	.219	6	49.67	0.51	48.5-52.0
.220	.229	2	53.00	2.00	51.0-55.0
.230	.239	5	52.10	0.83	50.0-54.5
.240	.249	5	54.50	0.84	52.0-57.0
.250	.259	5	56.10	0.86	53.5-58.5
.260	.269	4	55.50	1.19	53.0-58.0
.270	.279				
.280	.289	1			59.0

APPENDIX

Table 5c: Mean lower jaw length and body weight

A/J: Cortisone treated

BW	Number Embryo	Mean	Standard Error	Range
.090 .099	2	32.00	1.00	31.0-33.0
.100 .109				
.110 .119	1			36.0
.120 .129				
.130 .139	8	38.44	0.68	36.0-42.0
.140 .149	4	40.56	0.58	39.5-42.0
.150 .159	8	42.69	0.76	39.0-46.0
.160 .169	3	42.83	1..17	41.0-45.0
.170 .179	4	45.25	0.60	41.0-48.0
.180 .189	6	47.50	0.37	46.5-49.0
.190 .199	3	51.50	0.50	50.5-52.0
.200 .209	12	52.04	0.41	49.0-54.0
.210 .219	6	54.08	0.45	53.0-56.0
.220 .229	7	52.50	0.78	49.0-55.0
.230 .239	2	53.50	0.50	53.0-54.0
.240 .249	3	56.83	0.73	55.5-58.0
.250 .259	1			56.0
.260 .269	2	57.25	0.25	57.0-57.5
.270 .279	1			57.5
.280 .289	3	59.67	0.67	59.0-61.0
.290 .299	2	59.50	0.50	59.0-60.0

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Table 5d

Mean lower jaw length and body weight

A/J: Vitamin A treated

BW (g)	Number Embryo	Mean	Standard Error	Range
.090-.099	1			31.0
.100-.109	1			35.0
.110-.119	2	34.00	1.00	33.0-35.0
.120-.129	3	35.50	1.61	32.5-38.0
.130-.139	9	37.56	0.55	34.5-40.0
.140-.149	1			32.0
.150-.159	1			38.5
.160-.169	7	41.71	0.51	40.00-44.0
.170-.179	6	43.92	0.84	41.0-46.0
.180-.189	4	42.25	1.31	40.0-45.0
.190-.199	6	47.67	0.85	44.0-49.5
.200-.209	2	48.00	3.00	45.0-51.0
.210-.219	9	50.28	0.61	48.0-53.0
.220-.229	8	52.31	0.48	50.0-54.0
.230-.239	3	51.17	2.09	47.0-53.5
.240-.249	3	52.42	0.94	51.0-54.0
.250-.259	4	54.75	1.36	51.0-57.5
.260-.269	1			52.0

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Table 5e

Mean lower jaw length and body weight strain: C57BL control

BW (g)	number embryo	mean	standard error	range
.100-.109	1			32.5
.110-.119	2	33.50	0.50	33.0
.120-.129	7	36.21	0.95	32.0-40.0
.130-.139	6	37.92	0.37	37.0-39.5
.140-.149	14	39.79	0.50	37.0-44.0
.150-.159	34	40.93	0.34	37.0-45.0
.160-.169	30	42.82	0.30	38.5-45.0
.170-.179	23	43.39	0.29	41.5-46.0
.180-.189	21	45.29	0.30	43.5-48.0
.190-.199	18	46.97	0.40	44.0-50.0
.200-.209	20	48.10	0.41	45.0-51.0
.210-.219	6	49.83	0.61	48.0-52.0
.220-.229	6	51.00	0.73	48.0-52.0
.230-.239	15	53.10	0.31	51.0-55.0
.240-.249	11	54.23	0.55	52.0-57.0
.250-.259	4	54.88	1.01	52.0-56.5
.260-.269	3	54.67	0.33	54.0-55.0
.270-.279	1			50.0
.280-.289	1			56.0
.290-.299	4	56.50	2.22	51.0-61.0
.300-.309	3	61.17	0.93	60.0-63.0
.310-.319	1			62.0
.350-.359	2	62.00	1.00	61.0-63.0

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Table 5f

Mean lower jaw length and body weight strain: C57BL, 6 AN treated

BW (g)	number embryo	mean	standard error	range
.120-.129	1			34.5
.130-.139	2	40.50	2.50	38.0-43.0
.140-.149	2	38.75	1.50	37.5-40.0
.150-.159	2	40.75	0.25	40.5-41.0
.160-.169	5	41.90	0.51	40.0-43.0
.170-.179	11	43.77	0.42	41.0-46.5
.180-.189	6	44.75	0.54	43.0-47.0
.190-.199	12	46.50	0.45	45.0-50.0
.200-.209	7	46.50	0.60	45.0-49.0
.210-.219	15	48.73	0.47	45.0-52.0
.220-.229	11	49.95	0.39	48.0-52.0
.230-.239	8	51.63	0.38	50.5-53.0
.240-.249	8	53.65	0.47	51.5-55.0
.250-.259	9	52.44	1.32	45.0-56.0
.260-.269	4	54.38	2.82	46.0-58.0
.270-.279	3	56.50	0.87	55.0-58.0
.320-.329	1			62.0
.330-.339				
.340-.349	1			51.5
.350-.359				
.360-.369	1			69.0
.370-.379	1			58.0
.380-.389	1			72.0
.390-.399	1			70.0
.430-.439	1			72.0

APPENDIX

Table 5g

Mean lower jaw length and body weight strain: C57BL Cortisone treated

BW (g)	number embryo	mean	standard error	range
.120-.129	2	38.00	1.00	37.0-39.0
.130-.139	1			41.0
.140-.149	6	42.58	0.30	41.5-43.5
.150-.159	7	44.50	0.35	43.5-46.0
.160-.169	9	45.22	0.54	43.0-48.0
.170-.179	7	46.79	0.55	44.5-49.0
.180-.189	9	49.00	0.45	47.0-51.0
.190-.199	13	50.62	0.32	48.0-52.0
.200-.209	6	51.83	0.71	49.0-54.0
.210-.219	8	53.13	0.41	51.0-54.5
.220-.229	11	54.18	0.47	52.0-57.0
.230-.239	6	56.33	0.24	55.0-57.0
.240-.249	3	55.67	1.33	53.0-57.0
.250-.259				
.260-.269	1			59.0
.270-.279	2	60.00	5.00	55.0-65.0
.280-.289	1			64.0
.290-.299				
.300-.309				
.310-.319	3	64.67	1.33	62.0-66.0
.320-.329	1			61.0

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Table 5h

Mean lower jaw length and body weight strain: C57BL Vitamin A treated

BW (g)	number embryo	mean	standard error	range
.110-.119	1			33.0
.130-.139	3	36.17	0.44	35.5-37.0
.140-.149	1			38.0
.150-.159	3	40.33	0.93	38.5-41.5
.160-.169	11	41.91	0.57	39.0-45.0
.170-.179	7	41.50	1.10	37.0-44.5
.180-.189	8	44.25	1.01	39.5-49.0
.190-.199	10	44.85	0.45	42.0-46.5
.200-.209	3	49.33	0.88	48.0-51.0
.210-.219	6	48.92	0.84	47.5-52.0
.220-.229	2	48.50	0.50	48.0-49.0
.230-.239	7	49.36	1.79	45.0-54.5
.240-.249	2	45.00	2.00	43.0-47.0
.250-.259	2	50.00	4.00	46.0-54.0
.260-.269	3	47.67	3.28	43.0-54.0
.270-.279	7	56.00	0.53	54.0-57.0
.280-.289	4	56.75	0.43	55.5-57.0
.290-.299	3	57.33	0.88	56.0-59.0
.300-.309	1			46.5
.310-.319	7	61.00	1.27	59.5-62.0
.320-.329	4	60.00	1.47	56.0-63.0
.330-.339	3	61.33	1.20	59.0-63.0
.340-.349	2	63.00	2.00	61.0-65.0

To follow...

Table 5h

(suite)

BW (g)	number embryo	mean	standard error	range
.360-.369	1			64.0
.380-.389	2	62.75	4.25	58.5-67.0
.390-.399	1			68.00
.400-.409	3	68.50	0.50	67.5-69.0

APPENDIX

Table 5i

Mean lower jaw length and body weight C57BL amniotic sac puncture

	PUNCTURE			CONTROL		
BW (g)	number embryo	mean	standard error	number embryo	mean	standard error
.190-.199	1	52.0		2	49.00	0.00
.200-.209						
.210-.219	1	52.0				
.220-.229	1	55.0		1	55.5	
.230-.239	2	57.0	1.00	1	55.0	
.240-.249	2	55.25	0.25			
.250-.259	1	58.0		3	55.00	1.93
.260-.269				2	59.75	0.75
.270-.279	1	58.0		1	57.0	
.280-.289	2	59.50	4.50			
.290-.299	3	59.00	1.53	2	59.25	1.25
.300-.309				1	60.00	
.310-.319	1	63.0				
.320-.329				2	62.00	0.00
.330-.339						
.430-.439	1	71.0				
.440-.449	1	69.0				
.470-.479	1	76.0		1	77.0	
.490-.499				1	76.0	
.500-.509				1	77.5	

To follow...

Table 5i

(suite)	PUNCTURE			CONTROL		
BW (g)	number embryo	mean	standard error	number embryo	mean	standard error
.520-529 .530-.539	1	79.0		1	79.5	
.570-.579	1	81.0		1	81.0	

APPENDIX

Table 6a

Student's t-test for comparison of the mean lower jaw length of A/J and C57BL control embryos using MR as parameter

MR	t-Value	Degree of freedom	Probability
2	0.93	4	0.5-0.4
3	0.83	12	0.5-0.4
4	0.85	48	0.4-0.2
5	1.71	23	0.2-0.1
6	2.02	26	0.1-0.05
7	2.86	25	0.01-0.001
8	3.36	26	0.01-0.001
9	7.24	42	< 0.001
10	4.49	31	< 0.001
11	4.71	22	< 0.001
12	4.97	24	< 0.001
13	9.30	7	< 0.001
14	3.77	16	0.01-0.001
15	1.27	9	0.4 -0.2

APPENDIX

Table 6 b

Test of the equality of variances among A/J and C57BL control embryos

Lower jaw length vs Morphological rating

MR	F-Value	Degree of freedom numerator I denominator	P
2	3.125	1/3	0.25-0.1
3	3.39	7/5	0.1 -0.05
4	1.17	36/12	0.5 -0.25
5	1.01	14/9	0.75-0.5
6	2.44	8/18	0.1 -0.05
7	5.46	5/20	0.005-0.001
8	4.37	10/16	0.005-0.001
9	2.19	27/15	0.1 -0.05
10	1.08	13/18	0.5 - 0.25
11	1.48	10/13	0.5 - 0.25
12	1.76	7/17	0.25-0.1
13	1.33	5/2	0.5 -0.25
14	3.43	7/9	0.05-0.025
15	1.45	18/1	0.5 -0.25

APPENDIX

Table 6 c

"Student's" t-test for comparison of the mean lower jaw length between A/J control and A/J treated embryos

MR	Treatment	t-Value	d f	P
3	6 AN	1.30	8	0.4-0.2
	Cortisone	0.007	8	> 0.9
	Vitamin A	2.70	11	0.02-0.01
4	6 AN	0.521	16	0.9-0.5
	Cortisone	1.48	15	0.2-0.1
	Vitamin A	0.47	25	0.9-0.5
6	6 AN	2.17	15	0.05-0.025
	Cortisone	0.66	11	0.9-0.5
	Vitamin A	0.89	11	0.4-0.2
7	6 AN	2.59	13	0.05-0.025
	Cortisone	1.06	9	0.4-0.2
	Vitamin A	1.92	7	0.1-0.05
8	6 AN	2.13	20	0.05-0.025
	Cortisone	0.29	12	0.9-0.5
	Vitamin A	2.30	11	0.05-0.025
9	6 AN	3.30	25	0.005-0.001
	Cortisone	0.82	17	0.5-0.4
	Vitamin A	6.70	16	<0.001
10	6 AN	2.37	18	0.05-0.025
	Cortisone	0.29	14	0.9-0.5
	Vitamin A	3.09	17	0.01-0.005
11	6 AN	2.23	21	0.05-0.025
	Cortisone	1.10	14	0.4-0.25
	Vitamin A	2.26	16	0.05-0.025
13	6 AN	4.17	8	0.005
	Cortisone	1.93	6	0.2-0.1
	Vitamin A	2.50	10	0.05-0.025
14	6 AN	1.93	17	0.1-0.5
	Cortisone	0.20	13	0.9-0.5
	Vitamin A	0.35	9	0.9-0.5

APPENDIX

Table 6 d

"Students" t-test for comparison of the mean lower jaw length of C57BL control and treated embryos

MR	Treatment	t-Value	d f	P
4	6 AN	6.76	46	<0.001
	Cortisone	5.83	42	<0.001
	Vitamin A	1.46	41	0.2-0.1
5	6 AN	1.97	13	0.1-0.05
	Cortisone	4.74	17	<0.001
	Vitamin A	0.47	23	0.9-0.5
6	6 AN	3.71	26	0.001
	Cortisone	3.52	22	0.005-0.001
	Vitamin A	0.77	26	0.5-0.4
7	6 AN	1.79	25	0.1-0.05
	Cortisone	1.17	22	0.4-0.2
	Vitamin A	2.73	29	0.025-0.01
8	6 AN	1.82	24	0.1-0.05
	Cortisone	1.59	19	0.2-0.1
	Vitamin A	4.12	21	<0.001
9	6 AN	0.99	35	0.4-0.2
	Cortisone	2.28	30	0.05-0.025
	Vitamin A	0.75	36	0.5-0.4
10	6 AN	1.73	27	0.1-0.0.05
	Cortisone	0.55	21	0.9-0.5
	Vitamin A	2.38	20	0.05-0.025
11	6 AN	0.22	23	0.9-0.5
	Cortisone	1.87	18	0.1-0.05
	Vitamin A	0.94	17	0.4-0.2
12	6 AN	0.25	33	0.9-0.5
	Cortisone	3.24	24	0.005-0.001
	Vitamin A	3.25	21	0.005-0.001
13	6 AN	0.60	8	0.9-0.5
	Cortisone	1.99	8	0.1-0.05
	Vitamin A	3.80	12	0.005-0.001
14	6 AN	0.45	14	0.9-0.5
	Cortisone	2.83	10	0.025-0.001
	Vitamin A	4.59	14	0.005-0,001

APPENDIX

Table 7

"Student's" t-test for comparison of mean body weight of A/J and C57BL control embryos during palate closure

PS	t-Value	d f	P
0	1.89	111	0.1-0.05
1	0.89	92	0.4-0.2
2	0.004	19	> 0.9
3	0.73	17	0.5-0.4
6	0.85	30	0.4
7	1.17	55	0.4-0.2

F test on body weight and palate stage; comparison of A/J and C57BL control

PS	F Value	d f	P
0	1.35	56/55	0.25-0.1
1	2.20	44/48	0.01-0.005
2	5.00	2/17	0.025-0.01
3	1.75	4/13	0.25-0.1
6	15.32	24/6	0.005-0.001
7	2.97	35/20	0.025-0.01

APPENDIX

Table 8 a

Analysis of variance of lower jaw length among control and treated A/J embryos within each body weight interval

BW(g)		Groups	F Value	d f	P
.120	.129	6AN	2.92	2/10	0.1
.130	.139	Vitamin A All three	4.36	3/35	0.025-0.1
.160	.169	"	1.49	3/37	0.25-0.1
.170	.179	"	0.94	3/36	0.5-0.25
.180	.189	"	7.46	3/23	0.005-0.001
.190	.199	"	2.91	3/25	0.1-0.05
.200	.209	"	9.00	3/23	< 0.001
.210	.219	"	8.81	3/23	< 0.001
.220	.229	"	0.086	3/18	> 0.75
.230	.239	"	0.56	3/8	0.75-0.5
.240	.249	"	1.91	3/10	0.25-0.1

APPENDIX

Table 8b

Analysis of variance of lower jaw length among control and 6 AN and vitamin A treated C57BL embryos

BW (g)	F - Value	df	P
.130-.139	5.04	2/8	0.05 - 0.025
.150-.159	0.13	2/36	>0.75
.160-.169	1.54	2/43	0.25 - 0.1
.170-.179	4.13	2/38	0.025- 0.01
.180-.189	0.12	2/32	0.5 - 0.25
.190-.199	5.88	2/37	0.01 - 0.005
.200-.209	3.31	2/27	0.1 - 0.05
.210-.219	0.79	2/24	0.5 - 0.25
.220-.229	14.57	2/16	<0.001
.230-.239	5.67	2/27	0.1 - 0.05
.240-.249	25.0	2/18	<0.001

APPENDIX

Table 8c

"Student's" t-test between control and treated group: mean lower jaw length vs body weight

Strain: A/J

Treatment	BW (g)	t value	df	P
6 AN	.130-.139	4.38	20	0.001
Vitamin A	.130-.139	2.69	24	0.02 - 0.01
Cortisone	.180-.189	3.85	12	0.01 - 0.001
	.200-.209	6.98	13	0.001
	.210-.219	3.46	10	0.01 - 0.001

Strain: C57BL

Cortisone	.120-.129	1.30	7	0.4 - 0.2
	.140-.149	4.77	18	0.001
	.150-.159	7.57	39	0.001
	.160-.169	3.87	37	0.001
	.170-.179	5.38	28	0.001
	.180-.189	6.90	28	0.001
	.190-.199	7.19	29	0.001
	.200-.209	4.52	24	0.001
	.210-.219	4.47	12	0.001
	.220-.229	3.66	15	0.01 - 0.001
	.230-.239	8.39	19	0.001
Vitamin A	.190-.199	3.52	26	0.01 - 0.005
	.130-.139	2.81	7	0.05 - 0.02
	.150-.159	0.53	35	0.9 - 0.5
	.160-.169	1.44	39	0.2 - 0.1
	.170-.179	1.67	28	0.1 - 0.05
	.180-.189	0.99	27	0.4 - 0.2
	.190-.199	3.50	26	0.01 - 0.001
	.200-.209	1.26	21	0.4 - 0.2
	.210-.219	0.87	10	0.4
	.230-.239	2.06	20	0.1 - 0.05
	.260-.269	2.12	4	0.1

pathogenesis of cleft palate was offered by Harris (1964), who observed a reduction in the weight of amniotic fluid associated with cortisone-treated embryos before and during the period of palate closure. The strain difference might thus be traced in part to greater reduction of amniotic fluid volume in A/J than in C57BL. Although Walker (1965) reported reduced amniotic fluid volume in cortisone-treated animals, his data included observations of some cleft embryos with larger volume of amniotic fluid than the control animals. In an experiment designed to produce 50% incidence of cortisone-induced cleft animals, the volume of amniotic fluid reduction was exactly the same in cleft animals as in their normal litter mates (Fraser et al., 1967). Thus, the role of reduction in amniotic fluid was ruled out as the causative factor in cleft palate formation.

There is also a difference in degree of flexion in the cranial base of the embryo in the region of the craniopharyngeal canal which divides the cranial base into anterior and posterior portions. The amount of this flexion was found to be much greater in rats than in mice. In mice, the angle is greater in the C57BL strain than the A/J (Harris, 1964). Reduction in amniotic fluid and a less flexed cranial base in the A/J strain at the time of palate closure compared to the C57BL embryo could account for the strain difference.

Rapid growth and lengthening of the cranial base could provide internal shelf force and play a role in palate