INNER EAR DEVELOPMENT IN MATERNAL HISTIDINAEMIA IN THE MOUSE

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ABSTRACT

The work described in this thesis is primarily concerned with the development of the inner ear of embryos from histidinaemic mothers. The inner ear abnormalities were first examined in adults. The adult inner ear of behaviourally affected animals showed various structural inner ear defects such as shortening of the posterior vertical canal, narrowing of the canals, shortening of the crus commune, absence of otoliths and abnormality of the cochlea.

This was followed by the study of embryos from 9½ days gestation stage to new born animals. Abnormalities of the neural tube and hind brain were found from 9½ day embryos to 11½ day embryos. The first sign of abnormalities of the inner ear was the absence of minute calcium carbonate crystals in the otoliths of 14½ day embryos and the distension of the whole endolymphatic system became apparent from 16½ days embryonic stage and persisted up till the adult stage. The severely affected animals were found to be deaf and they were found to have abnormalities of the cochlea.

Variable expressivity of both behaviour and inner ear defects was found in two stocks, the Edinburgh stock and the Cambridge stock. Histidine injection experiments of histidinaemic animals showed that elevation of histidine levels produced no increase in expressivity or penetrance. Breeding experiments showed that the genetic background of the offspring plays a role in the defect.

Possible explanations for the mechanism producing the abnormalities and for the difference in penetrance and expressivity are discussed.

CONTENTS

INTRODUCTION

In 1973 a mouse mutant was discovered which appears to be closely similar to that causing human histidinaemia. The stock of mice with high levels of histidine in urine, liver, plasma and brain was first obtained from Cambridge and this stock was derived from wild trapped mice from Peru in 1962. Due to low histidase activity, with less than 5% of normal, the histidine values in the tissues of adult histidinaemic mice were at least 10 fold higher than normal, except for skin. The high histidine character is inherited as a single autosomal recessive. The allele is designated as his (Kacser, Bulfield and Wallace, 1973). See Table 1.

Table 1 (from Kacser, Bulfield and Wallace, 1973)

Concentrations of Histidine in Various Tissues at 7 Weeks

Tissue	Mean value and s.e. of histidine High (*his/his*)	Normal (+/)	Ratio = $\frac{H}{N}$
Liver *	21.16 ± 0.94	0.97 ± 0.025	21.8
Brain *	2.10 ± 0.19	0.17 ± 0.007	12.2
Plasma †	3.29 ± 0.35	0.12 ± 0.005	28.7
Urine †	0.86 ± 0.16	0.04 ± 0.003	21.5
Skin *	0.49 ± 0.08	0.23 ± 0.050	2.1

All determinations were carried out by column chromatography and ninhydrin reaction.
* For brain, liver and skin the units are μmol g^{-1}.
† For plasma and urine the units are μmol ml^{-1}.

The stock in which this histidase mutant occurred also showed a high incidence of balance defective animals. In fact this behavioural phenotype was observed long before the biochemical lesion was discovered. It was, however, apparently not possible to obtain a strain where all animals showed the effect.

There was also variation in incidence between litters from the same mating. Wallace (1970) concluded from this that the balance defective character was controlled by an imperfectly penetrating

recessive gene under non genetic as well as polygenic control. With the discovery of segregation at the his locus in derivatives of the original stock, a relationship between the his/his, his/+ and +/+ genotypes and the balance defect was investigated. It was established that the balance defect syndrome appeared only in animals born from histidinaemic mothers and did not depend on the offspring's genotype. The animals with balance defects from this stock presented circling and head tilting behaviour, deafness, inability to swim, lack of orientation after spinning and poor maze learning. Lyon discovered various degrees of shortening of the posterior vertical canal and the crus commune and absence of otoliths in one or both ears in these animals (Kacser, Bulfield and Wallace, 1973).

It is therefore clear that the behavioural abnormality, associated with malformation of the inner ear, is caused not by some action of the animal's genes but due to a maternal effect of histidinaemic mothers. High levels of histidine or its derivatives apparently act as teratogens on the developing embryo.

Since most information about normal and abnormal development of the inner ear of the mouse has been obtained from an analysis of various mutants (i.e. cases where the offspring's own genotype is the cause of the lesions), it is useful to review the evidence before comparing it with the maternally caused condition. Before starting to review the mutants with inner ear defects, it is useful to describe the structures of the inner ear, so that one can refer to the various structures.

Figure 1

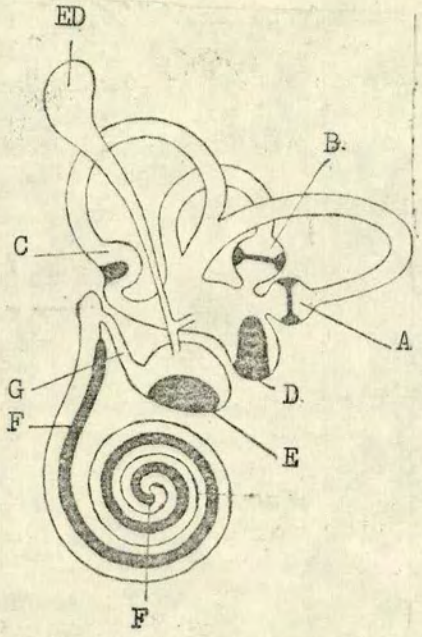


Diagram of the membranous labyrinth.
Neuro-epithelial areas black.

A, ampulla of anterior vertical canal.
B, ampulla of horizontal canal;
C, ampulla of posterior vertical canal;
D, utricular macula;
E = saccular macula;
ED = endolymphatic duct;
G = ductus reuniens;
F = organ of Corti in the cochlear duct.

Redrawn and slightly modified from Maximow and Bloom (1948).

The inner ear is called the labyrinth because of its complex structures. It comprises a series of canals and cavities which are hollowed out of the bone and are known as the osseus labyrinth. According to their position in space relative to the skull they are named as anterior, posterior and horizontal canals. The anterior semicircular canal is orientated vertically and placed transversely to the long axis of the pars petrosa of the temporal bone. The posterior semicircular canal is also vertical and positioned at right angles to the anterior canal. The horizontal canal as its name implies is horizontal. At the end of each canal there are dilations, called the ampullae. The inner ear also consists of bony cochlea, a spirally coiled tube, resembling a snail shell (Fig. 1).

The interior of the osseous cavities is lined with a layer of periosteum and encloses a system of vesicles and canals with a fibrous wall the membraneous labyrinth. All parts of the latter are continuous and communicate with one another; they are filled with a clear fluid, the endolymph. The bony labyrinth is filled with a fluid perilymph and communicates with the arachnoid space of the brain cavity via a narrow channel, the perilymphatic aqueduct. The vestibular membraneou s labyrinth consists of the saccule, the utricle, three semicircular ducts and the endolymphatic duct and sac. It serves as a receptor organ for equilibrium (balance and orientation). The saccule and the utricle together occupy the one bony vestibule, whereas the semicircular membraneous ducts are lodged in similarly shaped semicircular bony canals. The anterior semicircular duct connects with the utricle via a widening, the ampulla and it joins the posterior semicircular duct to form the crus commune, which

in turn opens into the utricle. The posterior semicircular duct connects with the utricle via an ampulla and the opposite end of the duct. Each ampulla has areas of specialized neuroepithelial cells, the crista ampullaris. These are neuroepithelial layers in the utricle and saccule as well, they are macula utriculi and macula sacculi.

There are two types of cells in the neuroepithelium layer of cristae as well as of maculae. These are the hair cells and the supporting cells. The hairs of the hair cells of cristae are embedded in the cupula, a gelatinous protein and polysaccharides. In the utricle and saccule, the maculae are covered by an otolithic membrane, which is a gelatinous mass with many small crystalline bodies, the otoliths.

The sacculus is connected to the cochlear duct via ductus reuniens, and to the utricle via a Y-shaped tube, the fork giving rise to the endolymphatic duct and this duct terminates as an endolymphatic sac. The cochlea is the auditory part of the inner ear, innervated by the cochlear nerve of the eighth cranial nerves. Its bony labyrinth forms a spiral channel of one and a half turns in the mouse (and which in man has two and a half turns and in the guinea pig five turns). There is a central bony stalk, the modiolus. Numerous bipolar ganglion cells are located in a spiral canal within the modiolus, forming the spiral ganglion. The cochlear duct is the spiral membraneous labyrinth of the cochlea which terminates as a blind end at the apex of the cochlea. The basal turn of the cochlear duct communicates via the ductus reuniens.

In the cochlear duct there are three parts scala vestibuli, scala tympani and scala media. Both scala vestibuli and scala tympani are filled with perilymph, but the scala media is filled

TABLE 2 a) Degenerative type

Mutant	Behaviour	Hearing	Onset of Degeneration of inner ear						Other Systems and Other abnormalities
			T. membrane	Organ of Corti	Spiral ganglion	Ventricular ganglion	Utriculus	Sacculus	Cristae	
Varitint Waddler Va/+	Head tossing and circling	deaf	Club shape lost contact with hair cells (-)	- (2nd wk)	- (3rd wk)	- (2nd wk)	+	- (4th wk)	- (after 1 month)	Some heterozygotes sterile. Spotting most of the belly and some area white.
Jerker je/je	Head tossing and circling	deaf	Lost contact with hair cells	- (2nd wk)	- (2nd wk)	+ (at birth)	- (at birth)	- (at birth)	+	+
Shaker-2 sh-2/sh-2	Head tossing and circling	deaf	Thick and lost contact with hair cells (-)	- (2nd wk)	- (4th wk)	- (12th wk)	+	- (at birth)	+	+
Shaker-1 sh-1/sh-1	Head tossing and circling	deaf	+	- (2-8 wk)	- (2month)	+	+	- (3rd wk)	+	+
Waltzer v/v	Vertical head movement and circling	deaf	Thicker than normal	- (2nd wk)	- (4th wk)	+	+	- (2nd wk)	+	C.N.S.

Pirouette pi/pi	Vertical head tossing and circling	deaf	Thicker than normal	- (2nd wk)	- (4th wk)	- (12th wk)	+	- (6th wk)	- (after 8 months)	+
Spinner sr/sr	Vertical head shaking and circling	deaf	- Lost contact with hair cells	- (2nd wk)	- ?	- ?	+	- ?	+	+
Deafness dn/dn	Head tossing	deaf	(-) Lost contact with hair cells	- (after 10 days)	- (after 50 days)	- (after 50 days)	+	- ?	+	+
Mocha mh/mh	Head tilting	deaf	Curled back	- ?	- ?		- ?	- ?		Eye and coat colour
Snell's Waltzer sv/sv	Jerking and circling	deaf	- Lost touch with hair cells	- (after 12 days)	- (after 18 days)	- (after 18 days)	- (after 3 months)	- (after 3 months)	- (after 3 months)	+

+ = normal

- = affected

? = unknown

TABLE 2 b) Morphogenetic type

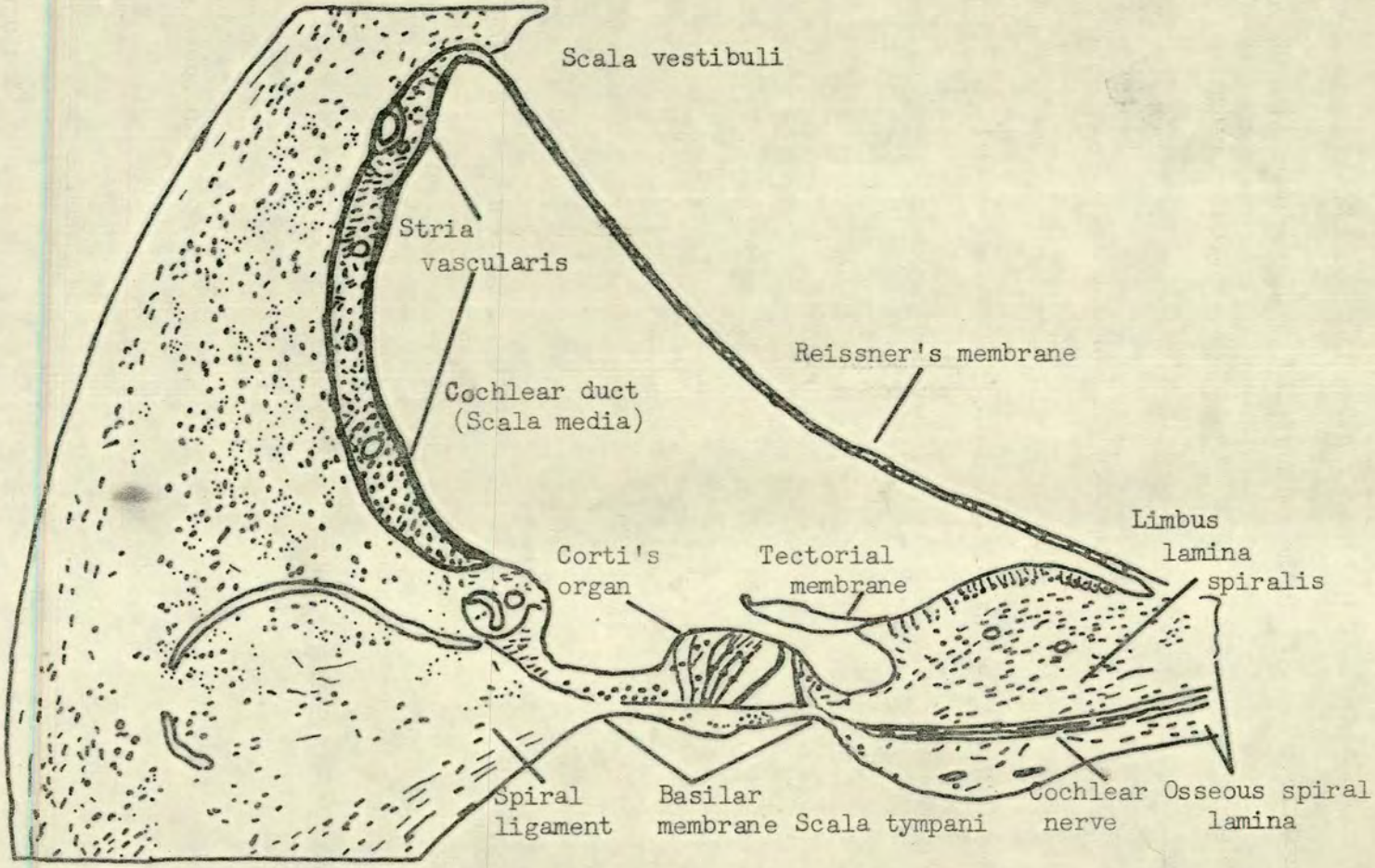
Mutant	Behaviour	Hearing	Canals			Cochlea	E.duct	Utriculus	Sacculus	Cristae	Other Systems and Other Abnormalities
			AVC	PVC	HC						
Shaker Short st/st	Circling & head shaking	deaf	-	-	-	-	-	+	+	+	Sterile, shortening or absence of the tail
Kreisler kr/kr	Head shaking & Circling	deaf	-	-	-	-	-	-	-		At early embryonic stages neural tube abnormal
Fidget fi/fi	Head tossing & Circling	+	Rudi-mentary	Rudi-mentary	-	+	+	+	+	-	Eye, paraflocular lobe of cerebellum
Dreher dr/dr	Head shaking & Circling	deaf	-	-	-	-	-	-	-	+	C.N.S.
Twirler Tw/+	Circling	+	Uneven Outlines	Uneven Outlines	-	+	+	No otoliths or few crystals	No otoliths or few crystals	-	Homozygote lethal with hare lip and cleft palate
Zig-zag	Zig-zag movements	+	+	+	middle portion constr. or -	+	+	+	+	+	+
Waltzer type (Wt/+)	Head Shaking & Circling	+	+	-	-	+	+	+	+	+	Homozygotes die at 11th gestation day with distorted brain, deceased proliferative activity, retardation in development & pycnotic degeneration of nuclei
Nijmegen Waltzer nv/nv	Horizontal & Vertical Head Shaking	+	+	+	constr. or -	+	+	-	-	-	+
Dancer Dc/+	Horizontal & Vertical Head Shaking & Circling	+	-	+	-	+	+	-	-	-	Homozygotes lethal, bilateral or unilateral, hare lip and cleft palate
Sightless Sig/+	Head tilting & Circling	+	-	-	-	-	-	-	-	-	Eyes open at birth and slight hydrocephaly. Homozygotes lethal at birth.

Pallid pa/pa	Head Tilting	+	+	+	+	+	+	-	-	+	Pink eye and pale coat colour
Muted mu/mu	Head Tilting	+	+	+	+	+	+	-	-	+	Light eye with coat colour with muted brown shade
Unbalanced ub/ub	Head Tilting	+	+	+	+	+	+	-	-	+	+
Cocked co/co	Head Tilting	Deaf	+	+	+	-	+	-	-	+	Kinky tail
Rotating rg/rg	Circling & Head jerking	+	-	-	-	+	+	+	-	+	Kinky tail
Shaker with syndactylism sy/sy	Head tossing and head shaking with occasional circling	Deaf	-	-	-	-	-	-	-	-	Syndactylism, faulty classification of the whole skeleton

+ = normal

- = affected

Figure 2



A diagrammatic representation of a cross section passing through the cochlear duct in the mouse.

Redrawn and modified from Maximow and Bloom (1948).

with endolymph. In cross section the cochlear duct shows a trapezoid profile (Fig. 2). The roof of the cochlear duct, bordering on the scala vestibuli is referred to as the vestibular membrane (or Reissner's membrane). The floor of the cochlear duct is formed by the basilar membrane. The specific auditory receptor cells rest on the organ of Corti. The outer wall of the cochlear duct is lined by the stria vascularis. The spiral organ of Corti is the auditory receptor organ of the inner ear. The surface of the organ of Corti is covered by a peculiar ribbon-like structure of jelly-like consistency - the tectorial membrane.

The behaviour abnormality known as the "shaker waltzer syndrome" is remarkably frequent in the mouse. Over fifty genes affecting the inner ear in the mouse are known. The inner ear has been found to be affected in all mutants with the shaker waltzer syndrome. Gruneberg in his review (1956) divided 12 mutants with shaker waltzer syndrome into two groups: those with degenerative abnormalities and those with abnormalities during ontogeny. Deol (1968) in his review article added up some more morphogenetic and degenerative types of inner ear defect. In the degenerative group the development and fine differentiation of the inner ear appear to proceed normally up to a few days old and then the degeneration sets in to various structures such as cochlea, saccule, utricle and semicircular canals, vestibular ganglion, particularly the degeneration of the neural elements (Table 2). The degeneration of the inner ear in Varitint waddler, shaker-2 and jerker has been described in detail by Deol (1954).

Varitint waddler Va/Va is a semidominant condition which shows spotting with shaker waltzer syndrome. Va/Va and some heterozygotes are sterile (Cloudman and Bunker, 1945). In the inner ear the anomaly is observed starting from the 4th day after birth. The tectorial membrane is thickened and it loses contact with the organ of Corti. The spiral ganglion cells become irregular and the number of cells are reduced. The organ of Corti, stria vascularis, maculae and cristae all show degeneration in later stages (Deol, 1954). Shaker-2 (sh-2/sh-2) is recessive. The utriculus and sacculus are affected from the time of birth. The tectorial membrane is abnormal and it is not in contact with the hair cells thus does not function at all. The spiral ganglion is affected at 14 days, Corti's organ degenerates between 12 and 15 days, the stria vascularis at 15 days, but the cristae are normal. Jerker (je/je), another waltzer shaker, is recessive. The abnormalities of the maculae of the utriculus, and sacculus are present at birth. The tectorial membrane is normal in size, but it loses contact with the hair cells, Corti's organ shows degeneration at the 11th day and in the spiral ganglion the cells show irregular shape which becomes apparent at the 8th or 9th day. The stria vascularis shows histological abnormalities round about the 17th day. Apart from a slight affect on the hair cells the crista is normal (Deol, 1954).

Shaker-1 (sh-1/sh-1) is a recessive character. The behaviour of sh-1 mice has been described by Lord and Gates (1929). In the inner ear the degeneration of Corti's organ can be detected only at the age of 12 days. The tectorial membrane is slightly thicker than normal. In the spiral ganglion there is reduction in the amount of cytoplasm around the nucleus and later on the nuclei themselves

degenerate. Both the size and histological structure are affected in the stria vascularis which shows degeneration later than that of organ of Corti. The macula of the utriculus is normal but the macula of the sacculus is affected.

Waltzer (v/v) is a recessive and shows the shaker waltzer syndrome. Adult waltzers are completely deaf. The organ of Corti and the macula of the sacculus are affected as in shaker-1. Pirouette (pi/pi) is a recessive condition and this gene was described by Woolley and Dickie (1945). From the age of ten days onwards they show the waltzing syndrome. They are deaf. The organ of Corti and the macula of the sacculus are affected as are the cristae unlike shaker-1 and waltzer (Deol, 1956).

The spinner mouse (sr/sr) which is also a behaviourally affected mutant, shows vertical head shaking and circling behaviour and is deaf from birth. In the cochlea the first signs of abnormality can be detected at the age of 15 days where the organ of Corti is reduced in size. The tectorial membrane loses contact with Corti's organ and in the spiral ganglion the ganglion cells are reduced. The stria vascularis is abnormal in cell size and is often vacuolated. The macula of the utriculus is normal though the cells of the macular epithelium of the sacculus is abnormal. The cristae are normal.(Deol and Rob 1962).

Snell's waltzer (sv/sv) shows deafness, hyperactivity, jerking movement of the head and circling, (Deol and Green, 1966). Degeneration of the organ of Corti, the two maculae and the three cristae are observed after the age of 12 days.

Mocha (mh/mh) described by Lane and Deol (1974) shows the waltzer shaker syndrome. Eye colour and coat colour are also affected. The inner ear of mh/mh show degenerative changes in the organ of Corti,

stria vascularis and spiral ganglion. The otoliths of both the sacculus and utriculus are also abnormal.

In the morphogenetic type there is a big range of abnormalities. In this type of defect the mutants show gross defects of the labyrinth which have arisen during the embryonic stages.

Shaker short (st/st) is now extinct. It was a recessive gene, had shortened tail, shaker waltzer syndrome and sterility (Dunn, 1934). Moreover many abnormal newborn had brain hernia (Bonnevie, 1936). The prenatal development of ear anomalies was studied by Bonnevie (1936). The inner ear was represented by an oval laterally compressed vesicle which lacks an endolymphatic duct, has no semi-circular canals and no division of the median part into separate chambers. The organ of Corti was rudimentary and the animals were completely deaf.

Dreher (dr/dr) a recessive gene was first described by Falconer and Sierts Roth (1951). The animals show circling behaviour, deafness, hyperactivity and head tremor. Embryology of the inner ear was investigated by Fischer (1956, 1957). The endolymphatic duct is dilated. The differentiation of the otic vesicles is incomplete and the folds which will separate the utriculus and sacculus are retarded. In the adult the cochlea is short and loosely coiled. The sacculus opens into the utriculus without any intervening ducts, forming one very big chamber. Deol (1964) reinvestigated dreher at the very early stages of development and discovered that the rhombencephalon was abnormal. In normal animals at 9 days of embryonic development, the thin roof of the rhombencephalon is kite-shaped whereas in dreher it is diamond-shaped and does not extend as far as the otic vesicle. Bierwolf (1956) studied

dreher and he noted 11% of all newborn offspring are hydrocephalic. Severely affected hydrocephalics died within a few days of birth. The brain abnormalities found in the newborn animals varied. In some animals the 4th ventricle was considerably dilated, the cerebellum instead of lying as a compact bulge behind the mesencephalon was hollow and its wall was thinned. In some mild cases, only a thin roof of brain substance and pia mater was present in the region of the vermis. Embryology of the brain was studied by Bierwolf (1958) and he found that in the 11 day embryonic stage the caudal region of the roof of the 4th ventricle became thickened and sealed the 4th ventricle and he suggested the dilation of the 4th ventricle and cerebellar malformation caused the newborn animal's hydrocephalus.

Kreisler (kr/kr), a recessive mutant was induced by x-rays. The behaviour of this mutant is also a shaker waltzer type. Pathology and development value studied by Hertwig (1944) showed that kreisler has one or two cysts situated under the rhombencephalon. The paraflocculer lobe of the cerebellum which is situated in the subarcuate fossa is also absent. Kreisler shows a completely unrecognisable inner ear. The endolymphatic duct is absent, no separation of utriculus and sacculus is observed and the semicircular canals are imperfect. In the cochlear part, the ductus cochlearis remains a wide slightly curved sac and does not form a spiralized cochlea. There is no organ of Corti and no spiral ganglion (Hertwig, 1956). They are also deaf.

Another mutant which shows morphogenetic changes in the inner ear is fidget (fi/fi) which is a recessive mutant first described by Gruneberg (1943). It shows pleiotrophic effects. The membraneous

labyrinth, auditory capsule, cerebellum, eyes and lachrymal gland, numerous structures in the skull and the mandible are all affected. In addition the pelvic girdle and feet are affected as well. In the inner ear of this mutant the horizontal canal and its crista ampullaris are missing; the superior and posterior canals are rudimentary. The cochlea is completely normal and the animal can hear (Truslove, 1956).

The mutant twirler (Tw/+) which was first recognised by its waltzing behaviour was later found to have the morphology of the inner ear affected and, when homozygous, to cause death of new born animals through harelip and cleft palate. The abnormalities found in the bony labyrinth are the reduction or absence of the horizontal canal, absence of otoliths and uneven outlines of the vertical canals. The cochlea is normal (Lyon, 1958).

Zig-zag, a behaviour defect mutant inherited polygenically (Lyon, 1960) which walks in a zig zag path shows either reduction or absence of one or both horizontal canals. The defect varies from complete absence of the canal to normality, but the crista and the ampulla of the horizontal canal as well as the remainder of the inner ear are normal.

The semi-dominant gene waltzer type was first described by Bangham and Kelly (1955) after spontaneously occurring at Oak Ridge. It is dominant with incomplete penetrance. It was later studied in more detail by Stein and Huber (1960). Wt/+ mice show behaviour abnormality, from slight nervousness to rapid circling and vertical head shaking. The affected animals show circling in either direction and can be detected with certainty at the age of 3 weeks or more. Swimming is impaired. They are not deaf. Morphology of the inner ear shows various abnormalities.

Either the posterior vertical canal or horizontal canal is affected. The anterior vertical canal is never affected. In some of the affected ears there is a slight reduction in size of the canal or sometimes only part of the canal near the ampulla is present and the rest of the canal is missing. The most severely affected canal is the horizontal canal. Apart from the abnormality in the semicircular canal, no abnormality has been reported of the rest of the inner ear. The abnormality of the semicircular canal can be detected at the age of 13 days gestation.

Nijmegen waltzer (van Abeelen and van der Kroon, 1967) shows circling as well as horizontal and vertical head shaking. They are not deaf. The horizontal canal is either constricted in the middle or incomplete or altogether missing, Deol (1974).

Dancer (Dc/+) a semidominant is lethal in the homozygote. Homozygotes have a cleft lip and cleft palate and die within a few hours of birth. Heterozygotes show jerking movements of the head in both horizontal and vertical planes. Some mice show tilting behaviour as well. In addition to abnormal behaviour dancer mice have a white spot in the middle of the head (Deol and Lane, 1966). The inner ear of the dancer was found to be a morphogenetic type. The horizontal canal is constricted, discontinuous or absent. The utriculus and sacculus are not in separate chambers and the sacculus is reduced in size. The posterior semicircular duct and canal are unaffected as are the ampullae and the cristae.

Sightless (Sig/+), a semi-dominant gene which shows abnormal behaviour in the position of the head, was first described by Searle (1965). They have both eyes closed at birth and slight hydrocephaly. The homozygotes die at birth, showing more severe

hydrocephaly, sometimes with hind foot anomalies, especially syndactyly. The developmental process of this mutant was investigated by Khaze'I (1974). In the inner ear there is only one semicircular canal. The utricle, saccule and cochlea even though present cannot be clearly identified. The cochlea is loosely coiled. The three cristae and utricular macula are missing and there is no endolymphatic duct.

The last affected inner ear in the shaking waltzing group of mice is that of pallid.(Lyon, 1951). They have pink eyes and pale coat colour. The animals show head tilting behaviour and the abnormalities in the inner ear are the absence of otoliths in both utriculus and sacculus. Lyon also studied the development of otoliths in normal as well as in pallid animals, and she reported that the otolith matrix and crystals were first visible in the mouse between $15\frac{1}{2}$ and $16\frac{1}{2}$ day of gestation. Erway (1968) studied the development of the otolith in manganese-deficient mice and he showed a similar pattern to Lyon's finding in pallid. Erway, Fraser and Hurley (1971) did experiments on pallid by feeding manganese to pregnant females on the tenth and eleventh day of gestation and established that the defect could be prevented.

Muted (mu/mu) homozygotes have light eyes at birth and coat colour with a muted brown shade. They show head tilting behaviour. This balance defect was found to be due to the absence of otoliths from one or both ears (Lyon and Meredith, 1965).

Another mutant which shows balance defects and is similar to pallid or muted is unbalanced (ub/ub) (Lyon and Meredith, 1965). It is an autosomal recessive and has complete penetrance and the otoliths are always lacking from both ears. The remainder of the

inner ear is normal.

Shaker with syndactylism (sy/sy) was produced by x-rays (Hertwig 1942). Abnormal young are recognized at birth by fusions between the toes. The toes of the hind feet are abnormal in that they are fused, either by soft tissue webs or by bony fusions. These animals also show the shaker waltzer syndrome, head tossing and shaking with occasional circling. They are deaf. sy/sy show abnormalities of the inner ear, syndactylism and abnormal ossification including the whole skeleton (Gruneberg, 1962). The development of the inner ear has been analysed in detail by Deol (1963). The inner ear develops normally up to the 12th day of gestation. In the abnormal sy/sy embryo, around the otic duct there is an excessive formation of loose mesenchymal tissue. This tissue lies between the utricle and saccule, so that they are held apart abnormally. In this way these two structures lie in abnormal positions. At later embryonic stages, the spaces in the spongy mesenchyme are larger and coalesce forming a continuous cavity whereas in the normal embryo the mesenchyme becomes spongy in the whole periotic labyrinth, especially in the region of the future vestibule. After birth, histological abnormalities appear in the cochlea, utricle and saccule. In the cochlea abnormalities can be found in the stria vascularis, vestibular membrane and organ of Corti, whose components are all abnormal. The saccule is smaller than normal before birth, but after birth its chamber shrinks as a result of thickening or swelling of its wall. The otolithic membrane and macula become abnormal.

Cocked (co/co) an autosomal recessive arose in a line that was being inbred for the open eyelids (oe) mutant. co/co animals show head tilting behaviour. Cleared otic capsules show large crystals

or no otoliths at all in the utriculus. In the sacculus they show variable abnormality of the otoliths. (Peterson, 1970).

Rotating (rg/rg) with the shaker waltzer syndrome e.g. hyperactivity, circling without any preference for either right or left, head jerking but without deafness was first discovered by Deol and Dickie (1967). Some of the rotating mice show a white spot in the centre of the abdomen, and some of the animals have a kinky tail. Both morphogenetic and degenerative types of abnormalities are found in the rotating mice (rg/rg). The maculae of the saccule show degenerative abnormality. The macula appears normal up to the age of 40 days. At a later stage degeneration sets in. The epithelium is thinner and the hair cells become reduced and the shape of the cells becomes abnormal. The otolithic membrane is also affected and later the whole sensory epithelium degenerates. The semicircular ducts and canals show the morphogenetic type of abnormality. In newborn, the horizontal canal is the most affected part. Constriction or closure of the lumen occurs and some of the horizontal ducts are shorter than normal.

In amphibians many investigators e.g. Zwilling (1941); Detwiler and Van Dyke (1950), Yntema (1950), Jacobson (1966) have demonstrated experimentally the importance of the medulla for differentiation of the otic vesicle.

In mice Deol reinvestigated kreisler (kr/kr) and he found out that in $8^{3}/_{4}$ day embryos the rhombencephalon is abnormal, the 4th to the 6th neuromeres do not develop, the facial acoustic ganglion complex is abnormal in position and the otic vesicle is removed from the neural tube. He suggested that the abnormalities of the inner ear could be due to those abnormalities (Deol, 1964a, 1968).

In dreher (dr/dr) at 9 and 10 days of embryonic development the rhombencephalon is abnormal, it is diamond-shaped whereas in the normal it is kite-shaped and in the abnormal embryo the rhomb encephalon comes to an end in front of the otic vesicle (Deol, 1964b). Deol suggested that the malformations of the inner ear are consequent on this abnormality of the neural tube.

Splotch (Sp/+), a dominant gene causes spotting in heterozygotes and prenatal death in homozygotes (Russell, 1947). The embryology of the malformation was studied by Auerbach (1954). She stated that homozygous splotch embryos can be recognized at $9\frac{1}{2}$ days and die on the 13th day due to a disturbance of the region of neural crest and dorsal part of the neural tube. Deol found out that the inner ear in the Sp/Sp was abnormal from its first appearance. The position and shape of the endolymphatic duct was abnormal. The cochlea and vestibular part of the labyrinth were also abnormal. The whole labyrinth of Sp/Sp embryo is abnormal (Deol, 1966). He established that the abnormalities of the inner ear are due to faulty differentiation of the neural tube.

Looptail (Lp/Lp) (Strong and Hollander, 1949), is a dominant gene with incomplete penetrance. Homozygotes are alive in utero but eaten by the mother at birth. Homozygotes show open neural tube and often umbilical hernia. They show a behaviour defect different from the waltzing shaking syndrome. Strong and Hollander described it as nervous rocking or wobbling of the head. Prenatal development of Lp/Lp was investigated by Stein and Rudin (1953). The earliest abnormality which was detected at $9\frac{1}{2}$ days was an open neural tube.

Apart from the gross morphological malformation of the neural tube histological differentiation of nervous tissue appeared normal. However, they found increased numbers of greater proliferative activity, between the optic stalk and the auditory vesicle and in the lower level of the spinal cord. At 10, 11, and 12 days of embryonic development Deol (1966) discovered that the endolymphatic duct is abnormal in position. The cochlea, utriculus and sacculus are abnormal. The semicircular canals, even though present, are abnormal in position. Deol suggested that the abnormality of the inner ear originated in the abnormality of the central nervous system.

In dancer, (Deol and Lane, 1966), which show waltzing behaviour and the inner ear defect, when traced back to the 10 day embryonic stage, the acoustic ganglion was found to be affected. It is either smaller or bigger than normal. Deol suggested that the primary abnormality in this mutant also concerns the nervous system.

In sightless the entire inner ear is malformed. There is only one abortive semicircular duct. In the 9 day prenatal stage the vesicle does not lie against the fifth neuromere but between the fifth and sixth neuromeres. Deol (1976) suggested that sightless mice also appear to have the original defect as a disturbance of the relationship of the otic vesicle to the neural tube and the acoustic ganglion.

Extra toes (Xt/+) is semidominant. Homozygotes have multiple abnormalities including feet with up to eight or nine digits, hemimelia, oedema and abnormal spinal cord, brain, nose, eye, ear. The inner ear of Xt/Xt has no horizontal canal and the anterior vertical canal is also abnormal. The heterozygotes have pre-and post-axial polydactylism of all four feet, but the inner ear and behaviour have not

been described (Johnson, 1967).

It is thus seen that there are large numbers of known mutants which have an effect on the inner ear. It is, however, clear that it is by no means the only effect of these genes and unlikely to be the primary defect. Its importance is that it results in fairly obvious behavioural abnormalities and therefore leads to the genetic identification of the allele as well as the description of the histology.

Furthermore the division into degenerative and morphogenetic classes, while convenient, is by no means exclusive, and it is probably better to regard all mutants as developmental with different times of onset of the major changes.

The work to be reported in this thesis is a histological study to find out the anatomical and neurological changes in the embryonic stages. It should be repeated at this stage that all previous work on behavioural mutants is concerned with the effect of the genotype of the animal carrying the alleles in question. This is not so in the phenomenon described here. Previous work (Kacser et al. 1973) has established that the effect was due to the maternal metabolism during pregnancy. Behaviourally abnormal offspring are obtained from his/his mothers which, owing to the absence of histidase activity, have greatly elevated levels of histidine (and its derivatives; imidazole pyruvic acid, imidazole lactic acid and imidazole acetic acid) in all tissues. Since this metabolic condition is, not unexpectedly, recessive (Kacser and Burns, 1973). heterozygotes (his/+) do not give abnormal offspring. It was however possible to make such heterozygous mothers temporarily

histidinaemic by appropriate additions of histidine to the diet only during the second week of pregnancy. Such treated mothers produced affected offspring. This indicated that the earliest time at which the morphogenetic effect can be brought about and the principal work therefore consisted of the histological investigation of embryos between $9\frac{1}{2}$ and $18\frac{1}{2}$ days of pregnancy.

A difficulty, present in the whole project, was the fact that in terms of the behaviour phenotype (at 3 weeks postnatal and later) the penetrance of balance defect was much less than 100%. It was therefore an important part of the investigation to determine whether at early stages of ontogeny any morphological abnormalities were present in all embryos which then regulated to give normal behaviour or whether even at these earlier stages differences in effect would be detected. Part of the work therefore consisted in investigating the possible existence of a metabolic threshold in determining the number of affected animals in the litter.

Finally it was known that the penetrance of the condition had declined to about 10% or less in the Edinburgh stock while a stock held at Cambridge (from which ours was derived) showed penetrance of over 80%. Part of the work therefore consisted in attempting to elucidate the difference and to discover its metabolic and genetic basis.

MATERIALS AND METHODS

Animals

The animals used for the present study were his/his, his/+ and +/+. Two stocks of animals were used.

Cambridge Stock (CAM): obtained from Dr. E. M. Wallace in August, 1976, which has about 73% penetrance of balance defective offspring and which was his/his . They are derived from wild mice trapped in Peru in 1962 (Wallace, 1970).

Edinburgh Stock (SEV) which has low and variable penetrance of balance defective offspring. In 1971 when it was first obtained from Cambridge it had 80% penetrance. It was maintained by random mating selecting only for the his allele. After seven crosses the stock was pure his/his. No selection for balance defect took place. A parallel stock was derived which was +/+ for histidase.

In the first part of the present work, (i.e. study of the inner ear development from 13½ days to newborn) most animals were used from Edinburgh stock.

Behaviour of the animals are classified as severely affected (bla^{+}) when the animal shows frequent tight circling, hyperactivity and deafness, mildly affected (bla^{+-}) when the animal shows very slight abnormal behaviour, tilting to left or right.

Timed Matings

Males and females were put together at 5 pm. The females were examined for vaginal plugs the next morning using a seeker. If a plug was found it was taken as day ½ of gestation. Pregnant females were killed on different days of pregnancy and their uteri removed. Embryos were dissected out in Ringer's solution before fixation. They were then checked with the aid of Gruneberg's (1943) chart.

Histological Procedure

Fixation and Embedding

Two fixation methods were used:

(1) Fixation in Bouin's fixatives for 24 hours.

(2) 80% alcohol at 4°C for 24 hours.

Otoliths are reported not to be preserved until two days after birth if fixed in Bouin, formalin or Susa fixatives (Lyon, 1955; Veenhof, 1969).

Mouse embryos of 13½ day to new born which were used for the study of development of inner ear, were fixed in 80% alcohol at 4°C to avoid decalcification of the material so that organic matrix and calcium carbonate crystals of otolith could be preserved more in its natural state.

Animals of 9½ to 11½ days gestation and some animals of 14½ days and 17½ days gestation which were used to look at the brain as well as the inner ears were fixed in Bouin's fluid, because Bouin's fixative preserved the brain better than 80% alcohol at 4°C. Otoliths are not present in the early stages such as 9½ to 11½ days. For the 14½ days and 17½ days gestation which were fixed in Bouin's fixative otoliths of the inner ears could not be scored.

In some embryos of 17½ and 18½ days gestation and in all new born which were used to study the inner ears, the skin was stripped from the skull, which was not too difficult because of the loose texture of the connective tissue between skin and skull. After this the skull was cut in a sagittal plane into two halves, after which the contents of the cranial cavity were removed. Then both halves of the skull were immersed in the fixative. After fixation the skulls were dehydrated in alcohol according to the following regime:

1. 70% alcohol - 24 hours.
2. 90% alcohol - 24 hours.
3. 95% alcohol - 8 hours.
4. absolute alcohol - 8 hours.
5. methyl benzoate run under alcohol and allow specimen to sink in 3 - 8 hours.
6. benzene, two changes - 4 hours (2 hrs each).
7. 50 benzene and 50 paraffin (52°C melting point) - 1 hour.
8. paraffin - (54°C melting point) - 2 changes, 1½ hrs each change were used and the specimens were embedded.

The same kind of dehydration procedure was used for the specimens that were fixed in 80% ice cold alcohol.

For the mice of 3 weeks and upwards the ears together with some surrounding bone are dissected out. The bulla was opened to prevent the accumulation of gas within it which would cause the specimen to float. They were then fixed in Bouin - 24 to 48 hours and then transferred into 2% nitric acid + 70% alcohol for 2 to 5 days, the medium being changed daily. The dehydration was carried out as stated above. After leaving the specimen in 50:50 methyl benzoate and absolute alcohol for 3 - 8 hours, the specimens were infiltrated with 1% colloidin in methyl benzoate, to give additional support to the tissue, and left in that medium for 24 hours, and then put into benzene for two changes (4 hours), 50:50 benzene and soft paraffin for 1 hour and then into paraffin (54°C melting point) for 3 hours (2 changes) and were embedded. The sections were cut transversely at 8μ.

Staining Methods

Haematoxylin and Eosin Stains

Before the sections were stained, the paraffin wax was removed by placing the slides in xylene for about 5 minutes and the xylene then washed off with absolute alcohol. The sections were hydrated through an alcohol series to distilled water. The sections were then stained with Delafield's haematoxylin (Raymond A. Lamb) for 4 minutes.

The sections were then washed in running water for 20 minutes and then counterstained with 1% watery yellow eosin (Gurr Ltd.) for one to five minutes, and dehydrated through the series of alcohol. The sections were cleared in xylene for about 5 minutes and were mounted in DPX mountant (slightly modified from Clayden, 1948).

Polysaccharides

Some of the $17\frac{1}{2}$ and $18\frac{1}{2}$ days gestation, newborn and 3 weeks old sections were stained to demonstrate polysaccharides. The Hotchkiss-McManus (PAS) method was used. This method is used for the oxidation of 1-2 glycol groups with periodic acid for 5 minutes. The sections are washed in tap water for 2 minutes then in distilled water for 2 minutes and stained with Schiff's reagent for 15 to 20 minutes. They were then placed in a sulphurous acid rinse for 2 to 3 minutes (3 changes 1 - $1\frac{1}{2}$ minutes each change), washed in running water and stained with haematoxylin for 2 minutes, washed in running water for 15 to 20 minutes and dehydrated through the alcohol series, cleared in xylene and mounted in DPX.

Control slides were placed in distilled water instead of periodic acid. Examination of these confirmed that the sections contained no substances which could recolourize Schiff's reagent without previous oxidation.

Stain for birefringence

The paraffin wax was removed from the sections by xylene and the slides were then washed in absolute alcohol for about 3 minutes. The slides were then placed in 1% Eosin in 95% alcohol for 3 minutes and then returned via absolute alcohol to xylene and mounted in DPX (Lyon, 1955). The slides were then examined with a polarizing microscope to detect birefringence of calcareous particles which could be seen as highly refractile crystals.

Whole Mount Preparation

Ears at 3 weeks or more

The ears at 3 weeks or more were cleared according to Lyon (1958). To prepare whole mounts of the bony labyrinth, parts of the skull including the labyrinth, were fixed in 70% alcohol for 24-48 hours and then macerated in 1% potassium hydroxide until the adherent soft tissue became transparent. They were then dehydrated in alcohol as follows:

70% alcohol)
90% alcohol) 1 day
95% alcohol)

50/50 benzyl alcohol/absolute alcohol 16-24 hrs.

100% benzyl alcohol. The cleared inner ear was then studied under dissecting microscope.

$17\frac{1}{2}$ to $18\frac{1}{2}$ day embryonic stage and newborn animals

Some of the inner ears at $17\frac{1}{2}$ and $18\frac{1}{2}$ days of gestation and newborn animals were prepared for scoring otoliths. The skin was removed from the skull and the skull was cut into two halves, the brain was removed and they were fixed in 70% alcohol, but the specimens at these stages were not decalcified with potassium hydroxide as in adults. They were dehydrated in an alcohol series as stated above and finally transferred into 100% benzyl alcohol. They were then observed under polarizing microscope for the birefringence of otoliths.

Injection Experiments

his/his females which were about 8 weeks old were used for the injection experiments. The animals were injected intraperitoneally with 0.5 ml of 5% histidine in saline at 10 am and 4 pm in one group and at 10 am and 10 pm in another group.

Blood Sampling

Blood was taken from the mice before each injection. The animal concerned was picked up by the tail with a tweezers and put into a plastic tube supported in a clamp stand, their entrance and exit was blocked with two metal pins so that the tail hung out of the end of the tube. Blood samples were obtained by cutting approximately one eighth of an inch off the tail and collecting 10µl or 20 µl of the blood in a heparinized capillary tube. After collection the blood was transferred to a plastic 2 ml tube, 0.2 ml of distilled water was immediately added to the blood samples to prevent them clotting and sticking to the sides of the plastic tube. This procedure also lyses the blood cells.

Histidine Estimation

After collection, 20 µl blood samples were mixed with 50 µl of an AGPA solution (1µmol/ml of AGPA L α-amino-β-guanidinopropionic acid, Calbiochem) and 50µl of 30% sulphosalicyclic acid were added to precipitate the protein then centrifuged. The relative amounts of histidine in the supernatant was estimated by automatic amino acid analysis involving separation of the amino acid and AGPA by automated ion exchange column chromatography and estimation of their relative amounts by the ninhydrin reaction. Two instruments were used, one a modified "Locarte" instrument, the other one developed for four columns in this Laboratory.

Amniotic fluid, foetal blood and liver

Amniotic fluid

Amniotic fluid was collected from the amniotic sac at $16\frac{1}{2}$, $17\frac{1}{2}$ and $18\frac{1}{2}$ days gestation. +/+ and his/his animals were used. Amniotic fluid was collected into 50 µl capillaries.

At $16\frac{1}{2}$, $17\frac{1}{2}$ and $18\frac{1}{2}$ days of pregnancy blood was taken from the mother by cutting the neck vein and 20 µl or 40 µl was collected. The liver was taken out as well. Then the foetuses with the amniotic sacs intact were removed from the pregnant females and were put on a blotting paper to remove the blood from the placenta. Amniotic fluid was collected by puncturing the amniotic sac with a 50 µl tube and allowing the fluid to drain into it. Sometimes the amounts of amniotic fluid drained into the tube was less than 50 µl. These volumes were recorded by measuring the length (as an indicator of volume) that went into the tube before transferring into the plastic tube. The foetuses were cleaned and blood was collected from the neck vein into heparinized capillary tubes. Livers of each foetus were taken out and put on ice in aluminium foil. The foetuses were then examined under a binocular dissecting microscope for externally visible malformations of the head and liver region and fixed and sectioned or prepared as whole mounts.

Histidine Estimation for Maternal Blood and Foetal Blood

The procedure was as stated above.

Histidine Estimation for Amniotic Fluid

In each tube of amniotic fluid 100 µl of a solution containing 0.4 µmol AGPA/ml and 100 µl of 30% sulphosalicylic acid were added and estimation of histidine was done as stated above.

Histidine Estimation for Maternal Liver and Foetal Liver

Maternal livers and foetal livers were weighed. They were then transferred into homogenizers, then three times volume/weight of a solution (0.4 µmol AGPA/ml) was added. The liver was homogenised in a teflon plunger homogenizer five times at setting 9. To the

homogenised livers was then added 0.1 ml of 30% sulphosalicylic acid to precipitate protein and other high molecular weight substances and mixed well with a Vortex mixer. It was then left in ice for about 5 minutes, vortexed again for 5 minutes. The liver homogenate was withdrawn by Pasteur pipette and put into a plastic disposable tube, labelled and stored in the deep freeze ($-20^{\circ}C$). The histidine was then determined as for blood samples.

Behavioural test

The animals were scored twice during the second and third week after birth. The animals were put into a big transparent plastic cage and observed for head tilting and circling. Affected animals showed frequent tight circling and sometimes they tried to walk backwards. These animals were scored as bla^{+}. Some animals were scored as bla^{+-} where the animals showed head tilting or shaking their body when held up by the tail. They tried to circle but did not make a complete circle.

Hearing was tested by making a sharp sound and twitching of the pinna or the body was looked for. Normal animals showed twitching of the pinna and/or the body when they heard the sound. No reaction was scored as "deaf".

NOTE The magnification given in the figures are the magnification of the microscope and not the actual magnification of the photographs.

SECTION I

Inner Ear Defects of Adult Animals Born from his/his Mothers

Lyon investigated whole mounts of the inner ears of some balance affected mice (reported in Kacser, Bulfield and Wallace, 1973) and found various degrees of shortening of the posterior vertical canal and the crus commune and absence of otoliths in one or both ears. In the present study an attempt was made to detect more details of the defect of the inner ear using histological methods. Moreover, since the affected animals were deaf, the cochlear part of the inner ear was studied as well.

Behaviour

The animals were scored twice during the second and third week. The grade of behaviour defect in the affected animals is variable, the severely affected and the mildly affected. The severely affected animals run in circles, especially when placed in strange surroundings, are disturbed, or after spinning by their tails. They try to walk backwards. They circle in both directions clockwise as well as anti-clockwise. They do not have a marked preference of direction. In addition to circling they show vertical head tossing and no response to hearing tests. They are completely deaf throughout their life. Both males and females are fertile but mothers do not nurse their young.

The mildly affected animals can be classified with certainty only at the age of three weeks whereas severely affected animals can be recognised at 14 days old. The mildly affected animals show tilting and/or head tossing and/or deafness. Sometimes they show circling behaviour but not as rapid as the severely affected ones.

ABBREVIATIONS USED IN TABLES 3 A AND B

Under Otoliths

U = utricular otoliths
S = saccular otoliths
\+ = normal otoliths
\- = absent
Dis = displaced
Big = big crystals

Under Canals

A = anterior vertical canal
P = posterior vertical canal
H = horizontal canal
\+ = normal
\- = shortened, narrowed or constricted

Under Crus Commune

Crus C. = Crus Commune
\+ = normal
\- = short

Under pigments

\+ = normal
\- = reduced or very few

Under Cochlea

\+ = normal
\- = distended

Under Endolymphatic duct

End.duct = endolymphatic duct
\+ = normal
\- = distended

Under Hearing

\+ = normal
\- = deaf

Under Behaviour

bla^{+-} = mildly affected
bla^{+} = severely affected

TABLE 3 A WHOLE MOUNTS OF INNER EAR OF BEHAVIOURALLY NORMAL ANIMALS

No.	Maternal Genotype	Genotype	Behaviour	Hearing	LEFT									RIGHT								
					Otoliths		Canals			Crus C.	Pigments	Cochlea	End. duct	Otoliths		Canals			Crus C.	Pigments	Cochlea	End. duct
					U	S	A	P	H					U	S	A	P	H				
A1-A3	+/+	+/+	Normal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A4	+/+	+/+	"	+	+	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A5-A6	+/+	+/+	"	+	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A7	+/+	+/+	"	+	+	+	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+	+
A8-A9	+/+	+/+	"	+	Dis	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10-A11	+/+	+/+	"	+	Dis	+	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+	+
A12	+/+	+/+	"	+	+	Dis	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+	+
A13-A19	+/+	+/his	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A20-A22	+/+	+/his	"	+	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A23	+/+	+/his	"	+	+	+	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+	+
A24-A32	+/his	+/his	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A33	+/his	+/his	"	+	Dis	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A34	+/his	+/his	"	+	+	+	+	+	+	+	+	+	+	Dis	Dis	+	+	+	+	+	+	+
A35	+/his	+/his	"	+	Dis	+	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+	+
A36-A43	+/his	his/his	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A44-A-53	his/his	his/his	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A54	his/his	his/his	"	+	+	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A55	his/his	his/his	"	+	Dis	Dis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A56	his/his	his/his	"	+	Big	+	+	+	+	+	+	+	+	Dis	+	+	+	+	+	+	+
A57	his/his	his/his	"	+	Big	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A58-A59	his/his	his/his	"	+	Big	+	+	+	+	+	+	+	+	Big	+	+	+	+	+	+	+
A60	his/his	his/his	"	+	-	+	+	+	+	+	+	+	+	Big	+	+	+	+	+	+	+
A61	his/his	his/his	"	+	+	+	+	+	+	+	+	+	+	-	Big	+	+	+	+	+	+
A62	his/his	his/his	"	+	Big	+	+	+	+	+	+	+	+	Big	Big	+	+	+	+	+	+
A63	his/his	his/his	"	+	-	Big	+	+	+	+	+	+	+	Big	Big	+	+	+	+	+	+
A64	his/his	his/his	"	+	Big	Big	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A65	his/his	his/his	"	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A66	his/his	his/his	"	+	Big	Big	+	+	+	+	+	+	+	Big	Big	+	+	+	+	+	+
A67	his/his	his/his	"	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
A68	his/his	his/his	"	+	Dis	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
A69	his/his	his/his	"	+	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-

TABLE 3 B WHOLE MOUNTS OF INNER EAR OF BEHAVIOURALLY ABNORMAL ANIMALS

No.	Maternal Genotype	Genotype	Behaviour	Hearing	LEFT									RIGHT							
					Otoliths		Canals			Crus C.	Pigments	Cochlea	End. duct	Otoliths		Canals			Crus C.	Pigments	Cochlea	End. duct
					U	S	A	P	H					U	S	A	P	H				
B1	his/his	his/his	bla^{+-}	-	-	-	+	-	+	+	-	-	+	Big	Big	+	+	+	+	+	+	+
B2	his/his	his/his	bla^{+-}	-	Big	Big	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
B3	his/his	his/his	bla^{+-}	-	-	-	-	-	-	-	-	-	-	Big	+	+	+	+	+	-	+	+
B4	his/his	his/his	bla^{+-}	-	-	Big	+	-	+	-	-	-	-	Big	Big	+	-	+	-	-	+	-
B5	his/his	his/his	bla^{+-}	-	-	-	+	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-
B6	his/his	his/his	bla^{+-}	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
B7	his/his	his/his	bla^{+-}	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-
B8	his/his	his/his	bla^{+-}	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
B9	his/his	his/his	bla^{+-}	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
B10	his/his	his/his	bla^{+-}	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
B11	his/his	his/his	bla^{+-}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B12	his/his	his/his	bla^{+-}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

															U	S	A	P	H	C	P_1	Co	E
B13	his/his	his/his	bla^+	-	-	-	+	-	+	-	-	+	-		Big	+	+	+	+	+	-	+	+
B14	his/his	his/his	bla^+	-	-	-	+	-	+	+	+	-	+		Big	Big	+	-	-	+	-	+	+
B15	his/his	his/his	bla^+	-	-	-	+	+	+	+	+	-	-		-	-	-	-	+	-	-	-	-
B16	his/his	his/his	bla^+	-	-	-	+	+	+	+	-	-	-		-	-	+	-	+	+	-	-	-
B17	his/his	his/his	bla^+	-	-	-	-	-	-	+	-	+	+		-	Big	-	-	-	+	-	+	+
B18	his/his	his/his	bla^+	-	-	-	+	-	+	-	-	-	-		Big	Big	-	-	-	-	-	-	-
B19	his/his	his/his	bla^+	-	-	-	+	-	+	-	-	-	-		-	-	+	-	+	-	-	-	-
B20	his/his	his/his	bla^+	-	-	-	+	-	+	-	-	-	-		-	-	+	-	+	-	-	-	-
B21	his/his	his/his	bla^+	-	-	-	-	-	-	-	-	-	-		-	-	+	-	+	-	-	-	-
B22	his/his	his/his	bla^+	-	-	-	+	-	+	-	-	-	-		-	-	-	-	-	-	-	-	-
B23	his/his	his/his	bla^+	-	-	-	-	-	-	-	-	-	-		-	-	-	-	+	-	-	-	-
B24	his/his	his/his	bla^+	-	-	+	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-
B25	his/his	his/his	bla^+	-	-	-	-	-	-	-	-	-	-		-	-	+	+	+	-	-	-	-
B26	his/his	his/his	bla^+	-	-	-	-	-	-	+	-	-	-		-	-	-	-	-	+	-	+	-
B27	his/his	his/his	bla^+	-	-	-	-	-	+	-	-	-	-		-	-	-	-	-	+	-	-	-
B28	his/his	his/his	bla^+	-	-	-	-	-	+	-	-	-	-		-	-	-	-	-	+	-	-	-
B29	his/his	his/his	bla^+	-	-	-	-	-	+	-	-	-	-		-	-	-	+	-	-	-	-	-
B30-B43	his/his	his/his	bla^+	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-

ABBREVIATIONS USED IN TABLE 3C

Under Utriculus

Otoliths

+ = normal

- = absent

Big = large crystals

Lumen

+ = normal

- = distended

Under Sacculus

Otoliths -

+ = normal

- = absent

Under Canals

A = anterior vertical canal
P = posterior vertical canal
(It is not possible to observe whether they are shortened or not)

H = horizontal canal

+ = normal diameter

- = narrowed diameter

Under Cochlea

+ = normal

- = abnormal

Under Pigments

+ = normal

- = less pigment granules

Under Nerves *

Nerves = nerves and ganglion cells of cochlea

+ = normal

- = distorted or disorganised

*Only the inner ear had been embedded and sectioned. Scoring was made only on those parts of the nerves and ganglion which happened to be present in the sections.

TABLE 3C SECTIONS OF THE INNER EAR OF 3 WEEK OLD MICE AFFECTED AND NON-AFFECTED

					LEFT										RIGHT									
					Utriculus		Sacculus		Canals						Utriculus		Sacculus		Canals					
No.	Maternal Genotype	Genotype	Behaviour	Hearing	Otoliths	Lumen	Otoliths	Lumen	A	P	H	Cochlea	Pigment	Nerves	Otoliths	Lumen	Otoliths	Lumen	A	P	H	Cochlea	Pigment	Nerves
C1	+/+	+/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2	+/+	+/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C3	his/his	his/his	+	+	Big	+	+	+	+	+	+	+	+	+	Big	+	+	+	+	+	+	+	+	+
C4	his/his	his/his	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C5	his/his	his/his	+	+	+	+	+	+	+	+	+	+	+	+	Few	+	+	+	+	+	+	+	+	+
C6	his/his	his/his	+	+	+	+	Big	+	+	+	+	+	+	+	—	—	—	—	d a m a g e d					
C7	his/his	his/his	bla+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-
C8	his/his	his/his	bla+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
C9	his/his	his/his	bla+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-
C10	his/his	his/his	bla+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3

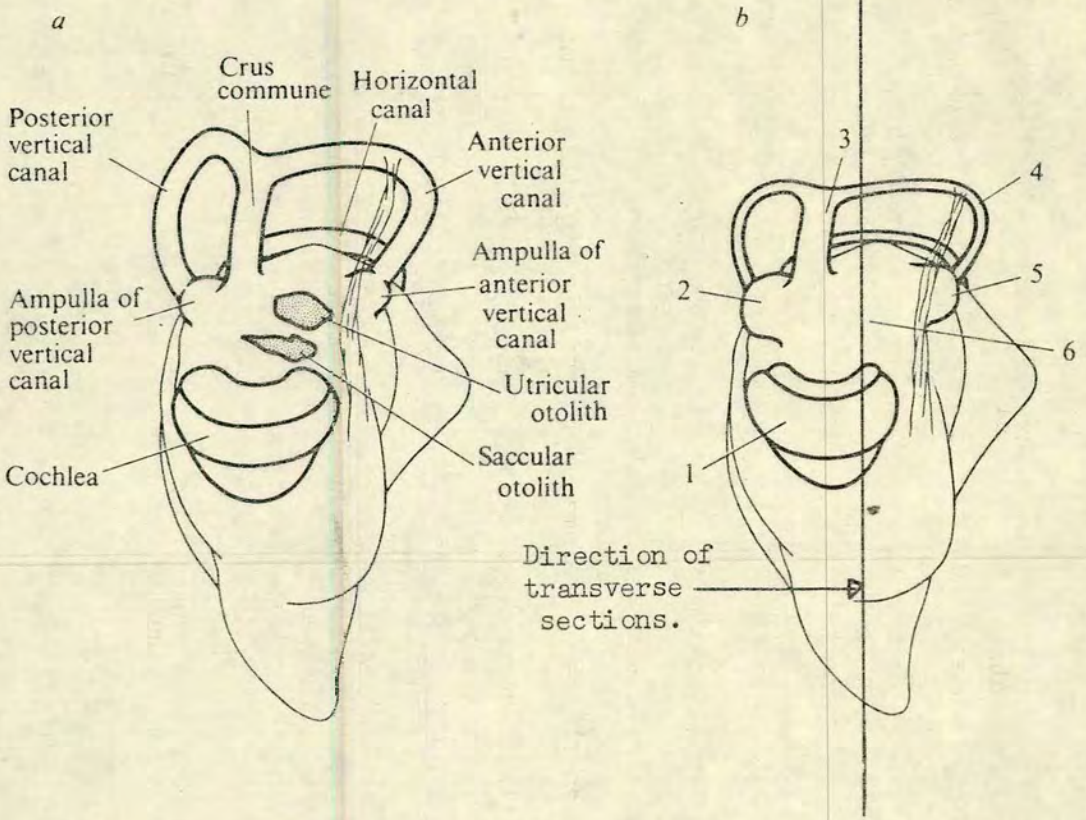
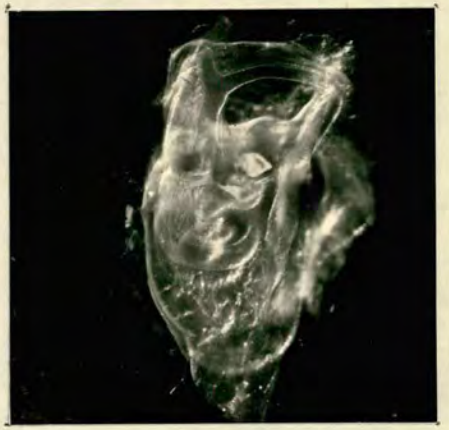


Diagram of the abnormalities seen in the whole mounts of adult ears. *a*, Normal; *b*, affected. The ears were dissected into 75% alcohol and eventually cleared in benzyl alcohol. They were examined under the normal light and polarised light microscope. Canals are considered abnormal if they are shortened, thin or misshapen; otoliths are altered if they are enlarged or thin. About half the offspring from both treated and untreated mothers have utricular or saccular otoliths displaced but not damaged. This occurs in animals of the Peru or hybrid Peru/C57BL strains but not with pure C57BL. Any one ear may show one or more of the following abnormalities. 1, Enlarged or distorted cochlea; 2 and 5, enlarged ampullae; 3, shortened crus commune; 4, canals thin or misshapen; 6, otoliths absent, or thin or large crystals.



(a)

(b)

From Kacser et al. (1977).

Whole mounts of adult mice.

(a) Left inner ear of normal mouse.

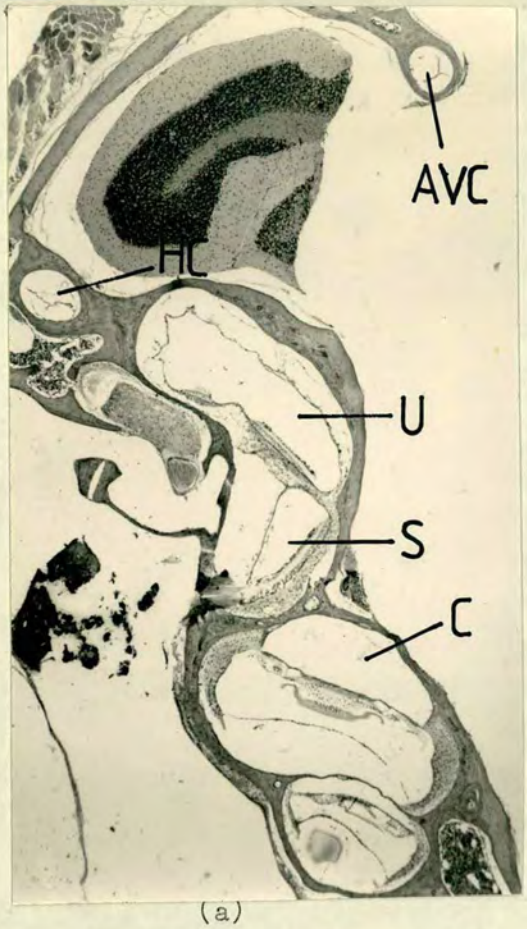
(b) Left inner ear of affected mouse. No otoliths in either utriculus or sacculus. Cochlea distended.

Whole Mounts of Adult Ears

Fig. 3 shows abnormalities found in the bony labyrinth. The abnormalities found in the bony labyrinth of the circlers varied. In severely affected animals two types of anatomical defect were found. The bony labyrinth of some of the affected animals had the posterior vertical canal shortened, accompanied by shortening of the crus commune and absence of otoliths in both utriculus and sacculus. The cochlea was enlarged and the endolymphatic duct widened. In some of the ears in addition, the horizontal canal was constricted where it passed the posterior vertical canal. In the other type, the diameter of the anterior vertical canal, posterior vertical canal and horizontal canal were all narrowed, the otoliths were missing, the cochlea, and the endolymphatic duct and ampullae of all three canals were enlarged. In both types of defect the pigment granules which are present in the utriculus, ampullae and crus commune were fewer than in the normal. The bony labyrinth of mildly affected animals showed one side with big crystals in the utriculus whereas the remainder of the inner ear was normal. On the other side the whole of the inner ear was abnormal. Some of the mildly affected animals show defects which were more or less the same as in the severely affected animals. 112 animals which were either behaviourally normal or behaviourally affected were observed as whole mounts.

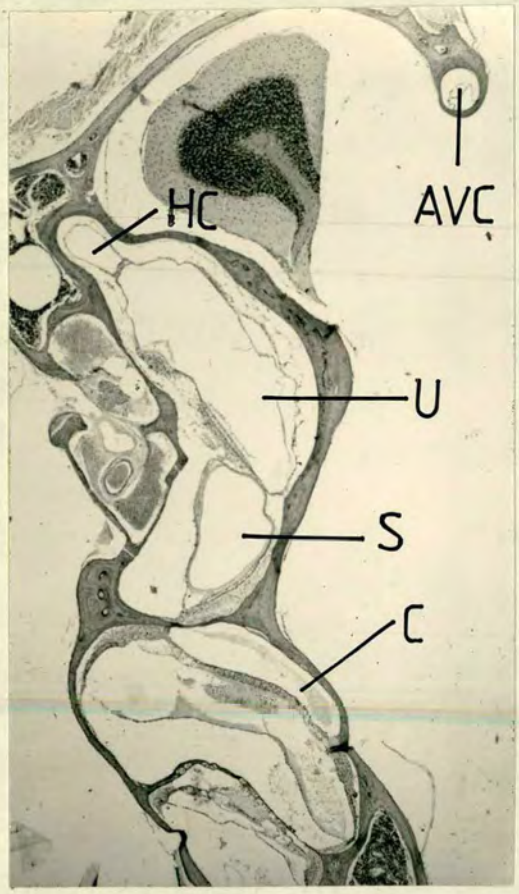
The genotypes of the normals were +/+, +/his or his/his and they were born from +/+, or +/his or his/his mothers (Tables 3A and 3B). There was no abnormality found in the canals and cochlea, apart from the displacement of some of the otoliths of both utriculus and sacculus, in animals born from either +/+ or +/his mothers. It is likely that the displacement of the otoliths was artifact. In the normals that

Figure 4

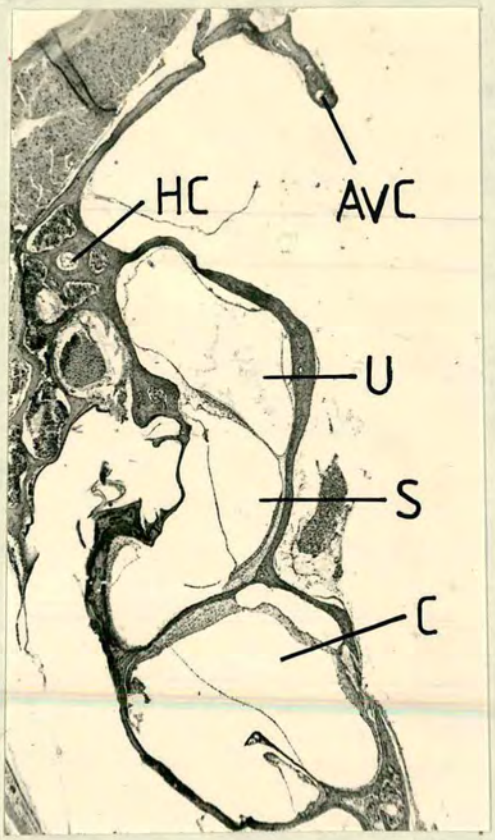


(a)

AVC = anterior vertical c
HC = horizontal canal
U = utriculus
S = sacculus
C = cochlea



(b)



(c)

Transverse sections of adult inner ears passing through utriculi, sacculi and cochlae X 33. The semicircular canals are indicated.

(a) Unaffected animal born from +/+ mother with normal canals.
(b) Affected animal with normal canals born from his/his mother.
(c) Affected animal with narrowed canals born from his/his mother.

were born from his/his mothers, two animals had the inner ear on one side of the head abnormal and 10 animals had absence of otoliths in utriculus and sacculus on both sides or big crystals were seen in both sides or in either side.

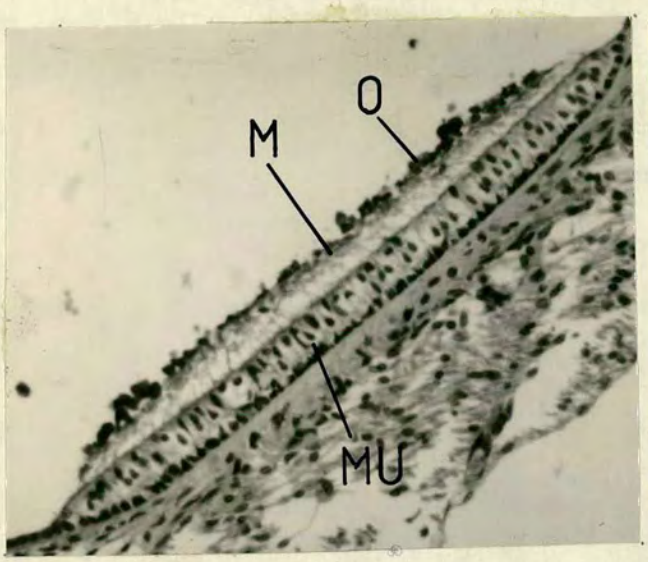
Sections

Three weeks old and older animals As in the whole mounts, two types of abnormalities were found (Figs 4b and 4c). In the first type, the diameter of the semicircular canals was normal. In the second type, the lumens of the semicircular canals were very narrow. In these sections it was difficult to say whether the posterior vertical canal was shorter or not. The vestibular portion and the cochlear portion showed more or less the same kind of abnormalities in both types of inner ears. The utriculus, sacculus and endolymphatic duct and cochlea will be described separately.

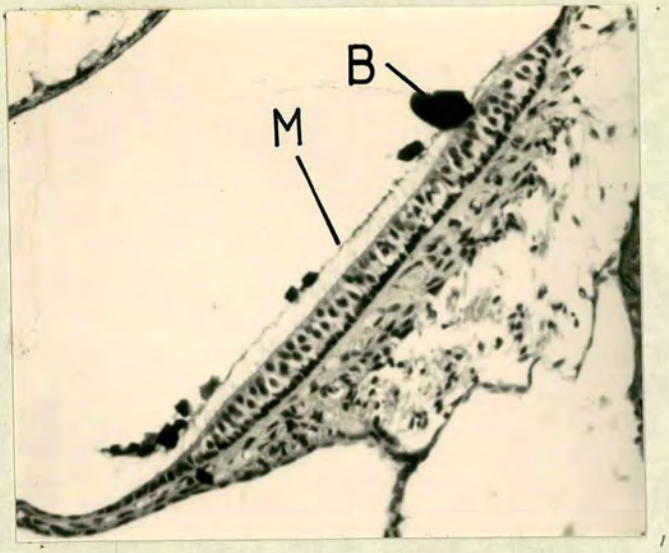
(i) Utriculus

In both types of abnormal ear the otoliths were often missing and the lumen was distended. In the utriculus of the normal animal, the otolith lies on the hair of the hair cells of the macula. When stained with haematoxylin these otolithic crystals stained dark blue. The otolithic membrane showed positive reaction with PAS showing that it is mucopolysaccharide positive. In the behaviourally normal his/his animals which were born from his/his mothers there were some big crystals which stained very dark blue with haematoxylin lying in the lumen some of them lying near the macula. The presence, absence or size of crystals can be clearly seen in the sections before de-waxing. Passage through the stains and washing some times tends to dislodge them particularly the large ones. This artefact has been taken into account in all the following descriptions. The otolithic membrane was present in

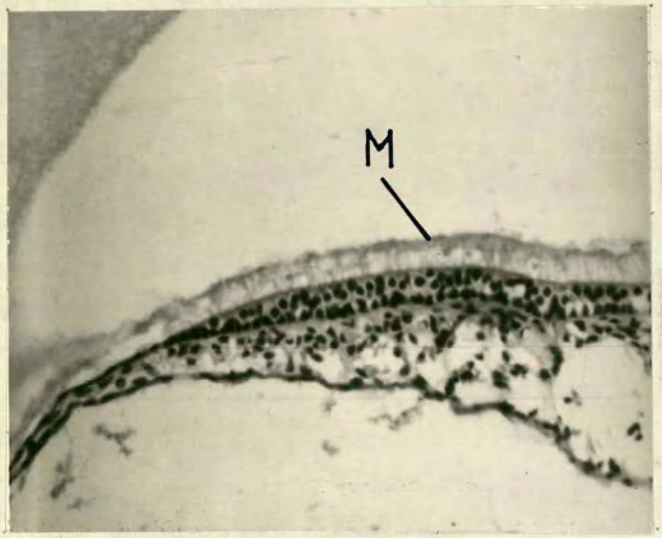
Figure 5



(a)



(b)



(c)

Transverse sections of adult utricular maculae X 250.

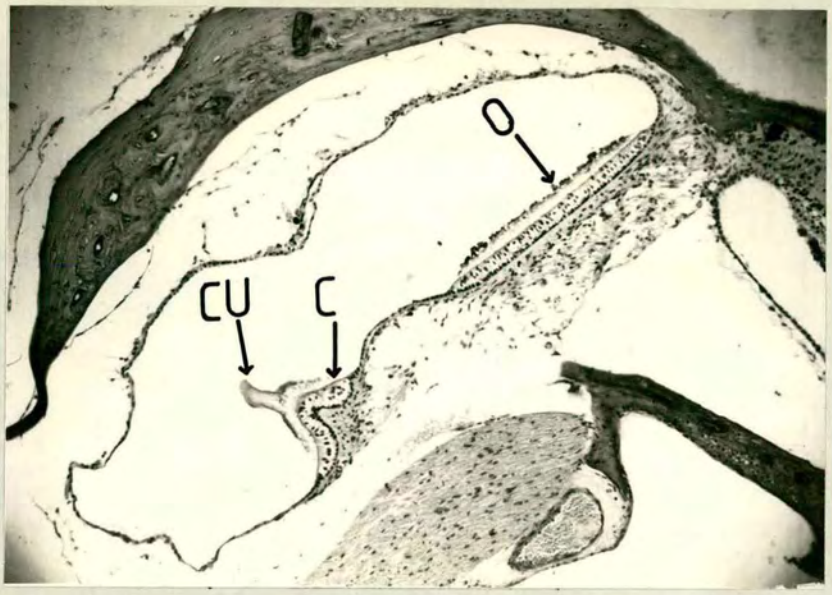
(a) Macula of normal mouse born from +/+ mother: otoliths normal.

(b) Macula of behaviourally normal mouse born from his/his mother: big crystals.

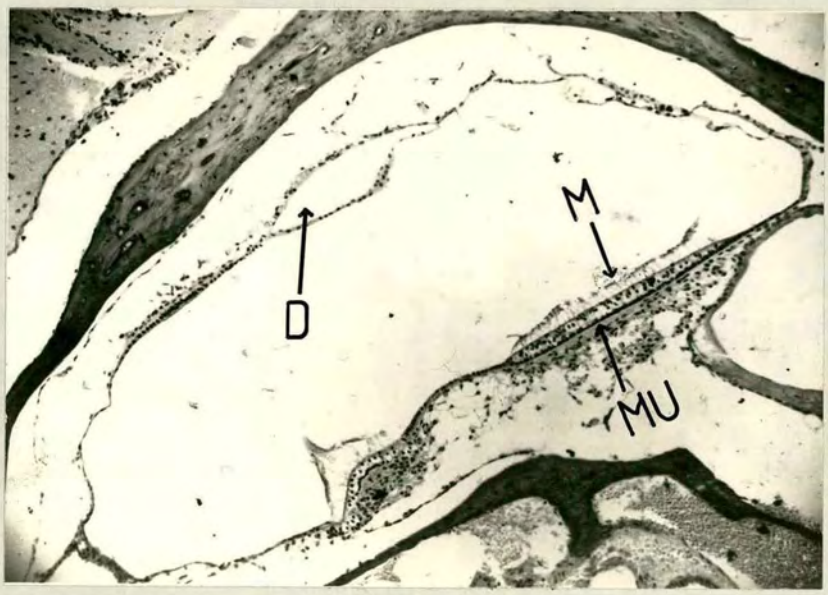
(c) Macula of behaviourally affected mouse born from his/his mother: otoliths lacking.

MU = macula of utriculus.
O = otoliths
B = big crystals
M = otolithic membrane

Figure 6



(a)



(b)

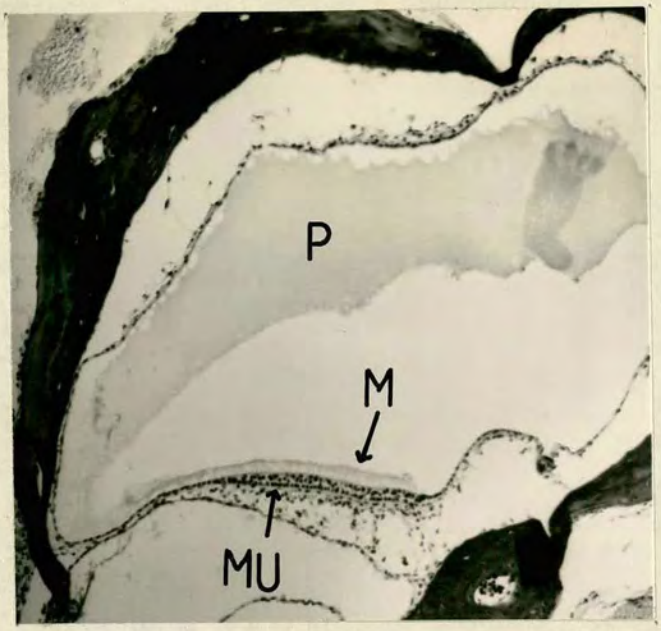
Transverse sections of adult utriculus X 100.

(a) Utriculus of normal mouse from +/+ mother.

(b) Utriculus of affected animal from his/his mother. Distended lumen with double cell layer. Otoliths missing.

O = otoliths.
M = otolithic membrane.
C = crista of horizontal canal.
D = double cell layers.
MU = macula.
CU = cupula.

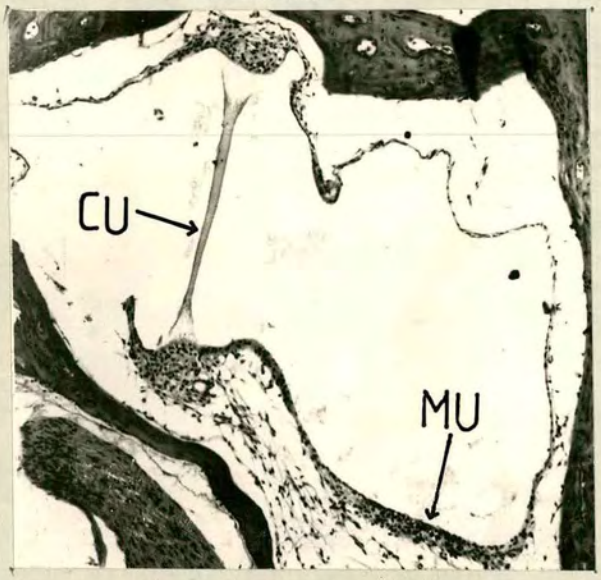
Figure 7



Transverse section of distended utriculus with thin macula. Adult X250.

P = PAS positive material.
M = otolithic membrane without crystals.
MU = macula

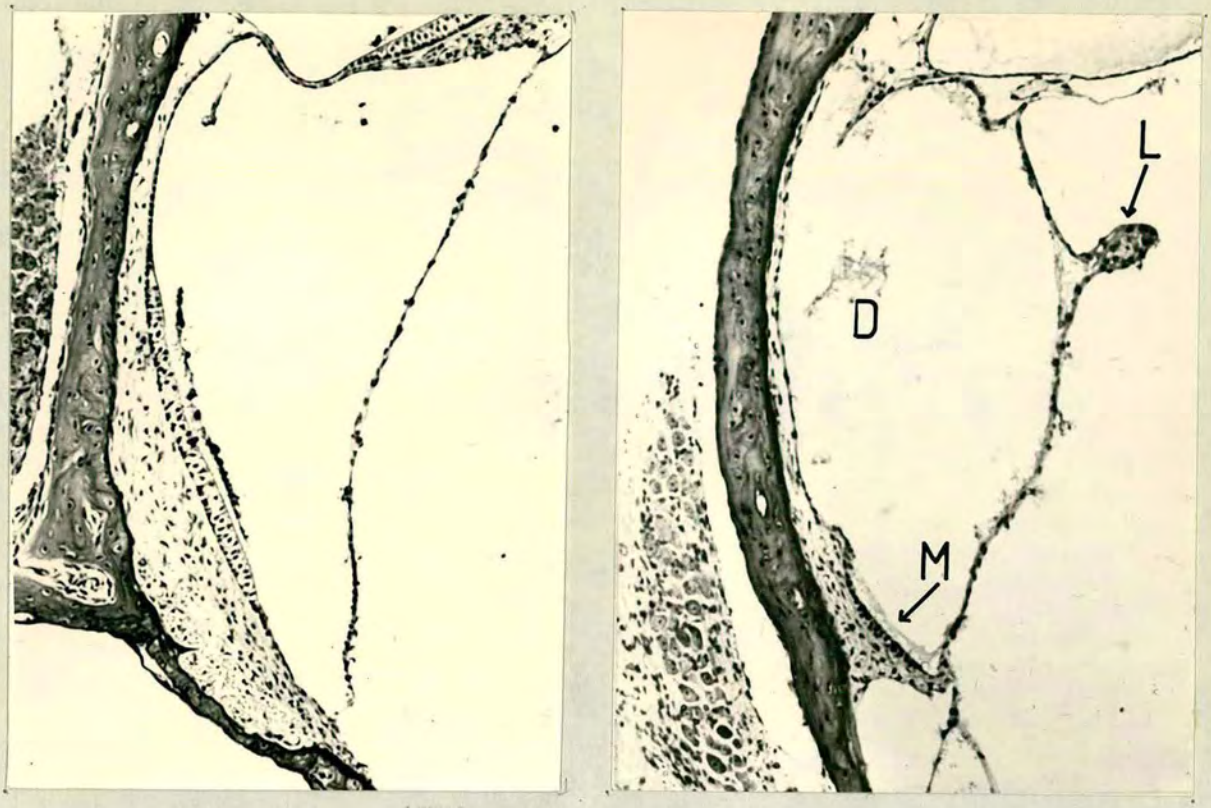
Figure 8



Transverse section of utriculus. Adult X 250.

MU = macula of utriculus. Supporting and hair cells disorganised.
CU = cupula of horizontal canal.

Figure 9



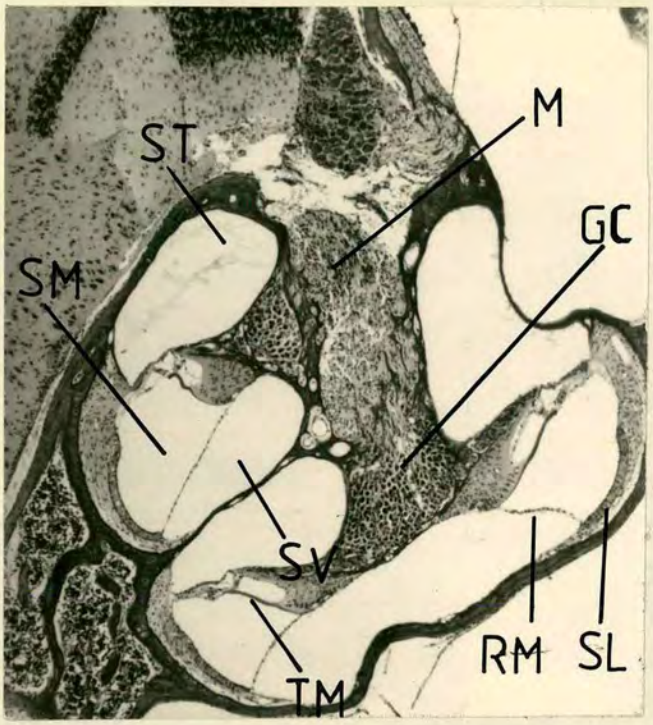
(a) (b)

Transverse sections of adult inner ears passing through sacculi X126.

(a) Animal born from +/+ mother.
(b) Animal born from his/his mother. Section showing loop in the saccular membrane.

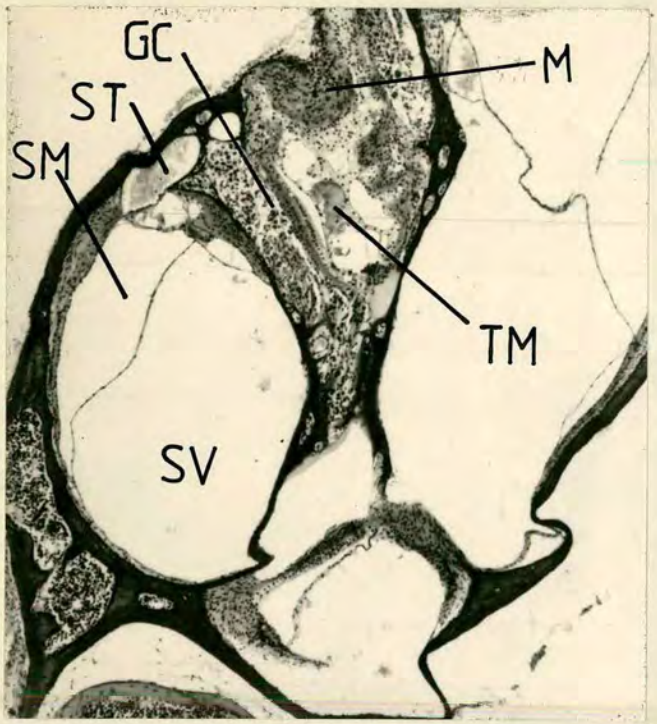
L = loop.
D = debris.
M = membrane with connecting strands.

Figure 10

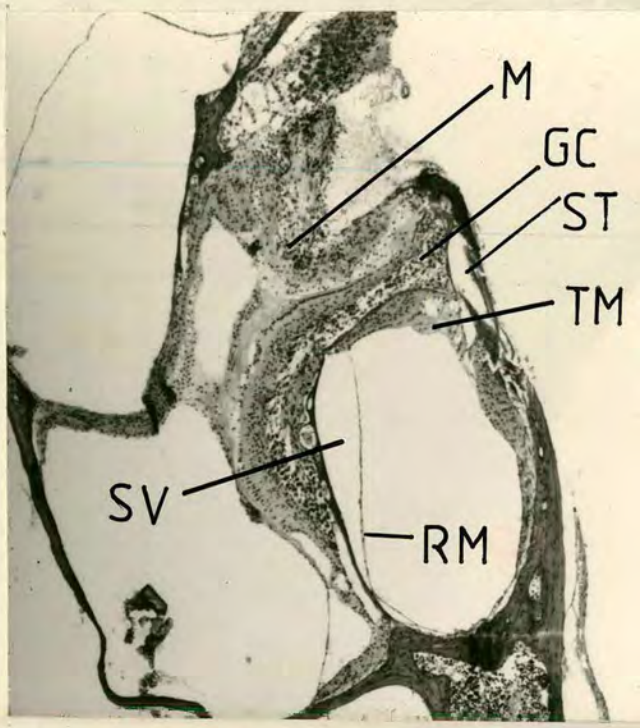


ST = scala tympani.
SM = scala media.
SV = scala vestibuli.
GC = ganglion cells.
RM = Reissner's membrane.
TM = tectorial membrane.
SL = stria vascularis.
M = modiolus.

(a)



(b)



(c)

Transverse section of adult cochlea X 55.

(a) Cochlea of animal born from +/+ mother.

(b) and (c) Cochlea of the left and right ear respectively of behaviourally affected mouse born from his/his mother. Scala tympani very narrowed. The modiolus of cochlea was abnormal in both ears.

the ears which had no crystals and in the ears which had big crystals (Fig. 5). In another case the roof of the utriculus had two layers of cells and the cell structure was abnormal with big nuclei with lots of cytoplasm around them (Fig. 6). In those ears there was very little debris present. The cells of the macula were abnormal and loosely arranged, the supporting and hair cells becoming disorganised. In one case (Fig. 7) both left and right utriculi had very thin maculae. The orientation of the macula of the utriculus was nearly horizontal. The lumen was half filled with PAS positive material. In another case the supporting and hair cells could not be differentiated (Fig. 8).

(ii) Sacculus

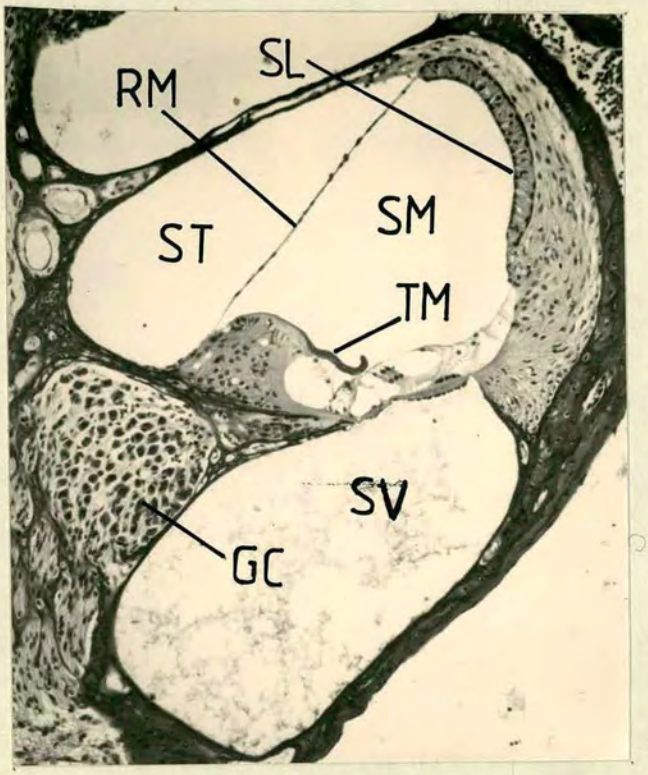
In both types of abnormal ear, the saccular otoliths were missing as frequently as in the utriculus. In some of the behaviourally normal animals, which were his/his and which were born from his/his mothers, there were big crystals in the sacculus. As in the utriculus, they too had an otolithic membrane. In some animals, there was debris like material present lying close to the macula and membrane of the sacculus which was PAS positive. The lumen was distended. In some cases part of the saccular wall was collapsed and lay close to the macula. In one case, the macula of the sacculus was very thin and seemed smaller. In the lumen there was lots of debris among which were minute structures which stained with haematoxylin. In another case the saccular membrane was partially folded and projected out forming a little loop (Fig. 9b). Between the macula and the gelatinous otolithic membrane there were strands of tissue connecting the two layers.

(iii) Cochlea

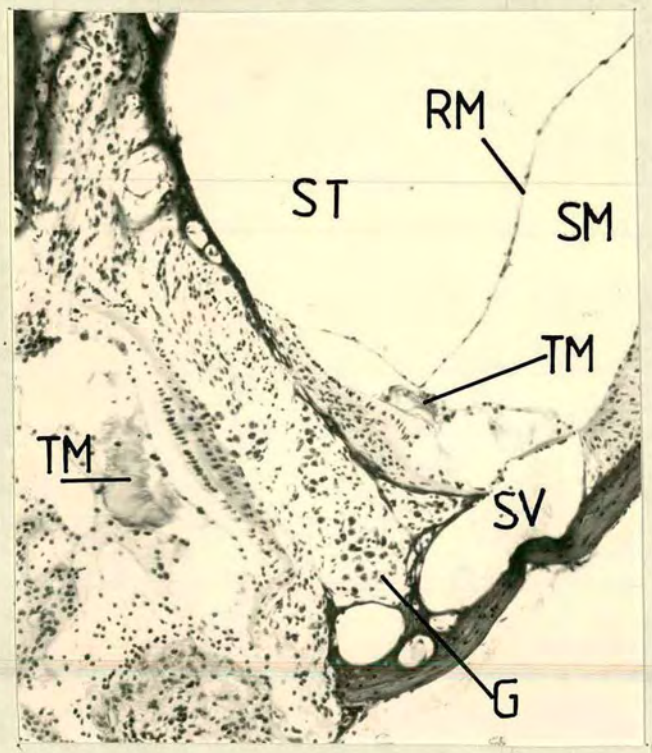
In all the deaf animals the cochlea was completely abnormal. The abnormalities of the cochlea were the same in both types of inner ear (Fig. 10) In each coil of the cochlea the scala tympani was very narrow so that

the remaining part of the bony coil was occupied by the dilated scala vestibuli and scala media. The position of the spiral lamina seemed to be abnormally displaced. The osseous spiral lamina was very thin. In each coil the spiral ligament which consisted of a mass of connective tissue was very thin and the stria vascularis was present as a strip of tissue in some cases and in other cases it was present as a thin layer of cells with some blood capillaries. The stria vascularis in the apical coil had bulges which protruded into the lumen of the cochlear duct. This abnormality was found only in the apical coils and the apical coil itself was smaller. This could be due to Reissner's membrane lying close to the limbus, which made the cochlear duct small. The scala tympani was also very narrow in this apical coil. The modiolus was abnormal and there seemed to be no osseous substance. In the normal ear, blood vessels with surrounding connective tissue and the cochlear division of the acoustic nerve penetrate through numerous openings into the bony substance, but in the abnormal ear bony substance was very small and all the ganglion cells and acoustic nerves and blood vessels were disorganised.
As will be discussed in the section on the prenatal embryos, it might have been worthwhile to look further into the nerve organisation in these animals. Since, however, only the ears were embedded it was not possible to pursue this with the material investigated. Basal coils seemed to be broken down and there seemed to be no osseous lamina. In some parts the basal coils were unrecognisable. In the modiolus region, the ganglion cells, acoustic nerve and blood vessels all were disorganised and in between them there was some tympanic-membrane-like structure and this was found to be PAS/positive. Reissner's membrane was thinner and longer than normal. The organ of Corti itself was disorganised, hardly any sensory part being recognised. The basilar

Figure 11



(a)



(b)

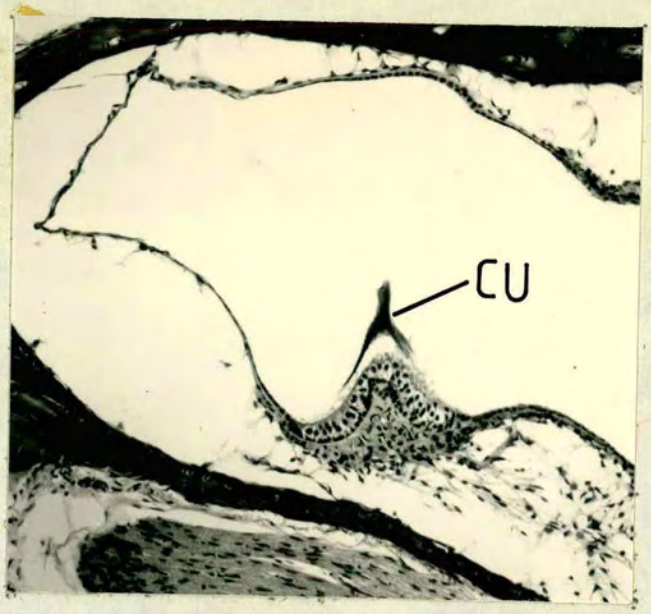
ST = scala tympani
SM = scala media
SV = scala vestibuli
GC = ganglion cells
RM = Reissner's membrane
TM = tectorial membrane
SL = stria vascularis

Transverse sections of adult cochleae X 135. (enlarged photograph of Figure 10a and b)

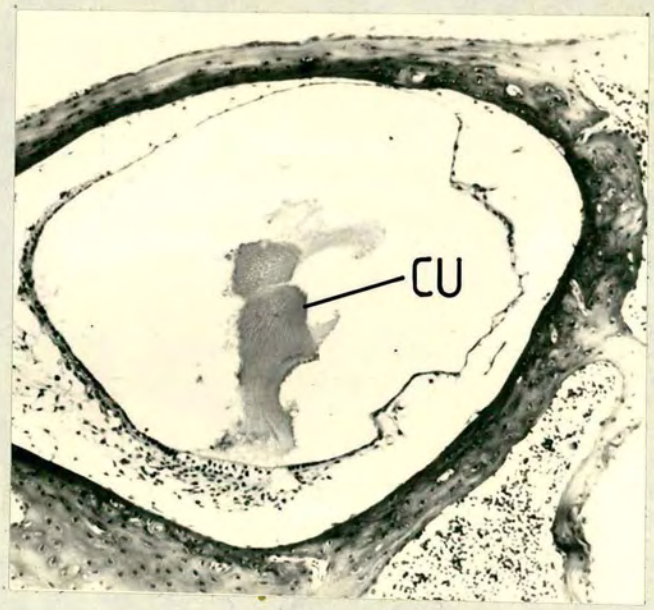
(a) Normal cochlea.

(b) Cochlea of behaviourally affected mouse. Note abnormal tectorial membrane.

Figure 12



(a)



(b)

Transverse sections of adult inner ears passing through ampullae of semicircular canals X 127.

(a) Ampulla of horizontal canal with normal cupula from +/+ mother.

(b) Ampulla of anterior vertical canal with very big cupula, from his/his mother.

membrane was present but the outer phalangeal and inner phalangeal cells were all broken down. Hardly any part of the cochlear duct was normal. The limbus was very small and the tectorial membrane even though it was present was completely abnormal. It was like several thin layers lying on top of one another (Fig. 11).

The disorganised tectorial membrane stained PAS positive as did the normal. In most of the affected animals, lots of debris was found in the scala media. In the spiral ganglion fewer ganglion cells were observed belonging to the mid coil, basal coil and apical coil. Basilar membrane showed no apparent abnormality.

(iv) Semi-circular Canals and Ampulla

In the second type of abnormality described in the Section on whole mounts, the semicircular canals were very narrow. The ampullae were found to be dilated as well. In one case where haematoxylin and eosin stain was used, the cupula of the horizontal canal was found to be like a long thin strand (Fig. 8), and in another case which was stained with PAS, though it showed the same kind of reaction as the normal, the cupula was big (Fig. 12). The cristae of all the canals were smaller and the hair cells and supporting cells were disorganised and loosely arranged.

(v) Pigments

In the completely abnormal ear where there were no otoliths in either utriculus or sacculus, pigment granules were present but these were very few in the crus commune and in the vestibular region. The pigments seen in the ears with big crystals in utriculus and sacculus were few but not as few as in those where there were no otoliths in utriculus and sacculus and where the inner ear was completely abnormal. In sections the same kind of difference in pigment granules distribution was observed.

DISCUSSION (Adults)

The bony labyrinth of 112 animals (Table 3A and 3B) which were normal as well as affected have been studied in whole mounts. It was found that whenever there was a behaviour defect, the inner ear was found to be affected as well. As stated above, in some of the severely affected animals the semicircular canals are all very narrow in diameter and in others all the diameters of the canals were normal but the posterior vertical canal was short. In three cases, where the animals had been scored as severely affected in behaviour, there were big crystals in sacculus, of the right inner ear, but the left ear of these animals lack otoliths in both utriculus and sacculus. It seemed that the absence of otoliths was the common abnormality in the inner ears of the affected animals. The subclinical defects were found to be varied in the animals that were born from his/his mothers. In some of these animals, even though the behaviour was normal, there were defects in the inner ear and in affected animals the defect of the inner ear varied. It also seemed that the animals which were very severely affected in behaviour were found to have severely affected ears.

In 43 animals which were born from either +/+ or +/his mothers, though they themselves were either +/+ or +/his or his/his, no abnormality was found in the canals or cochlea. The pigments were found to be normal as well. These observations are in agreement with previous findings (Kacser, et al. 1973).

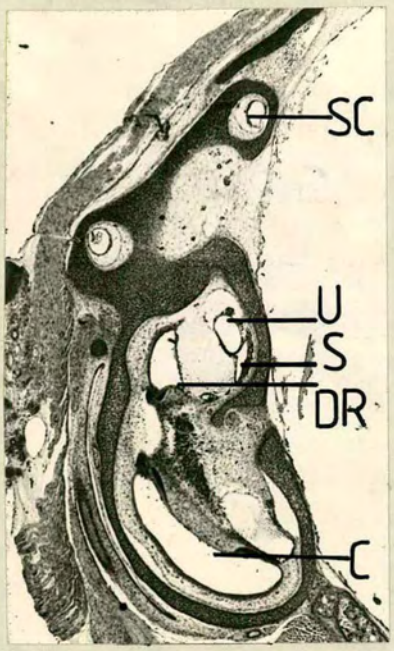
Of 30 behaviourally normal animals from his/his mothers investigated, 17 (57%) showed some inner ear lesions. 14 could be described as having 'mild' lesions while 3 (A67 - A69) showed lesions very similar to those in behaviourally affected (bla+-) animals (B1 - B3). Furthermore the pattern of defects found in bla+- (mild) animals was on the whole very similar to the bla+ (severe) affected animals.

It is therefore clear that behaviour (or behavioural scoring!) is less sensitive than histologically detectable abnormalities. This will be discussed in Section IV in connection with the quantitative aspects of penetrance.

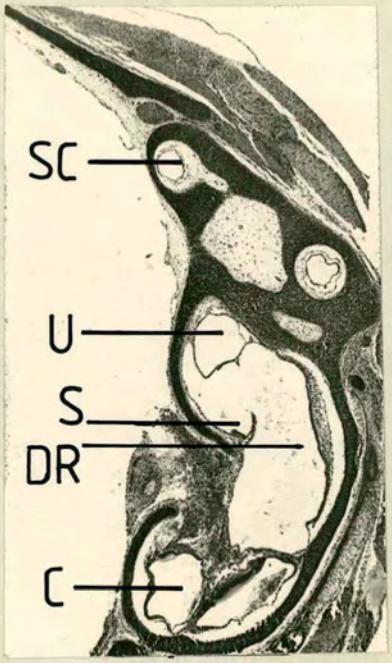
In the present study in all the animals which were scored as having abnormal behaviour, whether they were of very severely affected (bla+) or mildly affected (bla +-), the inner ear was found to be affected in both sides or on one side. Behaviour was severely affected in animals with severely affected inner ears except in 2 animals which were scored as bla+-, both sides being severely affected. A mild kind of defect was usually found in mildly affected animals. In 3 animals the cochleae of both sides were scored as normal but they were found to be deaf. Was this due to the crude test of hearing or if they had been sectioned would their cochlea be found to be affected? In all other deaf animals, the cochlea was found to be affected. As shown in whole mounts, the cochlear part was found to be dilated in one of the coils. In the sections, the ganglion cells, modiolus, scala tympani and the whole of the organ of Corti were found to be distorted.

Since the cochlea, and in particular the organ of Corti, is considered to be the hearing organ, it is significant that deaf animals had completely abnormal organs of Corti. In normal mice the lower surface of the tectorial membrane rests upon the end of the hairs which protrude from the hair cells, but in abnormal animals, the tectorial membrane seemed to be crushed down by the Reissner's membrane and looks as though it could not function. Both the limbus and hair cells, all of them, were small or disorganised. The abnormalities seemed to account for the cause of deafness. As for the abnormal motor behaviour it could be due to the abnormalities of the semicircular canals or the absence of the otoliths and the anomalies of the cristae. It was found in the present case that the behaviour defect and the inner ear abnormalities were strongly correlated. As will be seen from the Discussion later on this correlation does not necessarily imply a causal hierarchy of effects. Furthermore, how the metabolic state of the mother (i.e. either excess histidine or its derivatives) determines the distension of the endolymphatic system, absence of otoliths and faulty development of hearing organ in the embryonic stages is not revealed by the above results.

Figure 13



(a)



(b)

Transverse sections of inner ear of newborn animals passing through ductus reuniens and cochlea X 25.

(a) Newborn animal from +/+ mother.

(b) Newborn animal from his/his mother with distended utriculus, ductus reuniens and cochlea.

SC = semicircular canal.
U = utriculus.
S = sacculus.
DR = ductus reuniens.
C = cochlea

SECTION II

Embryological Development

Introduction

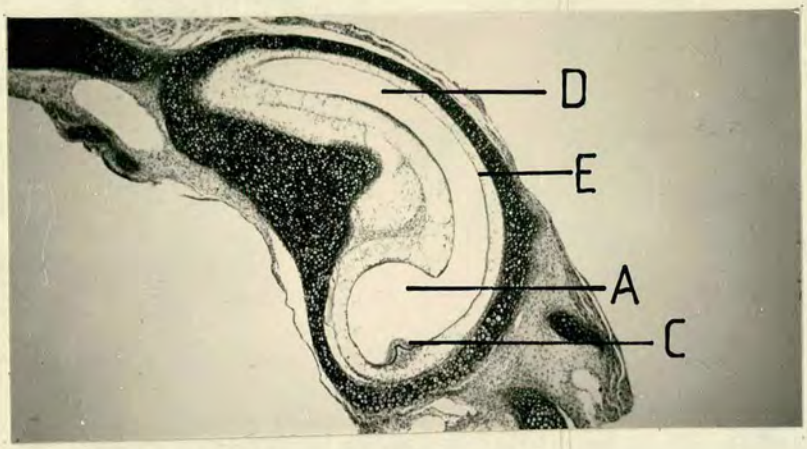
The results of the analysis of adult ears gave a reasonable explanation for the behavioural abnormality of affected animals. Two major questions, however, remain (1) What is the detailed developmental history of the condition? (2) What is the cause of variable penetrance? In this section results of observations on earlier stages of development will be presented. Since one of the principal aims was to discover the earliest manifestation of abnormality, the section is presented 'backwards' from adult to 9½ day embryos. Two further observations made these stages particularly important. It was known (Kacser, et al. 1977) that the second week of pregnancy (7-14 days) was the 'critical' period. Secondly due to the finding of subclinical lesions of the adult inner ear it was thought desirable to look from newborn to earlier stages, to see whether this kind of variability could be seen in embryological stages.

Embryos were taken from +/+ mothers as controls and embryos from his/his mothers were observed at different stages for differences in utriculus, sacculus, cochlea, ductus reuniens and semicircular canals. Fixing, embedding, section cutting and staining procedures are described in the Materials and Methods.

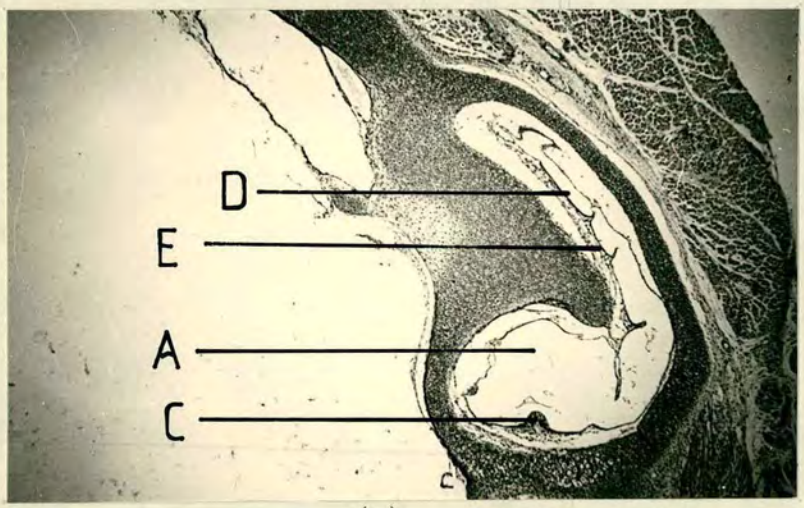
Day of Birth

In some newborn animals from his/his mothers, the whole endolymphatic system was affected. The utriculus, sacculus, cochlear duct, all three ampullae, crus commune, endolymphatic duct and endolymphatic sac were all distended (Fig. 13). There were no otoliths in either utriculus

Figure 14

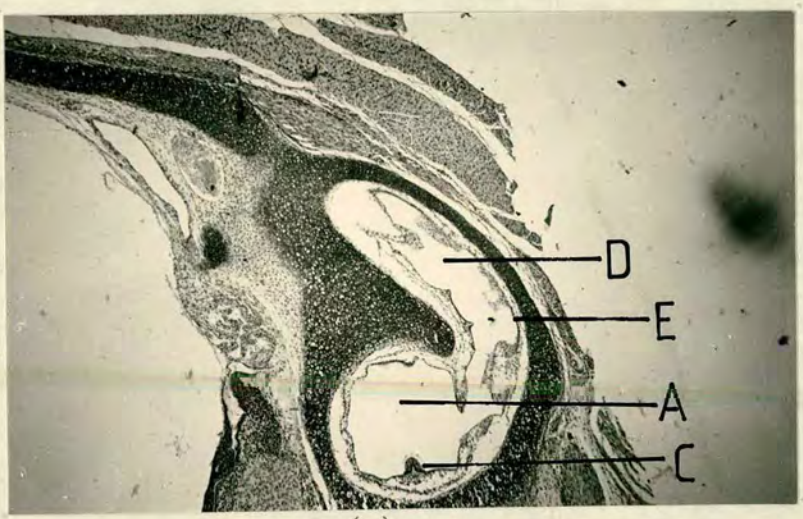


(a)



(b)

D = ducts
E = epithelial layer
A = ampulla
C = crista



(c)

Transverse sections of adult semicircular ducts X 32.

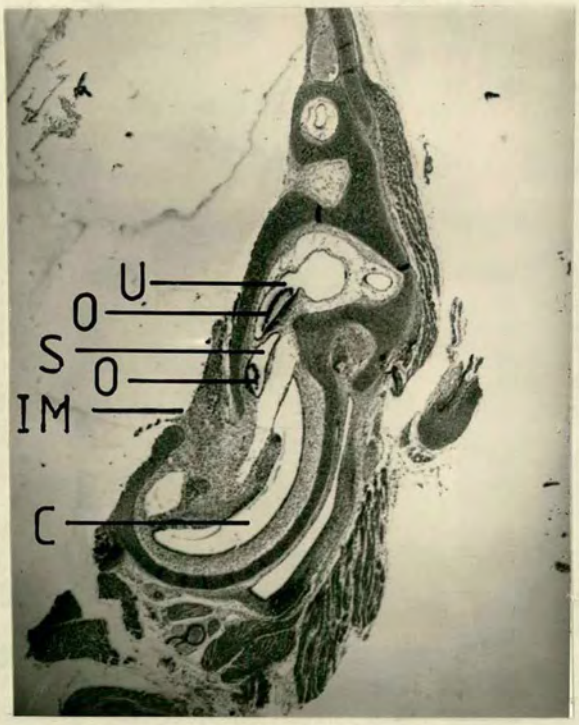
(a) Normal ampulla with normal diameter of semicircular duct. From +/+ mother.

(b) and (c) Constricted semicircular ducts with distended ampullae and convoluted epithelial layers. From his/his mother.

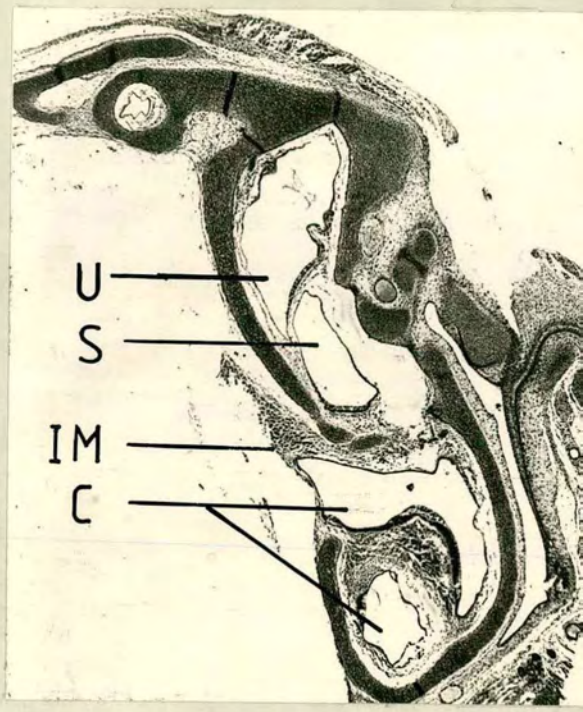
or sacculus and the maculae were very thin. In one case the macula of the utriculus was so thin that there seemed to be no layer of supporting cells, only the hair cells being found and the shape and size of the whole macula was abnormal. In the same animal, the crista of the horizontal canal was smaller but the cupula was found to be bigger and the ridge between the crista and the macula of the utriculus was abnormal. Some parts of the semicircular ducts become constricted or occluded. In some parts, the epithelial layer becomes folded and protrudes into the lumen of the duct thus creating a discontinuous duct (Fig. 14).

In the anomalous cochlea the whole of the periotic space had been taken by the distended cochlear duct. At this age the stria vascularis was still in the early stage of its differentiation but in the abnormal ear, it was thinner. At this stage, some part of the basal coil of cochlear duct was pushed out through the internal auditory meatus. Lots of debris was found in some of the distended cochleae, sacculi, utriculi, and endolymphatic sacs. This debris was absent when the lumens were not distended even though the otoliths were lacking. Pigment granules in the animals born from +/+ mothers could be seen near the wall of the utriculus, crus commune and ampulla of the horizontal canal. In the abnormal ear there were very few granules near the wall of the utriculus, crus commune and near the ampulla of the horizontal canal. The pigment granules were not entirely absent but very few were observed. Pigment granules found along the crus commune were fewer in those ears which had big crystals in the utriculus and sacculus. The stria vascularis has started differentiating. Pigment granules seem to be fewer in the ears which had big crystals in the utriculus and sacculus but very few in the ones where the otoliths were entirely missing.

Figure 15



(a)



(b)

Transverse section of the inner ear of 18½ day embryo X 25.

(a) 18½ day embryo from +/+ mother.

(b) 18½ day embryo from his/his mother, showing distended utriculus and sacculus. Basal cochlear duct pushed out through the internal auditory meatus.

U = utriculus.
S = sacculus.
IM = internal auditory meatus.
C = cochlea.

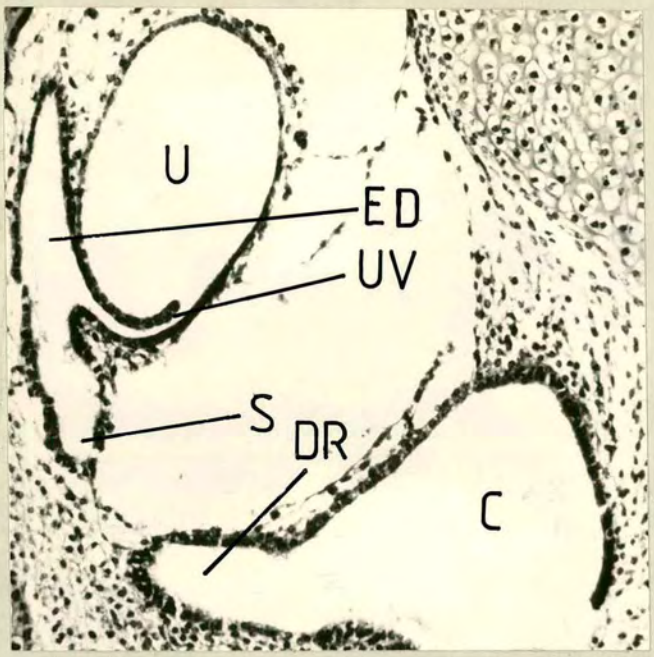
18½ Day Embryo

In ears, where there were no otoliths in either utriculus or sacculus, the lumens of the utriculus and sacculus, were distended as in newborn animals from his/his mothers, and the maculae of the utriculus and sacculus were thinner. In the severely affected ears all three ampullae, the endolymphatic duct and the endolymphatic sac were distended. As in the newborn animals lots of debris was found in the distended cochlear duct, sacculus, utriculus and endolymphatic sac. When the cochlear duct, sacculus and utriculus were of normal dimensions, no debris was found, but in some cases there were big crystals or the otoliths were entirely missing. Many blood cells were found in the perilymphatic spaces near the cochlear duct. The ampulla of the horizontal canal was distended and the crista was found to be smaller but the architecture of the supporting cells and hair cells appeared normal. The cupula which stained mauve with PAS was found to be bigger. In some of the affected embryos, the cochlear duct was so distended that it took up the whole of the perilymphatic space. As the whole of the perilymphatic space was taken up by the cochlear duct there seemed to be fewer ganglion cells and the caudal wall of the cochlear duct appeared thinner. At this stage in control animals the total number of cochlear coils had appeared, namely the apical coil, middle coil, and basal coil. In some severely affected embryos part of the basal coil was pushed out through the internal auditory meatus into the cranium (Fig. 15).

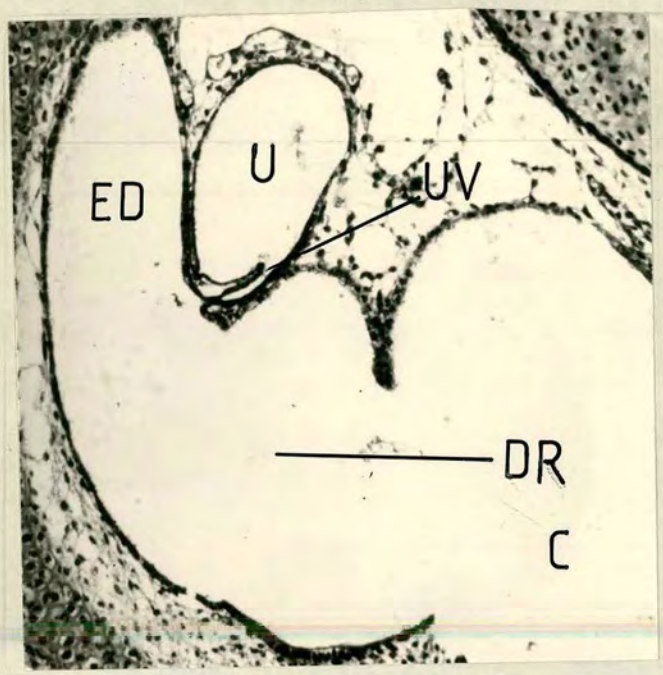
17½ Day Embryo

In normal embryos at 17½ days the otoliths were fully developed and the otolithic granules showed strong birefrigence, when observed

Figure 16



(a)



(b)

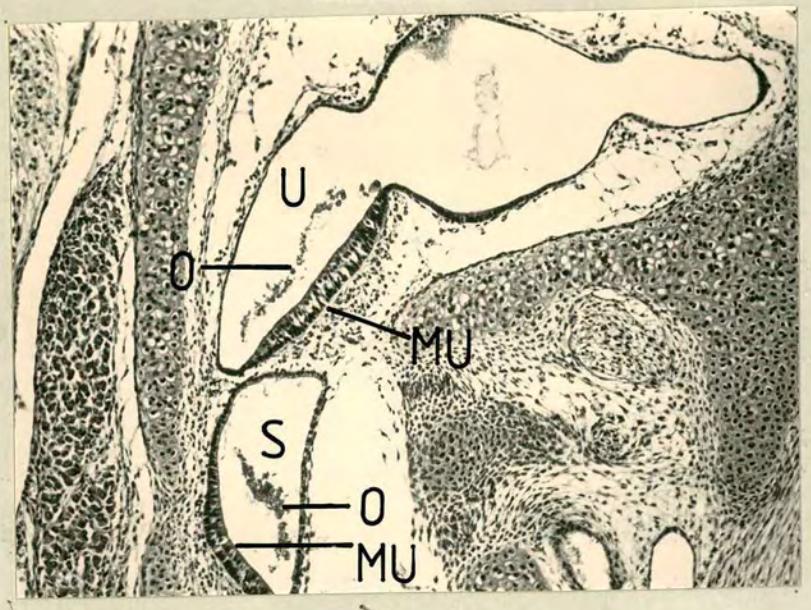
Transverse section through the region of ductus reuniens in the two ears of a 17½ day embryo X 175.

(a) Embryo from +/+ mother.

(b) Embryo from his/his mother. Abnormally distended endolymphatic duct, ductus reuniens and cochlea.

U = utriculus
ED = endolymphatic duct.
UV = utriculo-endolymphatic valve.
DR = ductus reuniens.
C = cochlea

Figure 17



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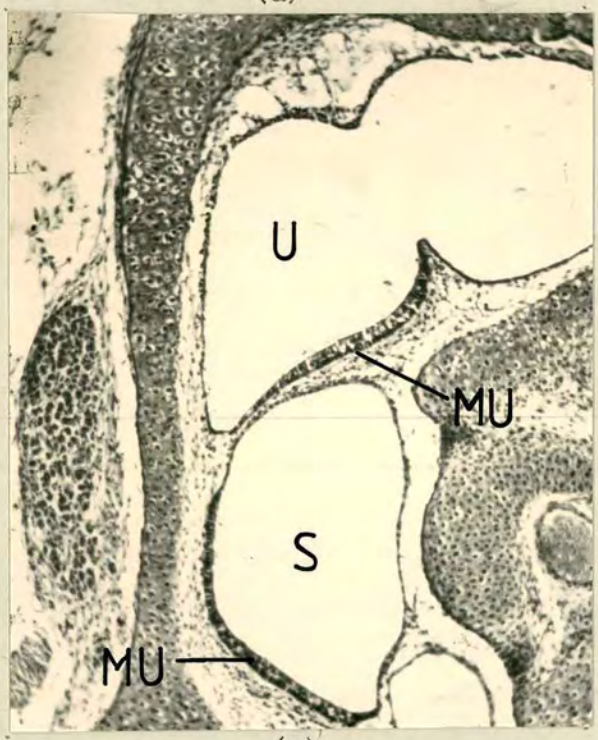


(b)

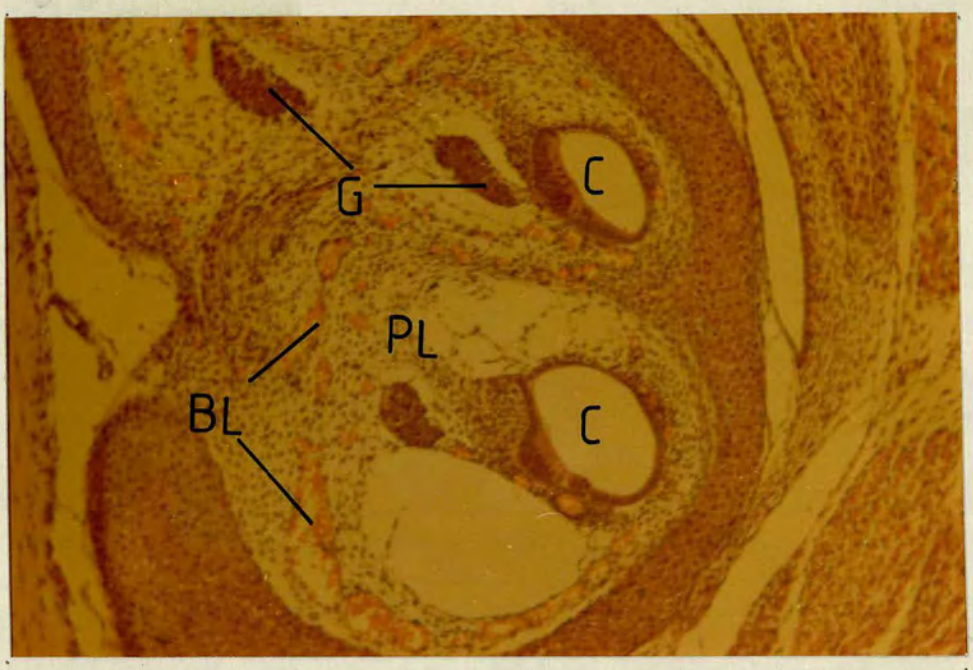
Transverse sections through utriculi and sacculi in the two ears of a 17½ day embryo from his/his mother X 80.

(a) Normal utriculus and sacculus with normal maculae. Otoliths present.
(b) Abnormally distended utriculus and sacculus with thinner maculae. Otoliths lacking.

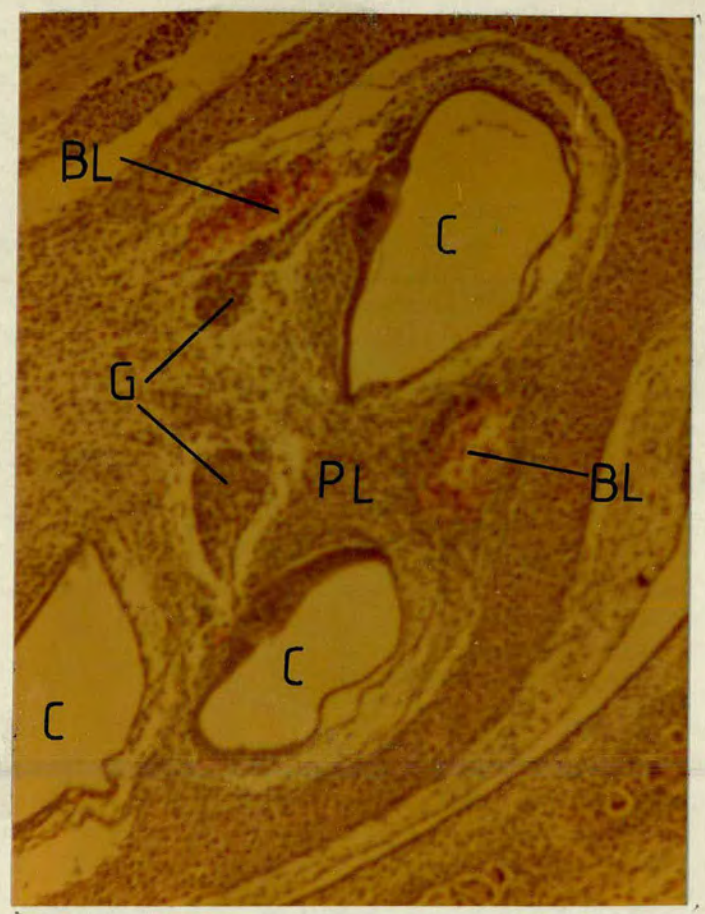
U = utriculus.
S = sacculus.
MU = macula.
O = otoliths.

under a polarising microscope. The abnormalities found at this stage were: lack of otoliths in both utriculus and sacculus and the lumens of both of these organs were distended; the cochlear duct joined the sacculus by a wide ductus reuniens (Fig. 16). The abnormalities seen at this stage varied from the abnormalities of the whole endolymphatic system to a lack of otoliths in either utriculus or sacculus, or with just big crystals whereas the rest of the inner ear was normal. In extreme cases, as in mice three weeks after birth or older, the maculae of the utriculus and sacculus were very thin (Fig. 17) and the cristae of the horizontal canal and posterior vertical canal were smaller and the ampullae were all distended. Instead of a non-cellular small cupula, there were bigger cupulae. As for the staining reaction, these big cupulae showed a positive reaction with PAS stain. The endolymphatic duct and sac were distended. In most, but not all, of the ears there was lots of debris in the distended cochlear duct, sacculus, utriculus and endolymphatic sac. In some animals, where the endolymphatic system was distended, the maculae of the utriculus and sacculus were not thin and the architecture of the cells appeared normal with supporting cells and hair cells. In only one case, where there were no otoliths in the utriculus and sacculus and where the lumens were distended, the macula of the sacculus seemed normal but the macula of the utriculus was abnormal. It had a bump near the ampulla of the horizontal canal and there seemed to be a ridge which invaginated into the lumen. In two cases the utricular otolith was missing but the diameter of the lumen was normal. In those same ears the lumen of sacculus was distended and instead of small crystals there were a few big crystals present. The otolithic membranes were present and they showed positive reaction with PAS as do normal membranes. The cochlea at 17½ days was in

Figure 18



(a)



(b)

BL = blood vessels.

PL = perilymphatic space.

C = cochlea

G = ganglion cells.

Sagittal sections of inner ears of 17½ day embryos X 80.

(a) Normal cochlear ducts with normal blood vessels from +/+ mother.

(b) Distended cochlear ducts with dispersed blood cells from his/his motner.

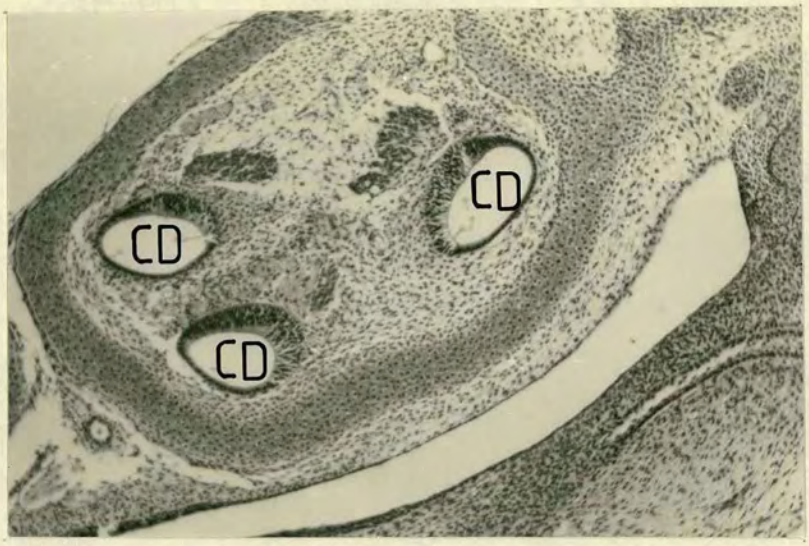
an early stage of development. In the affected abnormal ear the cochlear duct and ductus reuniens were hydropic. The architecture of the cells of the organ of Corti and Reissner's membrane looked normal. In some ears, there were more blood cells seen in the perilymphatic space of the cochlea and in the cochlear duct, and they were dispersed (Fig. 18), whereas in the normal ear, the blood cells lie in the blood capillaries.

16½ Day Embryo

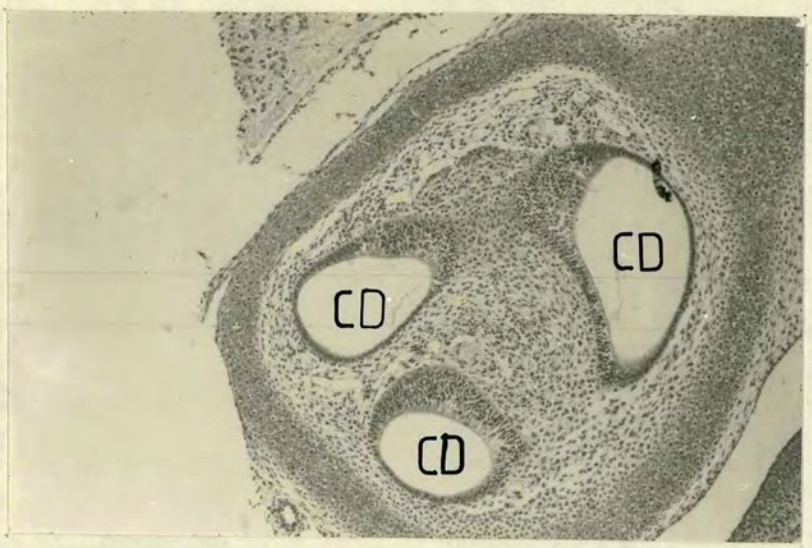
In the embryos that were taken from +/+ mothers, otoliths were found to be present in the utriculus and sacculus. The otoliths lay parallel to the maculae of the utriculus and sacculus but they were not lying on the hair of the hair cells as found in the adult ears. This could be due to fixation. These otoliths showed strong birefrigence when observed with a polarising microscope. In some animals which were taken out from his/his females, the otoliths were missing in both utriculus and sacculus, and the lumens of both utriculus and sacculus were distended.

The maculae of both utriculus and sacculus seemed to be of normal size. The otolithic membrane without crystals lay opposite the maculae of utriculus and sacculus. In one case all three semicircular ducts were narrow, but the perilymphatic spaces that surround the membranous ducts appeared to be the same diameter as normal. The endolymphatic sac was distended as were the ductus reuniens, utriculosaccular duct and cochlear duct. The cochlear duct had rugose edges. Apart from the distension and rugose edges, the cochlea did not show much difference from normal in cell structure. Some debris was present in the lumen of the distended cochlea and sacculus. In one embryo the cochlear duct, ductus reuniens, sacculus and utriculus, which lacked otoliths were all

Figure 19



(a)



(b)

Transverse sections of cochleae of $15\frac{1}{2}$ day embryos X 80.

(a) Normal cochlea (from +/+ mother).

(b) Distended cochlea (from his/his mother).

CD = cochlear ducts.

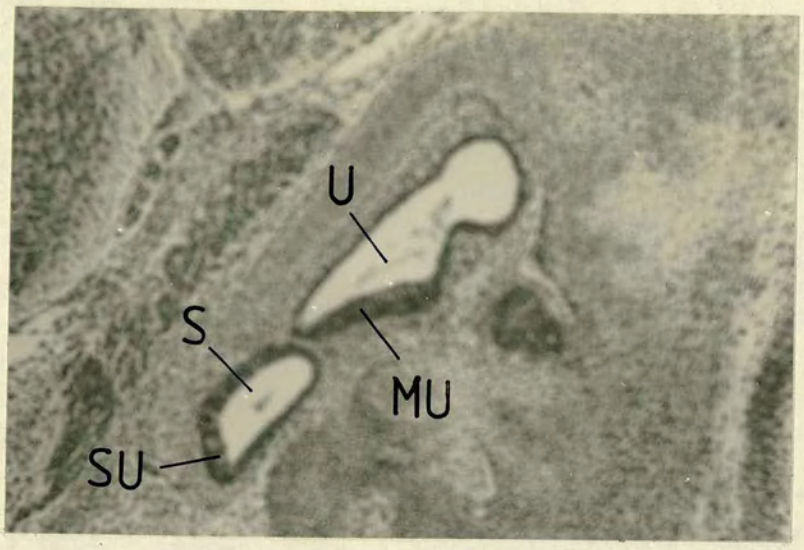
distended, but the endolymphatic sac and ampullae were of normal diameter.

15½ Day Embryo

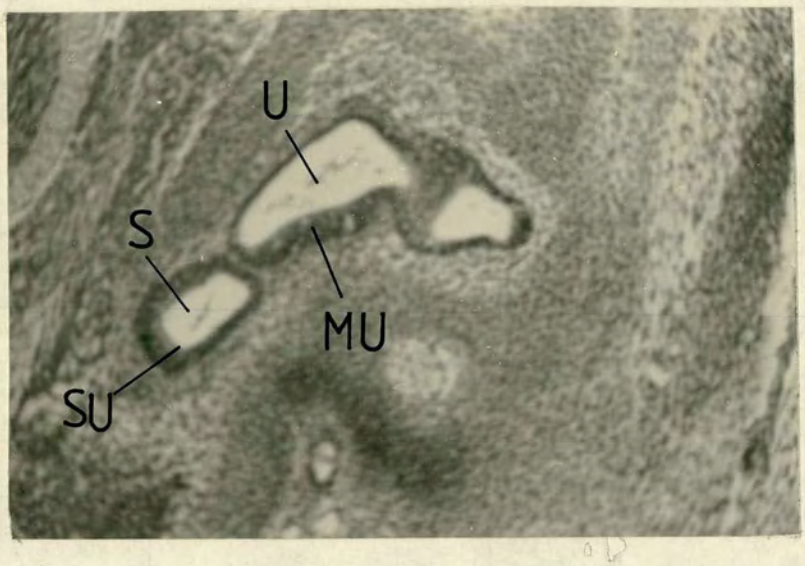
In the utriculus and sacculus, there were minute crystals which stained darkly with haematoxylin and when tested for birefringence showed as specks of birefringence along the fibrillar material in the lumen. In two embryos that were taken from his/his mothers, the calcium crystals were found to be bigger than those in the normal controls and their litter mates. In three embryos that were also taken from his/his mothers there were no signs of crystals at all. Two embryos were tested for birefringence and they showed no signs of crystals. At this stage, apart from the absence of crystals, there was no difference in diameter of the semicircular canals and the lumens of utriculus and sacculus of control embryos and embryos from his/his mothers.

In those three embryos which had no crystals in the utriculus and sacculus, the ductus reuniens and cochlear duct were slightly distended (Fig. 19). The endolymphatic ducts were swollen in the ears which had no traces of crystals. But as in 16½ day embryos, the endolymphatic sac also looked swollen in some of the embryos which were taken from +/+ mothers. In the embryos which had no crystals or which showed no birefringence, there was fibrillar material floating free in the lumens of the utriculus and sacculus. In ears which had slightly distended cochlea and absence of traces of otoliths the semicircular ducts were the same diameter as in normal.

Figure 20



(a)



(b)

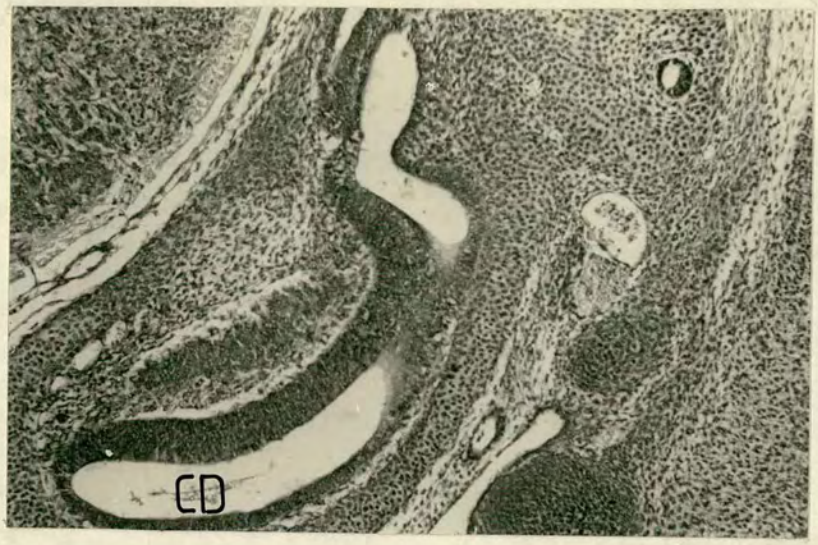
Transverse sections of inner ears of 14½ day embryos passing through utriculi and sacculi X 80.

(a) From +/+ mother.

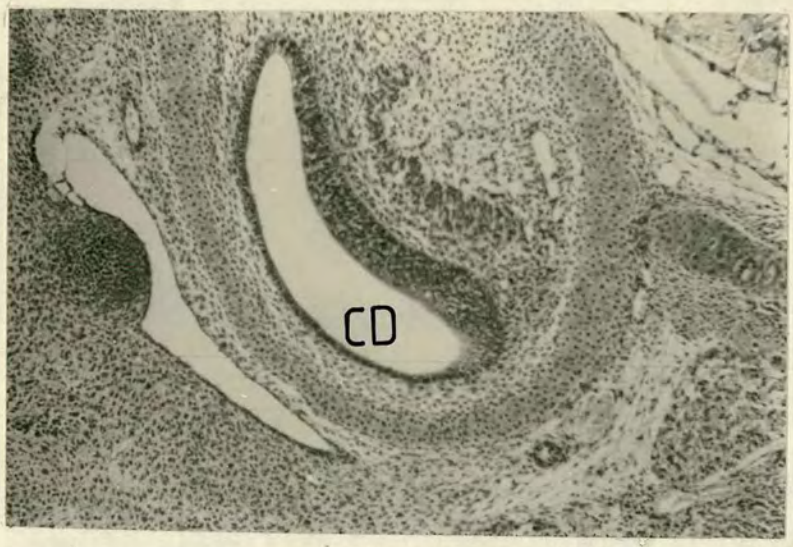
(b) From his/his mother.

U = utriculus
S = sacculus
MU = macula of utriculus
SU = macula of sacculus

Figure 21



(a)



(b)

Transverse sections of inner ears of 14½ day embryos passing through cochleae X 80.

(a) Right inner ear (from +/+ mother).

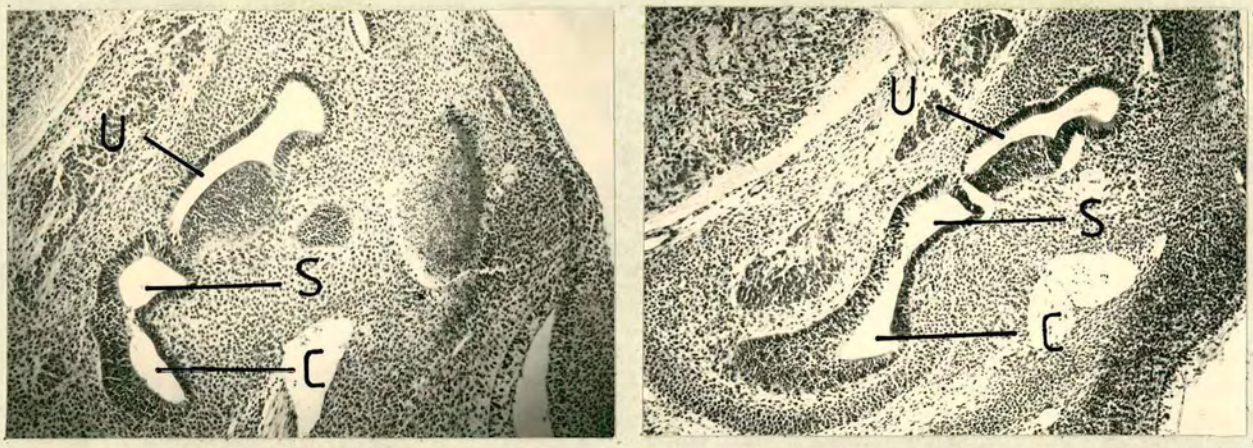
(b) Left inner ear (from his/his mother).

CD = cochlear duct.

14½ Day Embryo

At this stage all the main parts of the inner ear have been formed. The sensory epithelia of utriculus and sacculus were not fully developed but they had differentiated from the surrounding wall (Fig. 20). Five control embryos, taken from +/+ mothers, were stained with haematoxylin and eosin. In the utriculus and sacculus of these animals there were minute crystals which stained darkly with haematoxylin lying among the filamentous substance which was floating free in the lumens of both utriculus and sacculus. Two control embryos were stained for birefringence and observed under a polarising microscope. Pin points of birefringence were observed in the lumens of the utriculus and sacculus. The ampullae and cochlea had differentiated from the surrounding walls and the non cellular cupulae of the cristae of the three semicircular canals and the tectorial membrane of the cochlea were also present. The cochlea was in its initial stage of differentiation. Fifteen embryos from his/his mothers were studied at this stage. Six embryos, which had no traces of calcium crystals, had an undilated endolymphatic sac. These embryos, with small traces of crystal had the endolymphatic sac slightly distended. Two embryos were tested for birefringence but were negative. The endolymphatic sacs of these two embryos were dilated. Four animals which were stained with haematoxylin and eosin and which had no traces of crystals either in utriculus or sacculus, had the endolymphatic sacs distended. In one of the five control embryos the endolymphatic sac was found to be slightly distended. Apart from the swollen endolymphatic ductus reuniens, there was no difference in semicircular ducts, lumens of utriculus and sacculus and the cochlea between embryos from high penetrance his/his mothers and those of normal +/+ control mothers (Fig. 21).

Figure 21A



(a) (b)

Transverse sections of inner ears of $13\frac{1}{2}$ day embryos passing through utriculi, sacculi and cochleae X 100.

(a) From +/+ mother.

(b) From his/his mother.

U = utriculus
S = sacculus
C = cochlea

13½ Day Embryo

At this stage the three semicircular canals have been formed. Controls which were taken from +/+ mothers and from his/his mothers were looked at. There was no difference between the embryos from +/+ mothers and from his/his mothers. At this stage the maculae of the utriculus and the sacculus were in an early stage of differentiation. In the lumen of utriculus there was some precipitate-like material. This was found in lumens of both utriculus and sacculus. Two animals, one from a +/+ mother and one from a his/his mother were tested for birefringence and they showed a negative reaction. The cochlea too was in its early stage of development. None of the animals from his/his mothers showed any difference from +/+ mothers in either the cochlea or the ductus reuniens. No dilation was found in the utriculus or sacculus and no narrowing or dilation of semicircular canals was found in any of the embryos from his/his mothers. (Fig. 2) The ganglion cells and nerves supplying the inner ear appeared the same in the +/+ controls as in the animals born from his/his mothers.

SUMMARY

Otoliths

The first sign of abnormality found in the animals from his/his mothers could be detected as early as 14½ day embryos. Pin point birefringence and small crystals which were seen in the animals that were born from +/+ mothers were missing in some animals which were born from his/his mothers. But there was no sign in some 14½ day embryos taken from his/his mothers of the big crystals which were found in the 15½, 16½, 17½ day embryos and onwards.

Utriculus and Sacculus

The first sign of abnormality found in some of the animals that were born from his/his mothers was at 16½ days. At that stage as stated above the maculae of the utriculus and sacculus were very thin and the lumens were dilated. This kind of dilation and thinning of the macula was not found in all the animals that had distended cochleae and ductus reuniens, as it was in all these animals which had no otoliths. In some cases the otoliths were missing in both the utriculus and sacculus but the utriculus showed no sign of distension and no thinning of the macula. In most cases, but not in all, the lumen was distended and the macula found to be thin as well.

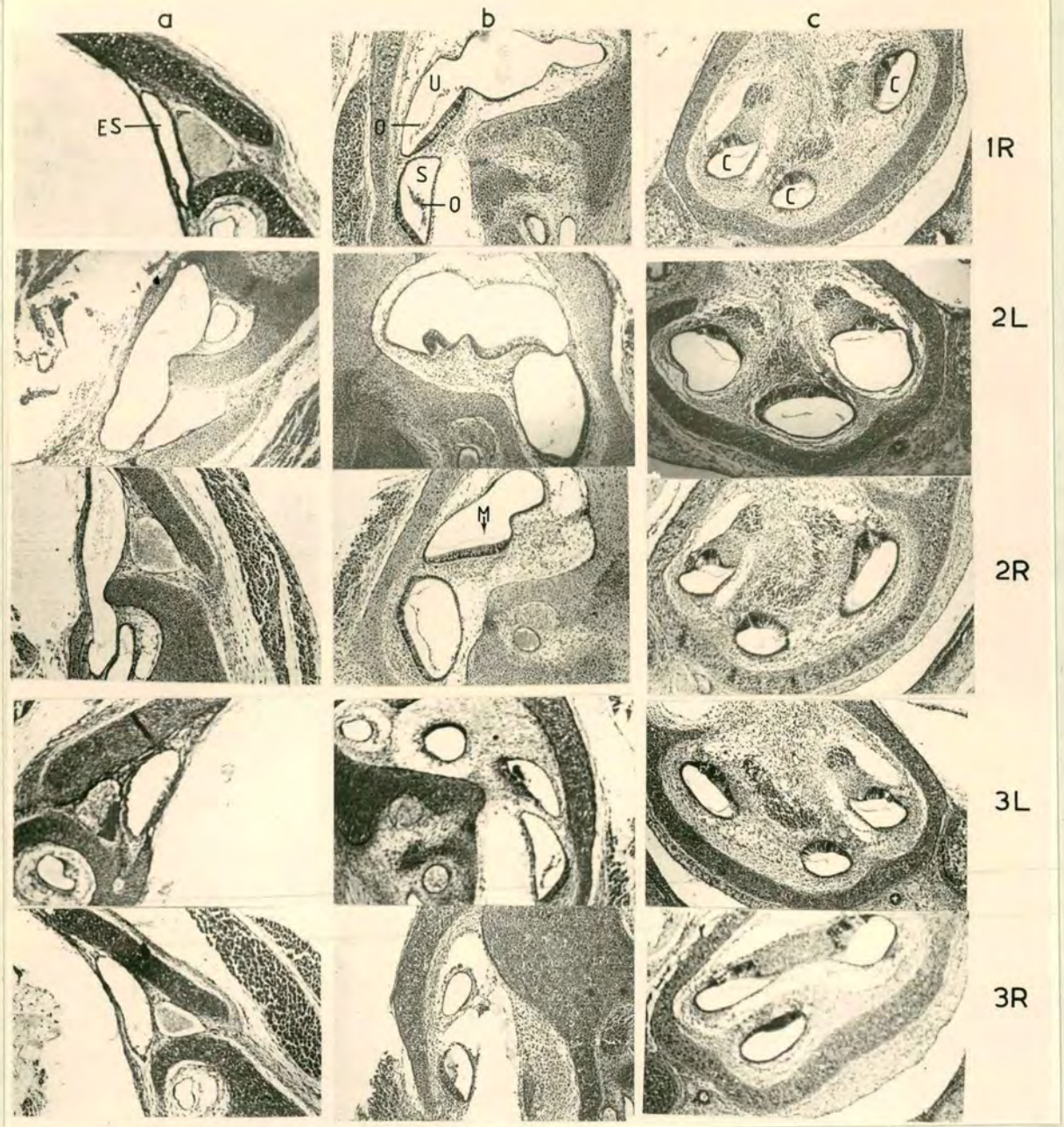
Canals

Even though there were narrowed semicircular ducts in sections as well as in whole mounts of adult ears, no narrowing or constriction or distension of the semicircular ducts were seen in 14½ and 15½ day embryos. In one embryo at 16½ days which was taken from a his/his mother the three semicircular ducts seemed to be narrowed but the surrounding perilymphatic space was of normal dimensions. In some cases where the cochlea, ductus reuniens, sacculus and utriculus were distended the semicircular ducts were found to be distended as well, these kinds of abnormalities were clearly seen in 17½ and 18½ day embryos and newborn animals.

Ductus reuniens

No sign of abnormality was found in the ductus reuniens at 14½ days in embryos. A slight distension was first seen at 15½ days in embryos of his/his mothers and from 16½ days onwards the distension was very obvious.

Figure 21B



(a) showing endolymphatic sac

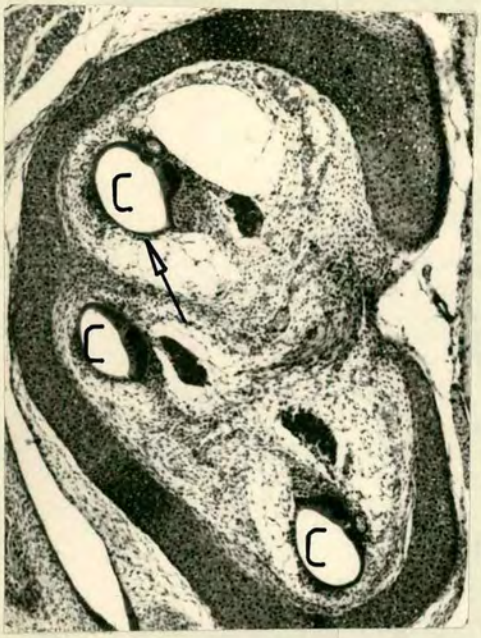
(b) showing utriculus and sacculus

(c) showing cochlea

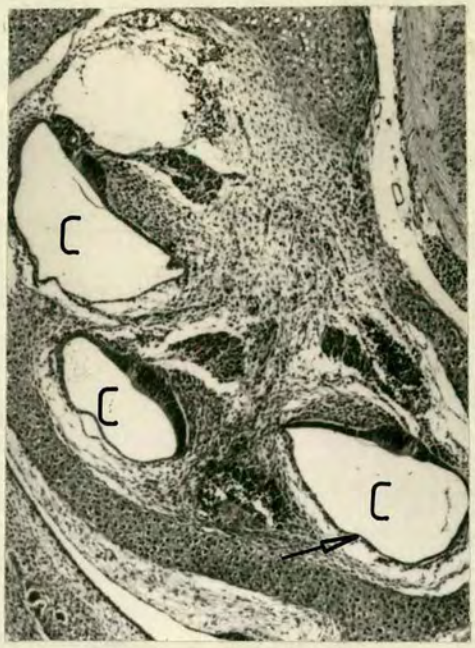
U = utriculus
S = sacculus
C = cochlea
B = big crystals
ES = endolymphatic sac

1 - 6 = embryo numbers
R = right inner ear
L = left inner ear

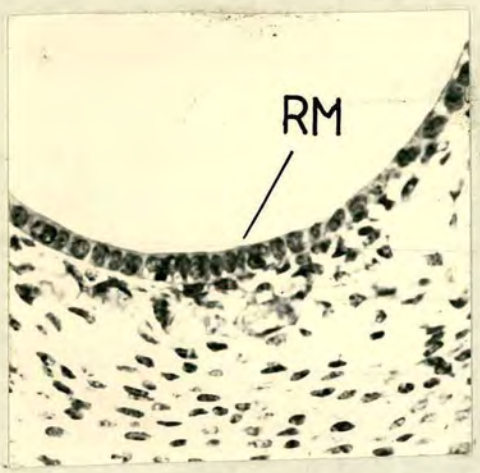
Figure 21C



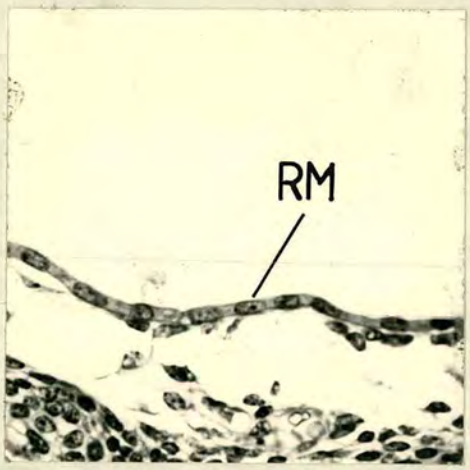
(a)



(b)



(c)



(d)

Sagittal sections of 17½ day embryos passing through cochleae ((a) and (b) X 63 and (c) and (d) X 630).

(a) and (c) From +/+ mother.
(b) and (d) From his/his mother.
(c) and (d) Showing high power view of anlage of Reissner's membranes. Arrows indicate the part that is shown in (c) and (d). Note in Fig. (d) the cells look further apart as though they are stretched.

RM = Reissner's membrane
C = cochlea ducts

Endolymphatic sacs

As there was variation in the endolymphatic sac even in animals that were taken from +/+ mothers it was difficult to say whether the endolymphatic sac was distended at 14½ or 15½ days. But from 16½ days onward the endolymphatic sac in some animals, which had distended cochleae, ducti reuniens, utriculi and sacculi and which were taken from his/his mothers, showed gross dilatation as shown in Fig. 21B. This kind of gross distension was found from 16½ days onwards.

Cochleae

No sign of abnormality was found in 13½ and 14½ day embryos born from his/his mothers. Starting from 15½day embryos the cochlea showed slight distension and from 16½ day onwards the dilation was very apparent in the cochlear coils. The architecture of cell structure appeared more or less the same except that in the dilated membrane it appeared as though the membrane had been pulled from both ends (Fig. 21C).

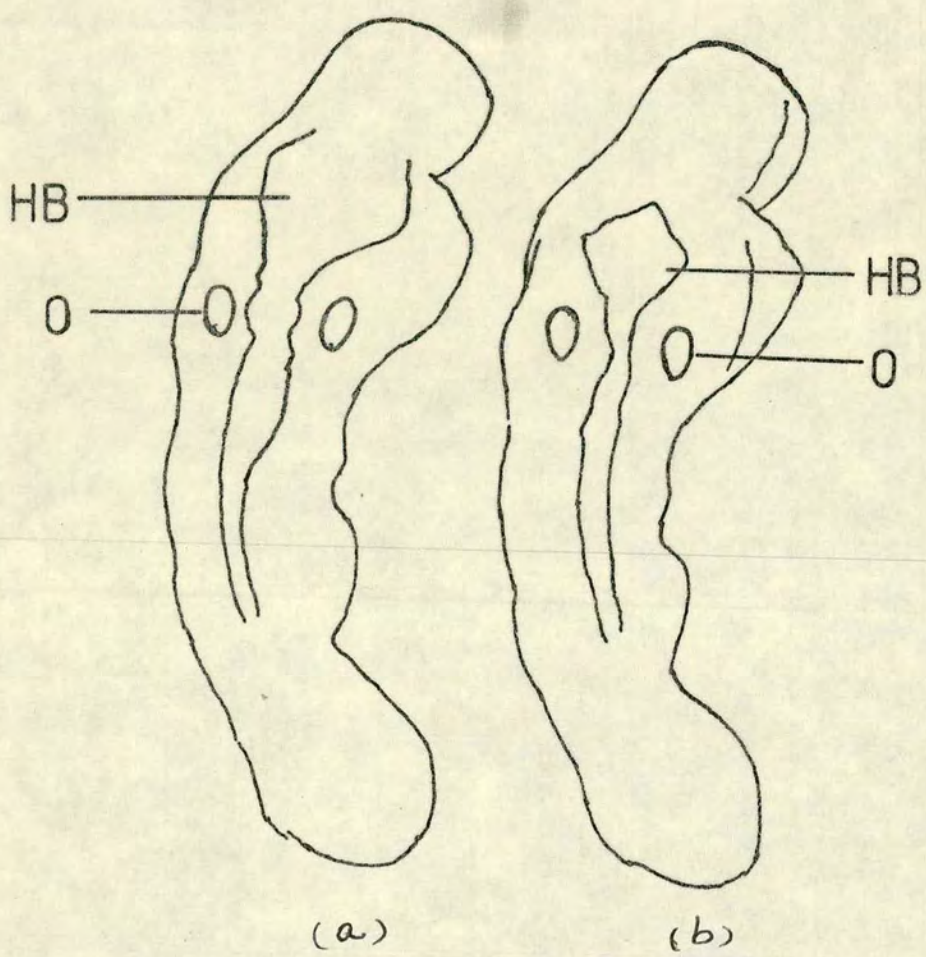
SECTION III

Development of Structures other than the Inner Ear

In the previous Section it was shown that the earliest morphologically detectable abnormalities were seen at $14\frac{1}{2}$ days. At $13\frac{1}{2}$ days very little difference from normal was detectable. This may be due mainly to the fact that many of the structures, seen later to be abnormal, are in very early stages of development and fine differences are difficult to distinguish. It was, however, known from histidine feeding experiments of his/+ mothers (Kacser et al., 1977), that the period from 7-14 days was critical in producing the developmental lesions. Furthermore evidence from early stages of certain mutants indicated that the malformation in the present study might not be a direct effect of the maternal metabolites on the structures of the inner ear but could be the result of changes or interferences of inductive events.

The inner ear is derived from the otic vesicle which develops from ectoderm. The otic vesicles are known to form by inductive influences arising from both the hind brain and the surrounding mesoderm. In amphibians, Detwiler and van Dyke (1950) and Yntema (1950) did transplantation experiments and found that the differentiation of the otic vesicle into a complex labyrinth depends on the inductive influence of the neural tube. In mice, Deol looked at kreisler and dreher at the very early stages and found that the thin roof of hind brain (rhombencephalon) was narrower and looked like the shape of a diamond rather than the shape of a kite. In kreisler the otic vesicle looked normal in shape but situated not in proximity to the neural folds but at some distance and in dreher the otic vesicle looked normal up till the 9 day stage but in later stages the inner ear was found to be

Figure 21D



Camera lucida drawings of dorsal views of the heads of 9 day embryos from his/his mother.

(a) Embryo with normal looking hind brain.

(b) Litter mate of (a) with narrowed hind brain which looked like the shape of a spade in a pack of cards.

HB = hind brain
O = otic vesicle

TABLE 4: Embryonic head morphology and days of gestation

Litter No.	Maternal genotype	Paternal genotype	No. of Embryos	Morphology of head		Days of Gestation
				N	Ab	
1	+/+	+/+	10**	5	0	$9\frac{1}{2}$
*2	his/his(L)	his/his(H)	10	8	2	$9\frac{1}{2}$
3	his/his(I)	his/his(H)	6	4	2	$9\frac{1}{2}$
4	his/his(I)	his/his(H)	10	6	4	$9\frac{1}{2}$
5	+/+	+/+	10***	9	0	$10\frac{1}{2}$
6	+/+	+/+	8	8	0	$10\frac{1}{2}$
7	his/his(I)	his/his(H)	10	8	2	$10\frac{1}{2}$
⁄8	his/his(I)	his/his(H)	10	8	2	$10\frac{1}{2}$
9	+/+	+/+	9	9	0	$11\frac{1}{2}$
10	+/+	+/+	9	9	0	$11\frac{1}{2}$
11	+/+	+/+	8	8	0	$11\frac{1}{2}$
⁄*12	+/+	+/+	10	10	0	$11\frac{1}{2}$
*13	his/his(L)	his/his(H)	9	7	2	$11\frac{1}{2}$
*14	his/his(I)	his/his(H)	10	6	4	$11\frac{1}{2}$
15	his/his(H)	his/his(H)	6	4	2	$11\frac{1}{2}$
16	his/his(H)	his/his(H)	6	4	2	$11\frac{1}{2}$

⁄ Fixed in utero before dissection.

* Some of the embryos were sectioned.

** 5 embryos damaged during dissection.

*** 1 embryo damaged during dissection.

% affected in cross I X H = 28%

% affected in cross H X H = 33%

% affected in cross L X H = 21%

completely abnormal. This abnormality could be due to those abnormalities of the hind brain (Deol, 1964a and 1964b).

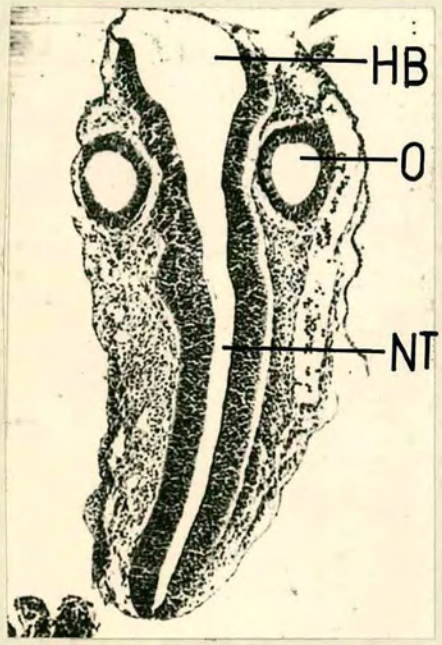
An attempt was made to find out whether at very early stages such as $9\frac{1}{2}$, $10\frac{1}{2}$, $11\frac{1}{2}$ days of embryonic life, before the otic vesicle showed much development, there were effects on the neural tube or hind brain. This might add some more information on the abnormalities of the inner ear if there was any morphological difference between the animals from +/+ mothers and from his/his mothers.

Most of the litters were dissected out from the membranes in Ringer's solution and examined under a dissecting microscope for externally visible malformations. Some of them were embedded in paraffin wax and sectioned. In two litters (Nos. 8 and 12, Table 4), the whole uterus was fixed before dissection of the embryos. In some litters (3,7,5,14,15,16), the positions of the embryos in the uterine horns were noted. Counting the one nearest the right ovarium as No. 1 and so on.

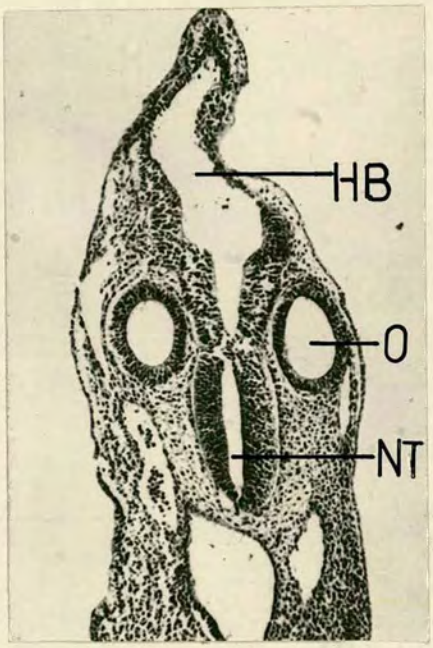
$9\frac{1}{2}$ Days

At this early stage, the inner ear was in its very early stage of development, the otic vesicle could be seen under the dissecting microscope without sectioning. In the animals that were taken from +/+ mothers, no abnormality was found in the neural tube or the hind brain. In two animals out of 26, (one from litter 2 and one from litter 4) taken from his/his mothers, the whole neural tube was found to be opened, and in some animals from litters 2 - 4 the hind brain (roof of the rhombencephalon) was found to be narrowed. It was like the shape of a spade in a pack of cards, rather than like the shape of a kite. (Fig. 21D.) Two morphologically normal embryos and one

Figure 22



(a)



(b)

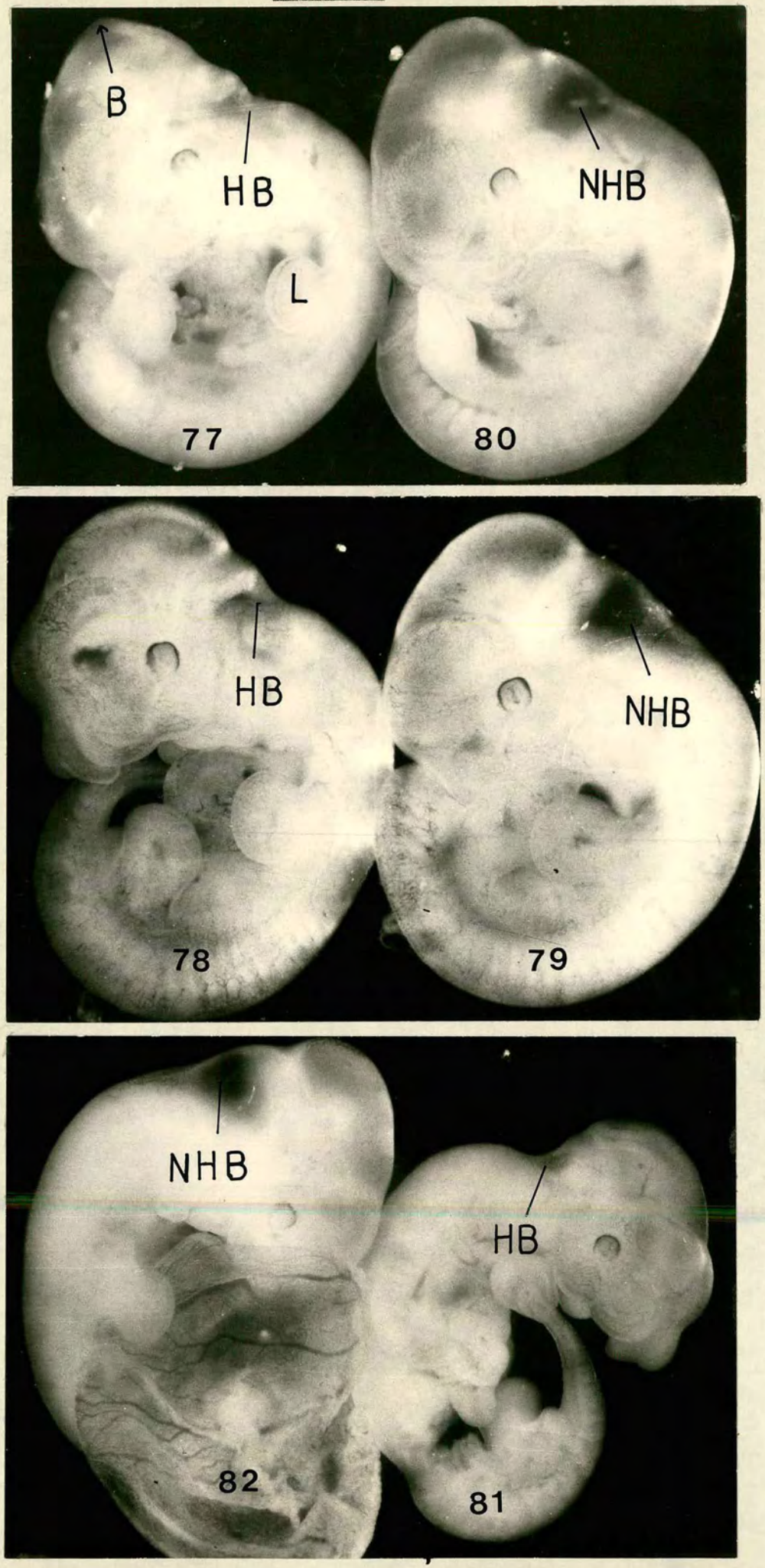
Transverse sections of 9½ day embryos passing through hind brain and otic vesicle X 32. There is no difference between the two sections. The apparent difference is due to slight differences in the planes of sectioning.

(a) Embryo from +/+ mother.

(b) Embryo from his/his mother.

HB = hind brain.
O = otic vesicle.
NT = neural tube.

Figure 23



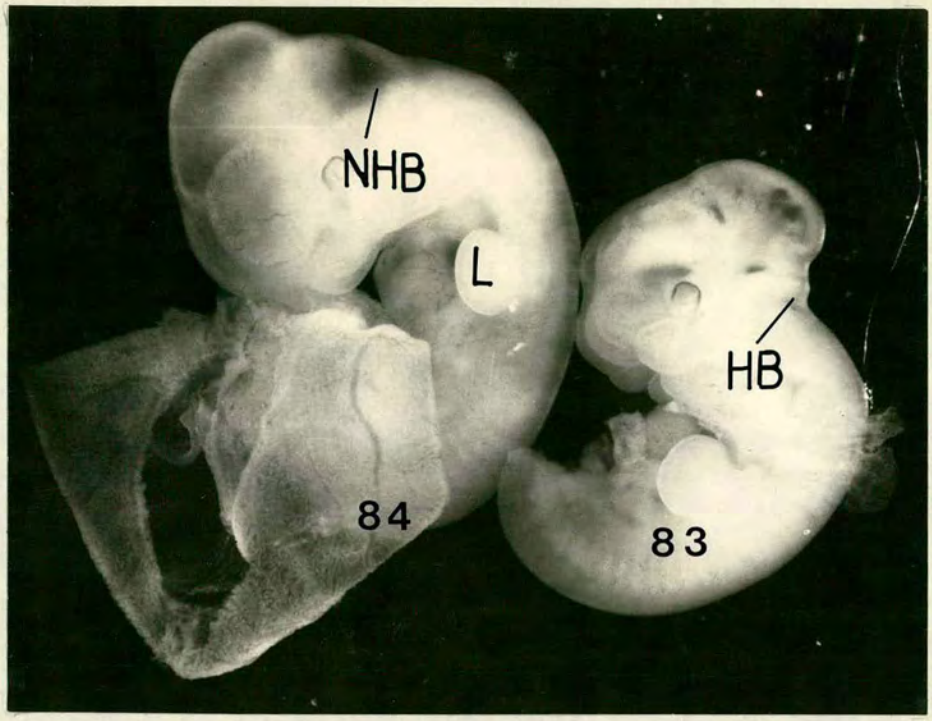
(a)

(b)

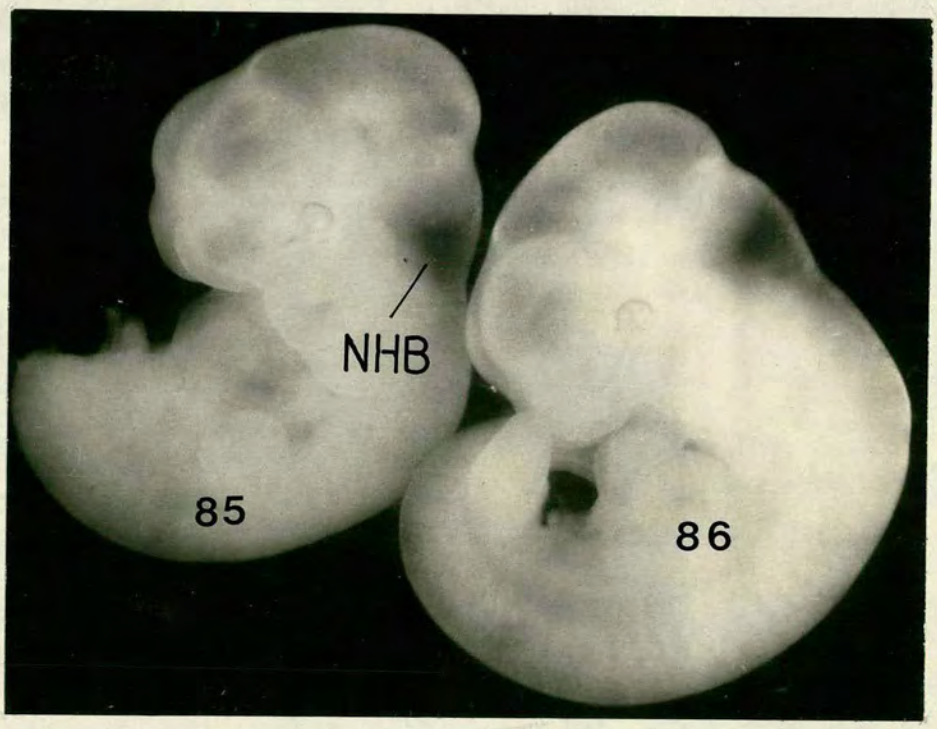
(c)

Figure 23 (ctd.)

(d)



(e)



11½ day embryos from litter No. 14, showing narrowed hind brains in No. 77, 78, 81 and 83. Bumps seen in No. 77 and No. 83.

HB = hind brain
B = bump in mid brain region
NHB = normal hind brain
L = fore limb

morphologically abnormal embryo with narrowed hind brain were sectioned, stained with haematoxylin and eosin and examined. The shape and size of the ear vesicle showed no apparent difference from the ear vesicle of morphologically normal looking embryos which were taken from +/+ mothers. (Fig. 22).

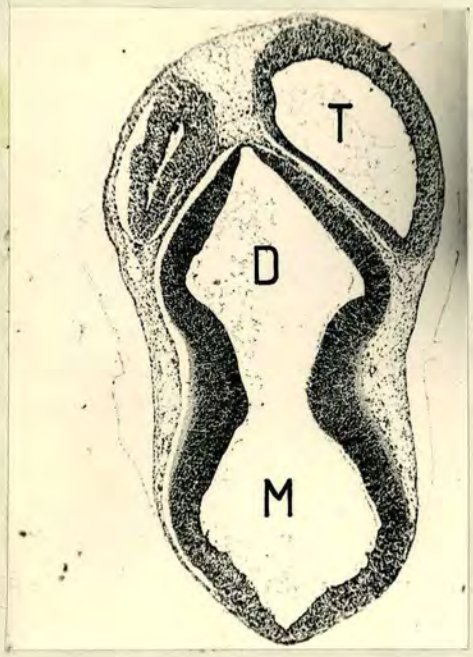
10½ Days

At this stage the animals were examined only as whole mounts and all the three litters were dissected out from the membranes before fixation. Four embryos out of 20 from his/his mothers, showed narrowed hind brain as in the 9½ day stage. No open neural tube was found in any of the embryos, no externally visible malformations were found. The otic vesicle was seen without sectioning as in 9½ days and from the whole mount view, the otic vesicles of the embryos with narrowed hind brain showed no difference from the otic vesicle of the embryos from +/+ control mothers.

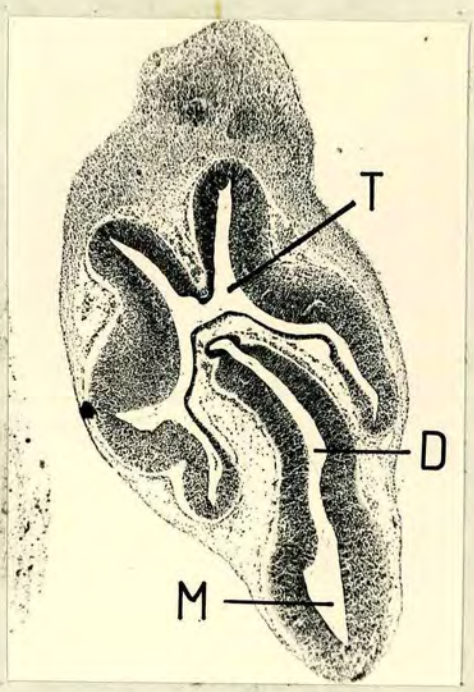
11½ Days

At this stage the positions of the embryos in the uterine horns were recorded and the embryos were removed and separated from their membranes in Ringer's solution. They were then examined for beating of the heart and the external appearance of head, limbs, eye and tail. From four litters giving 31 embryos from his/his mothers there were 10 morphologically abnormal embryos which had narrowed hind brain regions and some showed 'bumps' of the mid brain regions (Fig. 23). At this stage the inner ear was not as clearly visible as in the 9½ and 10½ day embryos. Under high power magnification the inner ear could be seen faintly as could the endolymphatic duct. Some embryos from litters Nos. 12, 13, and 14, both morphologically normal and with

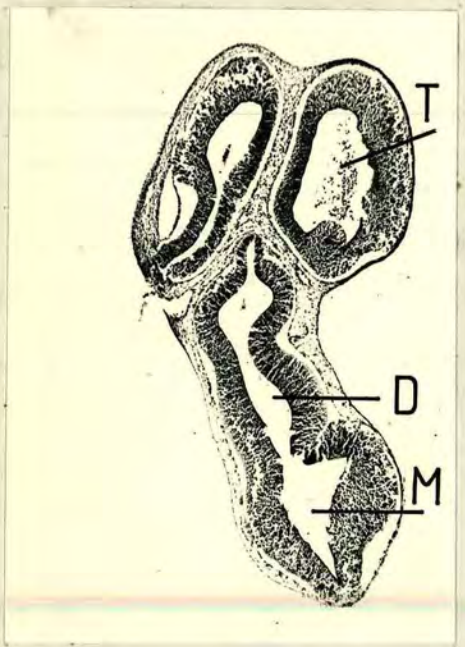
Figure 24



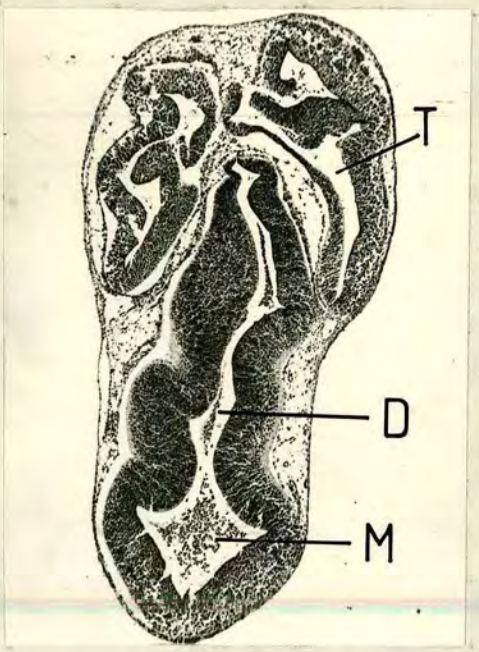
(a)



(b)



(c)



(d)

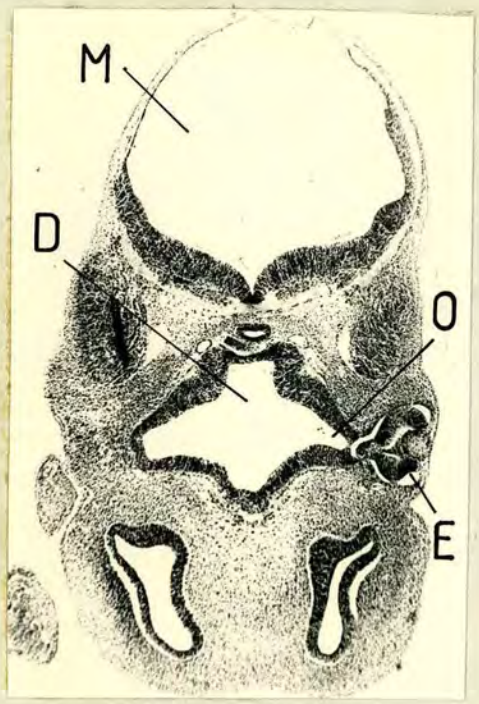
Sections of fore and mid brain regions of 11½ day embryos from Figure 23 X 33.

(a) No. 80. Normal.

(b), (c) and (d) 78, 81, 83 with narrowed fore brains and mid brains.

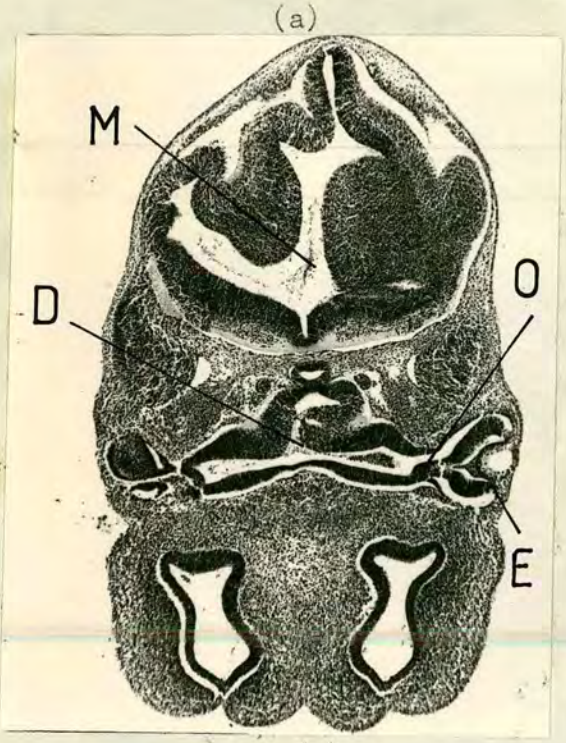
T = telocoele
D = diocoele
M = mesocoele

Figure 25

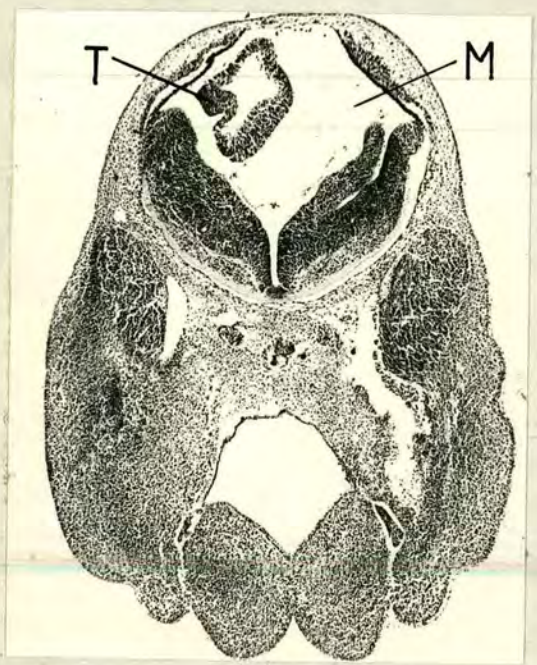


M = myelocoele
D = diocoele
O = optic stalk
E = eye
T = excessive tissue

(a)



(b)

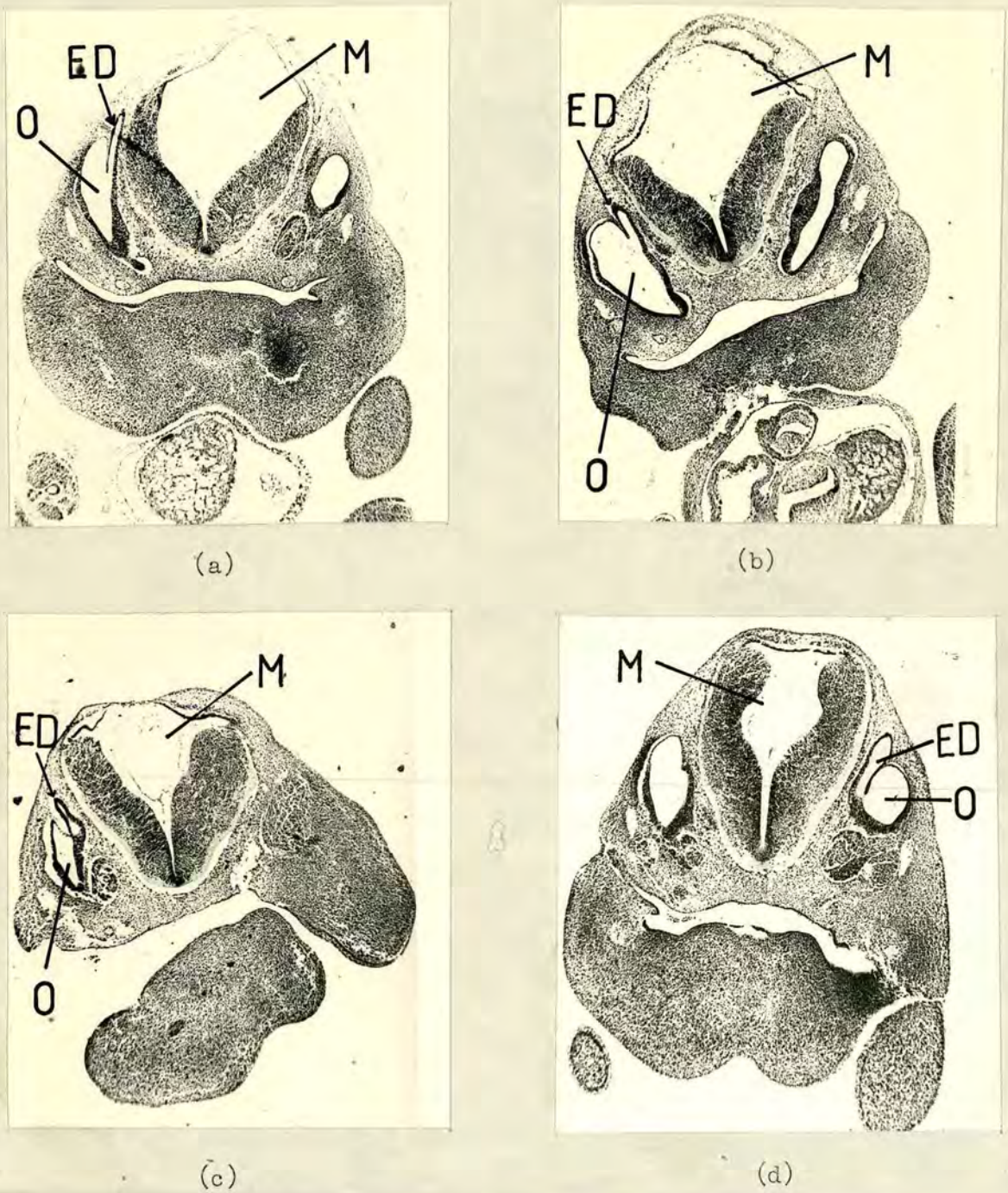


(c)

Transverse sections of mid brain and hind brain regions of embryos from Figure 23 (litter 14) X 33.

(a) Normal hind brain and mid brain.
(b) Narrowed hind brain, narrowed diocoele
(c) Section cut through hind brain showing excessive tissue floating in the brain cavity.

Figure 26



Transverse sections of $11\frac{1}{2}$ day embryos passing through hind brains and otic vesicles (litter 14) X 33.

(a) Normal hind brain with normal otic vesicle.

(b), (c) and (d) Narrowed hind brains.

M = myelocoele
ED = endolymphatic duct
O = otic vesicle

TABLE 5 : Head morphology and inner ear lesions with days of gestation

Date	Litter No.	Maternal genotype	Paternal genotype	No.of Embryos	Morphology of head		No. Sectioned	Inner Ear			Days of gestation
					N	Ab		N	Mild	Severe	
	1	+/+	+/+	11	11	0	0	0	0	0	12½
	2	+/+	+/+	10	10	0	0	0	0	0	12½
6.5.77	3	his/his(I)	his/his(H)	5	5	0	0	0	0	0	12½
2.6.77	4	his/his(I)	his/his(H)	6	6	0	0	0	0	0	12½
17.5.77	5	+/+	+/+	11	11	0	0	0	0	0	13½
	6	+/+	+/+	8	8	0	2	2	0	0	13½
5.5.77	7	his/his(I)	his/his(H)	10	10	0	2	2	0	0	13½
4.5.77	8	his/his(H)	his/his(H)	9	9	0	2	2	0	0	13½
	9	+/+	+/+	9	9	0	3	3	0	0	14½
	10	+/+	+/+	14	14	0	2	2	0	0	14½
8.12.76	11	his/his(I)	his/his(H)	11	11	0	11	6	4	0	14½
9. 5.77	12	his/his(I)	his/his(H)	4	4	0	4	0	4	0	14½
	13	+/+	+/+	9	9	0	3	3	0	0	15½
	14	+/+	+/+	10	10	0	2	2	0	0	15½
28.11.75	15	his/his	his/his	9	9	0	4	3	1	0	15½
	16	his/his(H)	his/his(H)	9	9	0	3	3	0	0	15½
8.12.76	17	his/his(I)	his/his(H)	8	8	0	3	2	1	0	16½
28. 8.75	18	+/his	his/his	8	8	0	7	7	0	0	16½
	19	his/his	his/his	8	8	0	8	7	1	0	16½
28. 2.77	20	his/his(L)	his/his(H)	8	8	0	2	2	0	0	16½
11. 5.77	21	his/his(H)	his/his(H)	2	2	0	2	0	0	2	16½
27.11.75	22	+/+	his/his	5	5	0	2	2	0	0	17½
25. 8.75	23	+/+	his/his	10	10	0	2	2	0	0	17½
26.11.75	24	+/+	his/his	11	11	0	3	3	0	0	17½
26.11.75	25	+/+	his/his	11	11	0	4	4	0	0	17½
25.11.75	26	+/+	his/his	5	5	0	3	3	0	0	17½
27. 8.75	27	+/his	his/his	8	8	0	3	3	0	0	17½
27. 8.75	28	his/his	+/+	9	9	0	3	2	1	0	17½
29. 9.75	29	his/his	+/+	10	10	0	3	1	2	0	17½
25. 8.75	30	his/his	+/+	11	11	0	7	3	4	0	17½
29. 5.75	31	his/his	+/+	10	10	0	7	2	5	0	17½
17. 3.77	32	his/his(I)	his/his(H)	10	10	0	10	0	0	10	17½
31.10.75	33	+/+	his/his	10	10	0	2	2	0	0	18½
18.11.75	34	his/his	+/+	10	10	0	10	10	0	0	18½
2.12.75	35	his/his	his/his	3	3	0	3	3	0	0	18½
31. 8.76	36	his/his(H)	his/his(H)	9	9	0	7	0	0	7	18½
28.11.75	37	+/+	his/his	9	9	0	2	2	0	0	Day of birth
6. 5.76	38	+/+	his/his	7	7	0	7	7	0	0	
8. 5.76	39	+/+	his/his	3	3	0	3	3	0	0	
21.11.75	40	his/his	+/+	7	7	0	7	7	0	0	
8. 9.76	41	his/his(H)	his/his(H)	7	7	0	3	0	0	3	

The designation (L), (H) and (I) refers to the 'Edinburgh' stock, the 'Cambridge' stock and their intercross respectively. These aspects will be discussed in Section IV (Penetrance).

narrowed hind brain, were sectioned and examined. The sections of Nos. 78, 81 and 83 from litter 14 showed narrowed fore brain, mid brain and hind brain. The telocoele, diocoele, mesocoele and myelocoele, were all very narrowed (Fig. 24 and 25). The fourth ventricle appeared to be very narrowed and the myelencephalon was distorted. In the normal embryo the fourth ventricle was wide with a very thin roof. The posterior choroid plexus had not formed yet. In the abnormal embryo the optic stalk was narrowed as well. The otic vesicle at this stage in normal embryos had the endolymphatic duct growing upwards (Fig. 26). Apart from otic vesicle of embryo No. 81, there was no difference seen between the otic vesicle and endolymphatic duct of normal and abnormal mice. The otic vesicle of No. 81 embryo (Fig. 26C), had an endolymphatic duct which seemed to be twisted and the otic vesicle appeared narrower. The ganglion cells and nerves supplying the otic vesicle did not show obvious differences from the morphologically normal looking litter mates or from embryos from +/+ mothers.

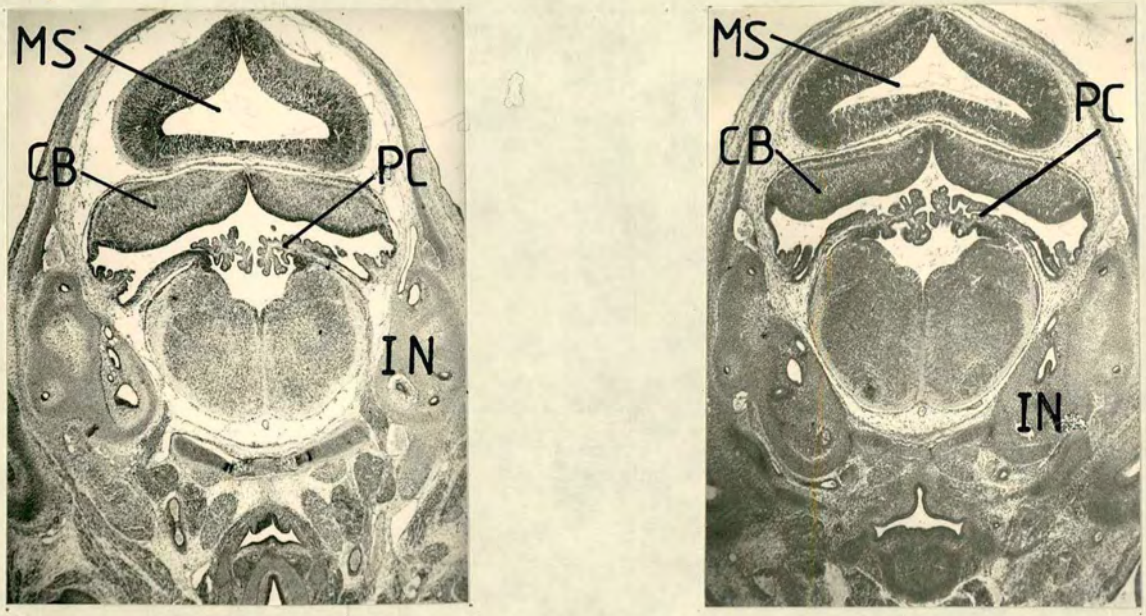
12½ Days

Only the external abnormalities were looked for at this stage. No sections were cut. Two litters from his/his mothers were looked at (11 animals) and they showed no apparent differences from the embryos from +/+ mothers. (2 litters, 21 animals). (For this and all subsequent ages, see Table 5).

13½ Days

Four litters from +/+ mothers and his/his mothers were looked at. There seemed to be no difference in the shape of the head or any other parts of embryos from his/his or +/+ mothers. Sections were cut of embryos at this stage to look for abnormalities in inner ear development of embryos from his/his mothers. The part of the brain that appeared in the sections was examined.

Figure 27



(a) (b)

Transverse sections of brains of 14½ day embryos passing through cerebellum and posterior choroid plexus X 25.

(a) From +/+ mother.

(b) From his/his mother.

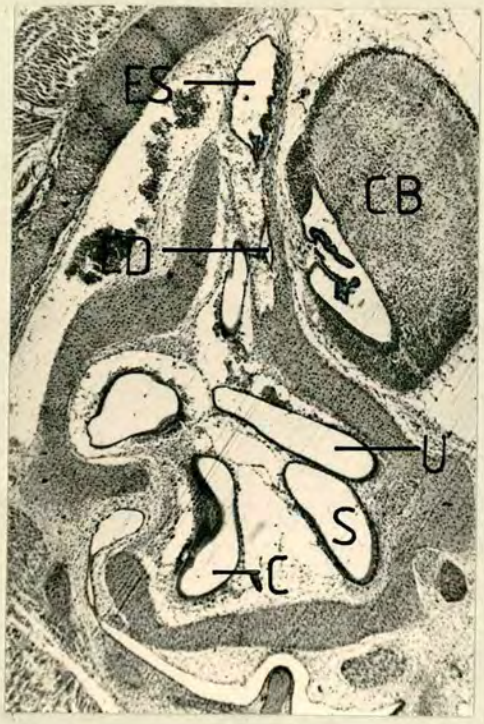
IN = inner ear

PC = posterior choroid plexus

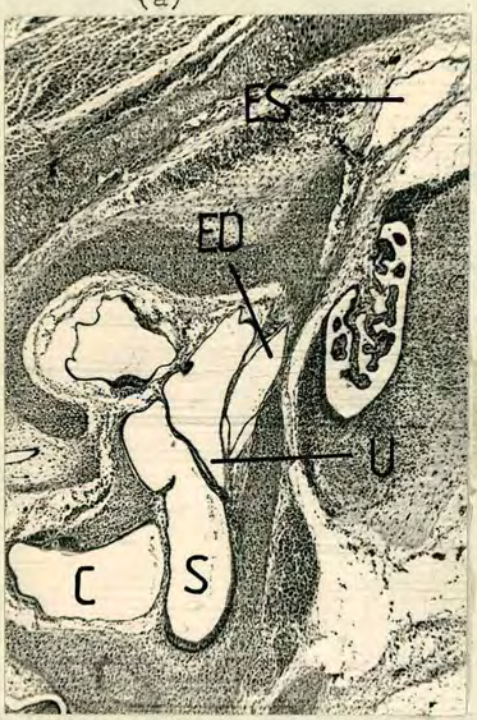
CB = cerebellum

MS = mesocoele.

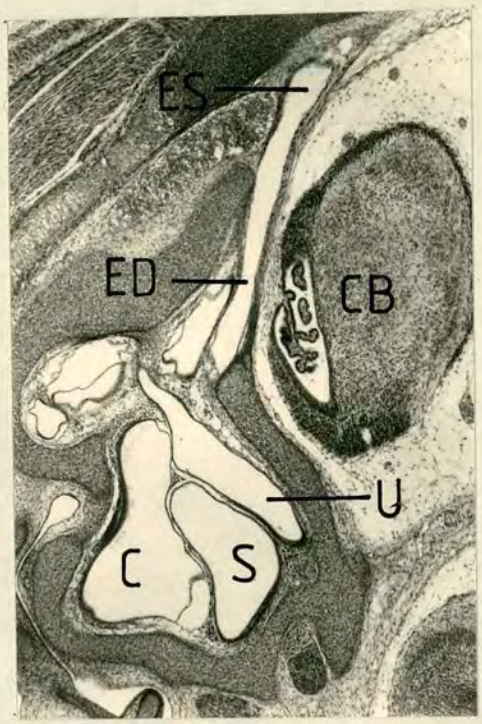
Figure 28



(a)



(b)



(c)

Sagittal sections of inner ears of 17½ day embryos. X 45.

(a) From +/+ mother.

(b) and (c) from his/his mother. Note dilated utriculi, sacculi, cochleae and endolymphatic ducts.

U = utriculus
S = sacculus
C = cochlear duct
ED = endolymphatic duct
ES = endolymphatic sac
CB = cerebellum

No difference of the shape of the head or any other structure could be seen in embryos from +/+ mothers compared with those from his/his mothers. The medulla (myelencephalon), cerebellum (mesencephalon) and posterior choroid plexus appeared the same in embryos from both his/his and +/+ mothers.

14½, 15½ and 16½ Days

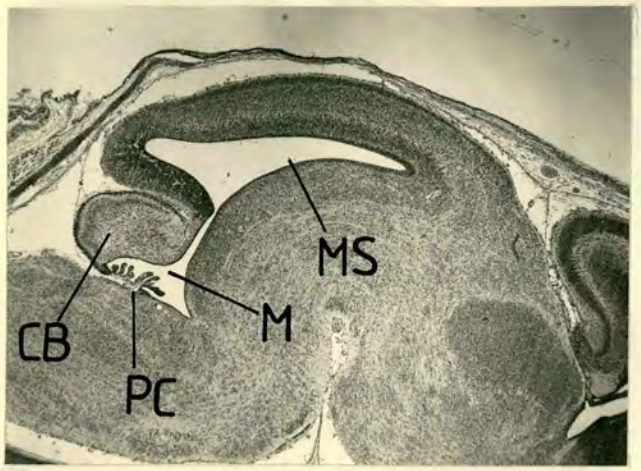
No difference in external appearance of embryos from +/+ mothers or from his/his mothers were found. In these stages like those in 13½ day embryos, parts of the brain was present in sectioned material, those were the cerebellum and the posterior choroid plexus. There seemed to be no differences in the tissues in these stages (Fig. 27).

17½ Days

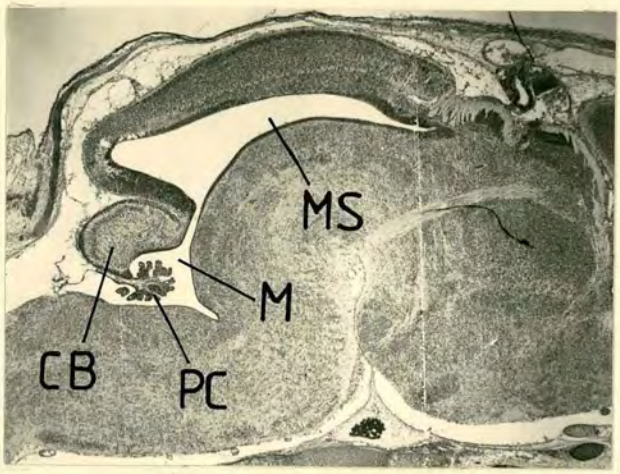
From the embryological study of the inner ear it was known that the abnormality could be clearly seen in 17½ day embryos when an abnormality of the whole of the endolymphatic system was found in some animals that were taken from his/his mothers. It was decided to look at the brain of the 17½ day embryonic stage and the inner ear to see whether the abnormality of the brain found in the early stages could be seen in the later in utero stages.

One litter from his/his mothers was sectioned, some in sagittal and transverse sections, and the inner ear and the brain were observed. Four animals from +/+ mothers were used as controls. The whole litter of 10 animals was found to have abnormal inner ears, as described in the previous Chapter; the utriculus, sacculus, ductus reuniens, cochlea and endolymphatic sac were all found to be dilated (Fig. 28). The brain

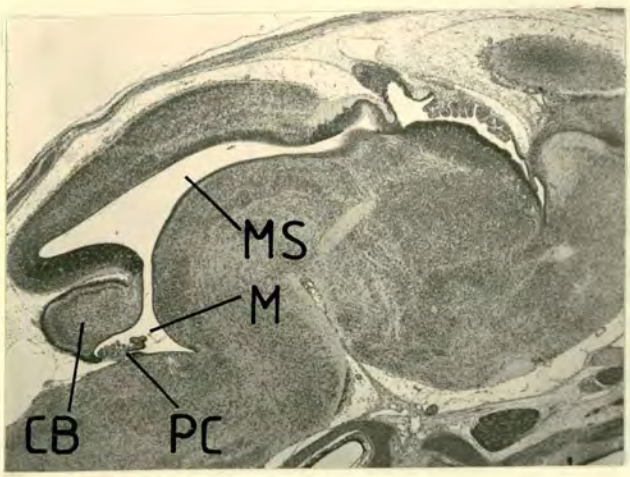
Figure 29A



(a)

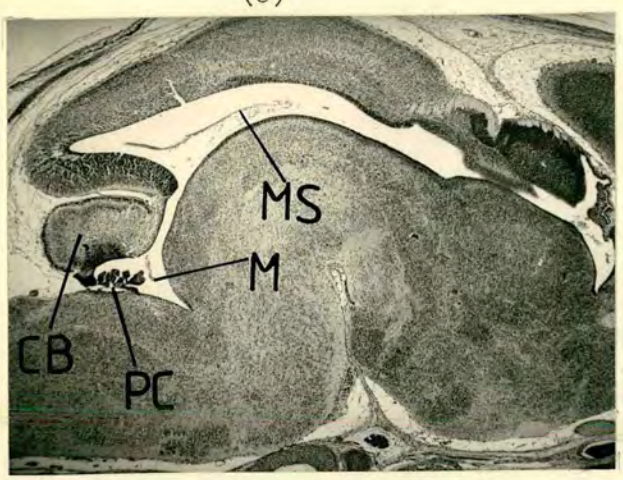


(b)



(c)

CB = cerebellum
M = myelocoele
PC = posterior choroid plex
MS = mesocoele



(d)

Sagittal sections of same embryos as Fig. 29, passing through mesocoele (mid brain) and myelocoele (hind brain) X 25.

(a) and (b) (same animal. (b) Approximately 80μ apart from (a). (From +/+ mother)

(c) and (d) Embryos from same his/his mother.

TABLE 6: Embryonic litter sizes (data from tables 4 and 5)

Days gestation	+/+ Mother L	(n)	N	his/his Mother L	(n)	N
9½	10	(10)	1	8.4	(26)	3
10½	9	(18)	2	10.0	(20)	2
11½	9	(36)	4	7.9	(34)	4
12½	10.5	(21)	2	5.5	(11)	2
13½	9.5	(19)	2	9.5	(19)	2
14½	11.5	(23)	2	7.5	(15)	2
15½	9.5	(19)	2	9	(18)	2
16½	(8)	(8)	1	6.5	(26)	4
17½	9.1	(55)	6	10.0	(50)	5
18½	10	(10)	1	7.2	(22)	3
Mean	9.3		23	8.2		29
s.e.	±0.40			±0.45		

L = Litter size (n) = number of animals N = number of litters

---

Post-natal litter sizes (data from Bulfield and Kacser, 1974).

Post-natal age	+/his Mother	N	his/his Mother	N
Birth	9.3	15	6.1	15
3 weeks	8.7		4.7	
7 weeks	8.1		4.1	

of those animals, however, showed no apparent difference from the brain of the embryos of +/+ mothers. (Figs 29 and 29a).

In normal embryos, the cerebellum had shown signs of lobulation and the choroid plexus had increased in size and number of convolutions. This kind of normal development of the cerebellum and the choroid plexus was also found in those animals which had abnormal inner ears. The 4th ventricle appeared to be the same in normal and abnormal animals.

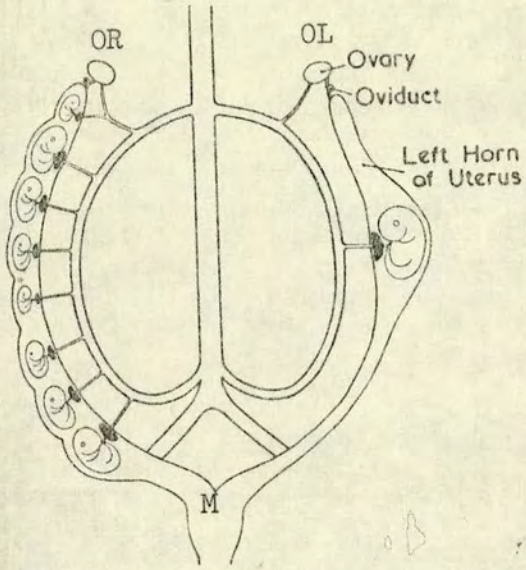
The diocoele and mesocoele, which were found to be narrowed in $11\frac{1}{2}$ day abnormal embryos, were found to be the same in the embryos with abnormal inner ears at this stage. Apart from the inner ear there seemed to be no apparent difference in the brain of embryos from his/his mothers and animals from +/+ mothers.

Litter size

Some of the observed embryonic abnormalities may be thought to have deleterious effects on the chance of survival in utero. From Tables 4 and 5 we can assess this effect by looking at the litter sizes at various ages. Table 6 gives this analysis.

There is greater heterogeneity in litters from his/his mothers than from controls and the difference of 8.2 compared to 9.3 is significant. Looking at any possible change in litter size with days of gestation, there does not appear to be a systematic drift to lower values as gestation proceeds. Such change would indicate progressive deaths and resorption of affected embryos. (No resorption sites (moles) were scored). The numbers are small but any severe intrauterine lethality would have been detected.

TABLE 7 Uterine Position and Head Abnormality



Diagrammatic representation of uterus with embryos in horns. (Slightly modified from McLaren and Michie (1960)).

N = normal Ab = abnormal D = dead

Litter No.	Days of gestation	OR ——— M ——— OL
3	9½	N N N Ab N Ab
7	10½	N Ab N N N Ab N
8	10½	N Ab N N N N D Ab N N
14	11½	N N Ab Ab N N Ab N Ab N
15	11½	Ab Ab N N N N
16	11½	N N Ab N Ab N

Uterine position

Uterine position in relation to growth and malformations has often been considered (see e.g. Brent and Jensh, 1967). The opportunity was taken to note the uterine positions of embryos when whole litters were dissected. The results are shown in Table 7. Only the early litters ($9\frac{1}{2}$ - $11\frac{1}{2}$ days) were so examined and therefore only head abnormalities were scored. It should be noted that only the relative positions of the embryos are recorded and not their distance from the ovaries or the cervical opening. It appears from these few observations that there is no relation between observed abnormality and position or crowding.

Discussion

The earliest difference that could be seen between animals from his/his and +/+ mothers occurred in the neural tube and the hind brain as early as $9\frac{1}{2}$ days, and later, at $10\frac{1}{2}$ and $11\frac{1}{2}$ days, there were differences in the shape of the head and the hind brain which were obvious. At $12\frac{1}{2}$ days these differences seemed to disappear. The question arises here, whether these animals with the morphologically abnormal shape of the head would be the animals, at later stage, with inner ear defects and, if they had been born and survived, would have shown balance defect. In the early stages such as $9\frac{1}{2}$, $10\frac{1}{2}$ and $11\frac{1}{2}$ days where there was a morphological defect in the neural tube or hind brain, the otic vesicles showed no difference from the controls which were taken from +/+ mothers possibly because there is little morphological differentiation. At $11\frac{1}{2}$ days, the animals which had narrowed hind brain, both externally and in sections, had otic vesicles and endolymphatic ducts indistinguishable from those taken from +/+ mothers. At $13\frac{1}{2}$ days unfortunately only part of the brain was included in the sections. This was the hind

brain (cerebellum), choroid plexus and 4th ventricle. There were no apparent differences found in the abnormal embryos compared with controls. It was difficult to see very slight differences as the development of the brain becomes very complicated from 13½ days onwards. As in the 17½ day embryos, where the whole litters from his/his mothers showed inner ear abnormalities, there was no apparent difference in the brain from embryos of +/+ mothers.

The embryos at 9½, 10½, 11½ days when they were dissected out of their membrane in Ringer's solution, tend to shoot out, and as all the tissues and membranes were very soft they usually recoil. One might argue that, the open neural tube and hind brain which were found at the 9½ - 11½ days were caused by such mechanical pressures. But in the present case, this was unlikely, even though the embryos were dissected out from their membrane in the unfixed condition. The narrowed hind brains were more likely to be a developmental abnormality rather than a mechanical artefact (Fig. 23 - 26). The animals with the narrowed hind brain were observed from a different angle and the thin membrane which is the roof of the rhombencephalon was still intact. Moreover the sections of these animals showed forebrain, midbrain, and hind brain distorted and in some hind brains, the walls of myelencephalon were malformed and there were excessive amounts of tissue present in the myelocoel. All externally visible malformed animals when sectioned show histological abnormalities as well. Nevertheless the possibility must be taken seriously that we were observing dissection artefacts (squashing, collapse) of the soft tissues in these early embryos.

This study left an open question, whether the inner ear defect was due to abnormalities of the hind brain, which was seen up to 11½ day embryos, or whether the animals with narrowed hind brain and abnormal shape of the head would have aborted at later stages. However, at the 11½ day embryonic stage, where the abnormality of the brain was still seen, the abnormal embryos were found to be alive. The heart beating was noted when the animals were observed in Ringer's solution under dissecting binocular microscope.

The results of the litter size survey indicated that there was no obvious lethality during the period 9½ to 18½ days. The morphologically abnormal animals of 9½ - 11½ days constituted about 35% (22 out of 75 total) with a litter size of 8.8. If all of these had died, the litter size at later stages should approach 5.7. From Table 6 it is obvious that this is not the case. This strengthens the 'damage-during-dissection' hypothesis.

The reduced litter size is apparent right from 9½ days and therefore any lethality must have occurred before that. The data are reasonably consistent with the post-natal litter sizes reported earlier by Bulfield and Kacser (1974) given for comparison in Table 6.

To sum up, it is entirely open to question whether there are changes in the neural tube and hind brain which precede the development of the otic vesicle. It is obviously very important that a full study of this problem will have to be undertaken.

SECTION IV

THE PENETRANCE OF THE DEFECT

The less than 100% penetrance of the balance defect in animals born of his/his mothers posed considerable problems in the work so far reported, particularly in the analysis of the effect in embryos. The correlation of any embryonic morphological lesion with the eventual phenotype, scored at 3 weeks plus, had to be largely a matter of conjecture. The variability from litter to litter (see Table 15 and 16) increased the problem of interpretation of the histological evidence. Whatever the relationship between behavioural and histological phenotypes, the last sections demonstrated that considerable variation in expression existed.

An important observation was that the penetrance had declined from around 80% when the stock was obtained from Cambridge in 1971 to about 37% in 1974 and now (1977) stands at about 7%. The high penetrance had been maintained in Cambridge. Since biochemical tests (of histidine) were not carried out there but crosses were made with the intention of retaining the balance defect, it was possible that the strong selection for balance defective character over several years in Cambridge may have accumulated modifying genes increasing the penetrance of the maternal effect on balance.

Conversely, at Edinburgh crosses were monitored mainly by testing biochemically for histidinaemia so that any selection for maternal effects would have been relaxed.

It was already known from early work at Cambridge that outcrossing severely reduced the incidence of balance defects.

Table 3 Effect of a 2% histidine-suplemented diet, normal diet and low histidine diet, during the second week of pregnancy on the offspring of homozygous (*his*/*his*) mothers

Diet	No. of litters	No. of animals	Effect on balance Severe	Mild	Normal	% Affected	χ^2	*P*
(−)2% Histidine	23	79	12	10	57	27.8	1.15	ns
Normal	22	94	11	13	70	25.5	—	—
Low histidine	20	65	0	2	63	3.1	25.2	< 0.001

Genetic background of the animals, diet and scoring of the balance defect were as for Tables 1 and 2. The number of offspring with their balance severely affected, mildly affected and unaffected were tested by χ^2 test for both the 2% histidine-supplemented diet and the low histidine diet against the expected numbers on the normal diet.
ns, not significant

Table 3 from Kacser et al. (1977)

Table 5 Effect of genetic background on the proportion of balance defective offspring produced by histidinaemic parents

Genetic background	No. of litters	No. of animals	Affect on balance severe	No. of mild	normal	% Affected	χ^2	*P*	Affect on hearing severe	No. of mild	normal	% Affected	χ^2	*P*
Peru	12	57	16	5	36	36.8	—	—	18	5	34	40.4	—	—
Hybrid Peru/C57BL	12	51	5	3	43	15.7	11.3	< 0.02	8	1	42	17.6	12.4	< 0.01

Some *his*/*his* mice from the Peru stock were crossed to C57BL and the offspring were intercrossed to produce *his*/*his* animals which were used as hybrid (50% Peru/50% C57BL) parents. The animals were scored for balance as in Table 1 and tested by χ^2 as in Table 3.

Table 5 from Kacser et al. (1977)

"Before the presence of the his allele was known, selection for BLA established a colony with 80% incidence of this condition. The defect disappeared on outcrossing to AG/Cam and had a very low incidence in the first few backcrosses to the BLA stock. There was also variation in incidence between litters from the same mating. It was concluded from this that the balance defective character was controlled by an imperfectly penetrating recessive gene under non-genetic as well as polygenic control",(Kacser, et al. 1973). Similarly in Edinburgh outcrosses to C57 resulted in a reduction of the penetrance (Table 5 of Kacser et al. 1977, see facing page). The histidine levels of the stocks showing different penetrance levels were not significantly different. There was, therefore, good evidence that the genetic background (in the presence of the his/his alleles) could influence in some way the eventual outcome of any intrauterine exposure.

Two general hypotheses to account for these phenomena are possible, one 'biochemical', the other 'developmental'. Both have experimentally testable consequences and both were investigated and reported in this section.

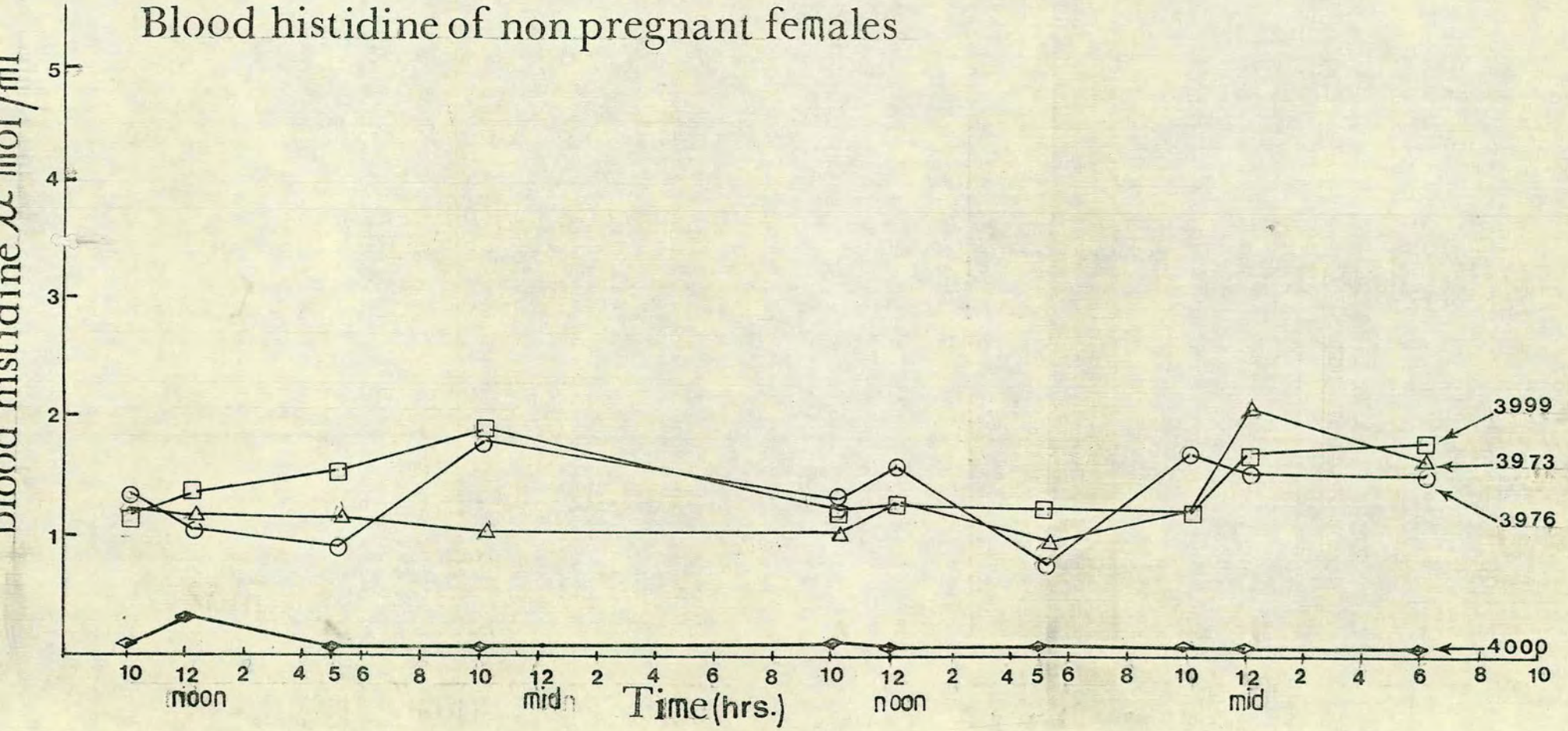
(1) The biochemical hypothesis. "The levels of histidine and its derivatives are near a threshold value which determines whether the effect on the vestibule will result in such malformation that behaviour is affected. Environmental fluctuations (e.g. diet) or differences in intrauterine position and exposure could account for the within and between litter variation. Differences in genetic background could alter the threshold slightly or alter the average level of the endogenous teratogen".

TABLE 8

BLOOD TAKEN AT DIFFERENT TIMES FOR 3 DAYS.
NON-PREGNANT, NON-INJECTED FEMALES.

Day	Time of Bleeding	Histidine, µmol/ml			
		3999 (his/his)	3973 (his/his)	3976 (his/his)	4000 (+/his)
1	10.00am	1.21	1.28	1.37	0.07
"	12.00noon	1.38	1.21	1.09	0.32
"	5.00pm.	1.56	1.21	0.94	0.07
"	10.00pm.	1.93	1.06	1.81	0.07
2	10.00am	1.23	1.05	1.34	0.11
"	12.00noon	1.25	1.30	1.62	0.07
"	5.00pm.	1.25	0.95	0.70	0.07
"	10.00pm.	1.17	1.19	1.71	-
"	12.00pm.	1.72	2.07	1.57	0.09
3	6.00am.	1.79	1.69	1.53	-

Fig. 30


Blood histidine of non pregnant females
Blood histidine µ mol /ml
5
4
3
2
1
3999
3973
3976
4000
10 12 2 4 5 6 8 10 12 2 4 6 8 10 12 2 4 5 6 8 10 12 2 4 6 8 10
noon
mid
Time (hrs.)
noon
mid

A substantial increase in histidine levels should, on this hypothesis, produce 100% penetrance. Preliminary experiments (Kacser, et al. 1977) had suggested that such treatment did not result in any increase. The method was a 2% histidine supplement to the diet. (see previous page), Table 3 of Kacser et al., 1977. It was, however, desirable to confirm this by more controlled experiments and in addition obtain information on the early histological consequences of such treatment. In the following section the results of this will be presented.

Results

Preliminary Injection Experiments

The early dietary experiments (Dunker, 1973) showed that it took 2 - 3 days after the switch to supplemented diets before the effect was noticeable in urine or blood. This may have been caused by temporary reduction in food intake consequent on the change to the new (less palatable?) diet. It was therefore decided to replace dietary supplements by intraperitoneal injections. From the diet results and the consumptions of about 5 gm/day of food, it was calculated that the equivalent would be 1 ml of a 5% histidine HCl solution per day. This was given in two equal doses of 0.5 ml.

It was first necessary to establish the diurnal variation within and between experimental animals. Table 8 and Fig. 30 show the results. There is no significant variation between animals. There is, however, a distinct rise for the values taken at night and early morning, 12.00 pm (midnight) and 6 am, consistent with the nocturnal feeding habits and the fact that the only source of histidine is dietary.

Non-pregnant female mice both 8 weeks old (3930) and (3931) were used for injection experiments. One (3931) was injected with 0.5 ml of 5% histidine HCl in isotonic saline at 10 am and 4 pm for

TABLE 9

BLOOD TAKEN AT 10.00AM. and 4.00PM. EACH DAY FOR 9 DAYS NON-PREGNANT his/his ♀♀. UNINJECTED CONTROL AND INJECTED ♀ AT TIMES STATED. EACH INJECTION: 0.5 ml of 5% HIS.

Day	Time of Bleeding	3930 Histidine µmol/ml	3931 - Histidine µmol/ml	Time of Injection
1	10.10am.	1.02	0.98	-
"	4.00pm.	1.28	1.42	-
2	10.00am.	1.16	1.19	-
"	4.00pm.	1.41	1.39	-
3	10.10am.	1.24	1.50	10.10am.
"	4.00pm.	1.25	3.30	4.10pm.
4	10.00am.	1.00	1.20	10.10am.
"	4.00pm.	1.49	4.35	4.10pm.
5	10.10am.	1.12	1.12	10.10am.
"	4.00pm.	1.64	3.17	4.10pm.
6	10.10am.	1.21	1.56	10.10am.
"	4.00pm.	1.58	3.77	4.10pm.
7	10.00am.	1.45	1.45	10.10am.
"	4.00pm.	1.11	3.85	4.10pm.
8	10.00am.	1.05	1.17	10.10am.
"	4.00pm.	1.34	2.82	4.10pm.
9	10.00am.	1.15	1.24	-
"	4.00pm.	1.37	-	-

Fig. 31

Blood histidine of non pregnant injected and non-injected females for 9 days. 10a.m. and 4p.m regime.

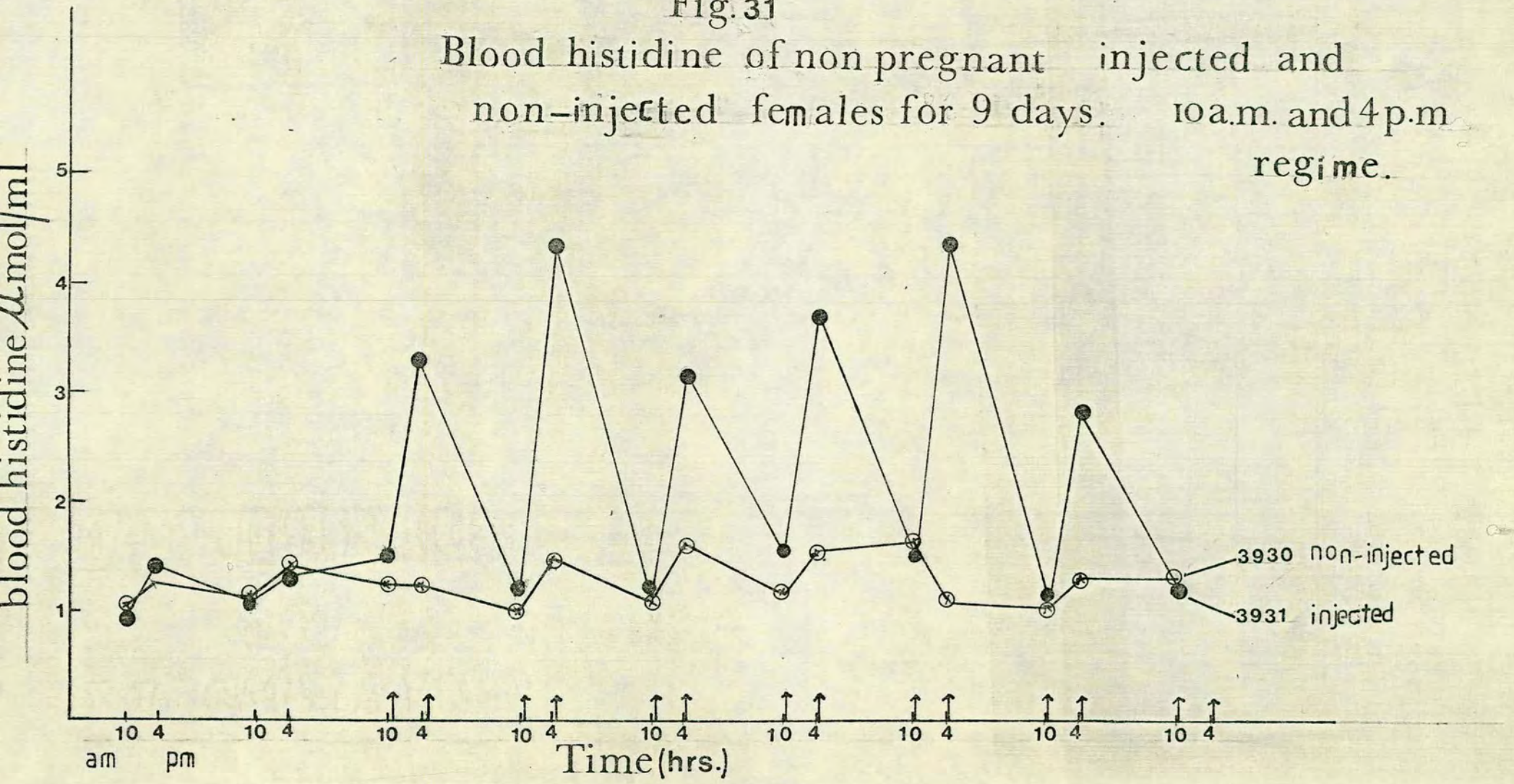


TABLE 10

NON-PREGNANT FEMALES INJECTED WITH 0.5ML OF 5% HISTIDINE AT 10.00AM. AND 4.00PM.

Day	Time of Bleeding	Histidine µmol/ml		Time of Injection
		3950 (his/his)	3956 (his/his)	
1	10.00am	1.29	1.17	10.10am
"	10.30am	4.19	5.69	-
"	11.00am	7.97	4.60	-
"	2.00pm	5.65	4.17	-
"	4.00pm	3.11	3.56	4.10pm
"	4.30pm	6.66	9.19	-
"	5.00pm	5.11	5.44	-
"	6.00pm	4.16	4.85	-
2	10.00am	2.47	1.96	-

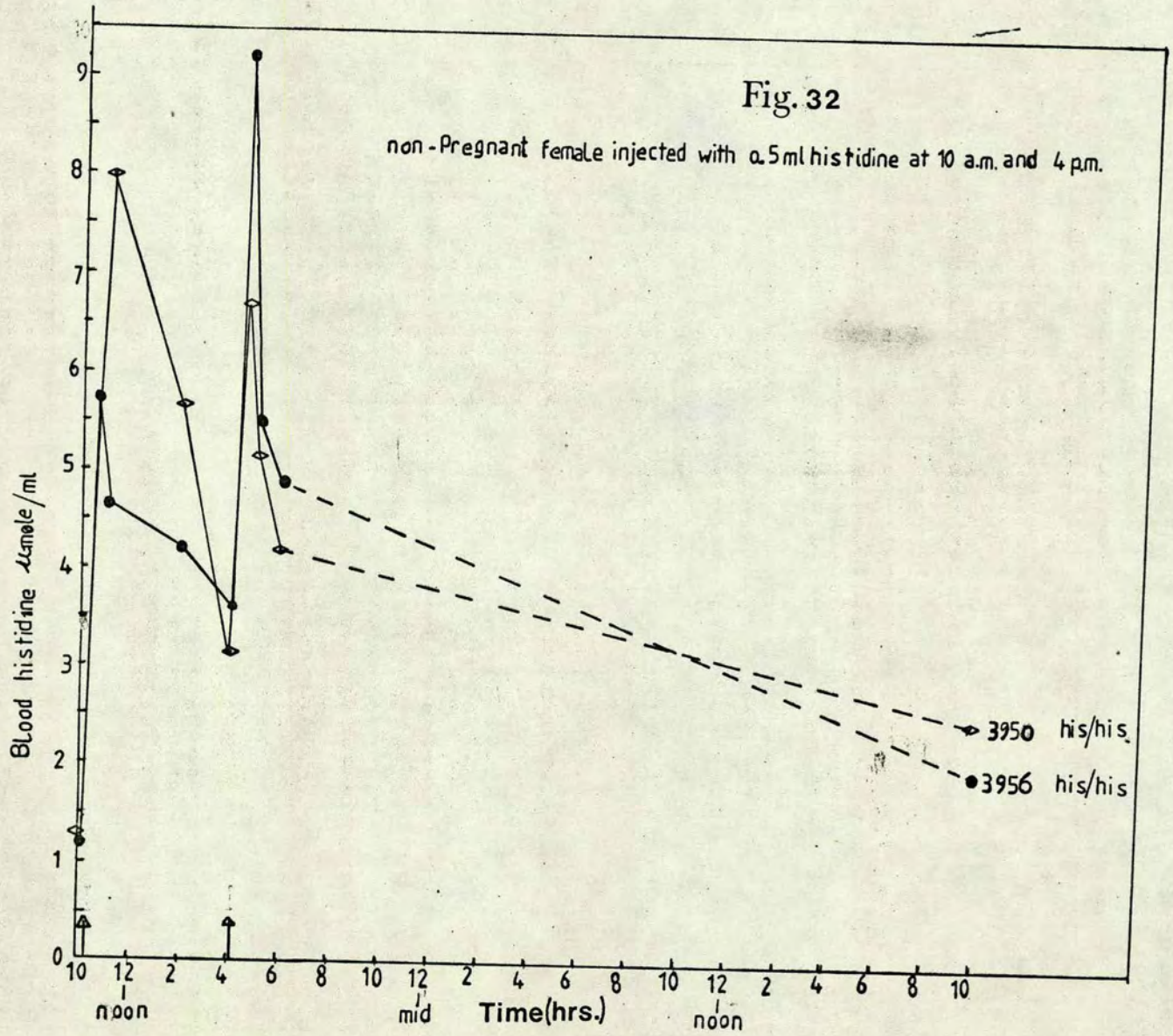


Fig. 32
non-Pregnant female injected with 0.5 ml histidine at 10 a.m. and 4 p.m.
Blood histidine μmole/ml
0
1
2
3
4
5
6
7
8
9
10 12 2 4 6 8 10 12 2 4 6 8 10 12 2 4 6 8 10
noon
mid
noon
Time(hrs.)
3950 his/his
3956 his/his

TABLE 11

PREGNANT FEMALES INJECTED WITH 0.5ML OF 5% HISTIDINE AT 10.00AM AND 4.00PM

Day	Day of Gestation	Time of Bleeding	Histidine µmole/ml		Time of Injection
			3931 (his/his)	3950 (his/his)	
1		10.00am	1.25	1.37	-
"		4.00pm	1.23	1.53	-
2		10.00am	1.46	1.64	-
"		4.00pm	1.22	1.70	-
3	7th	10.00am	1.21	1.46	10.10am
"		4.00pm	3.25	2.98	4.10pm
4	8th	-	-	-	10.10am
"		-	-	-	4.10pm
5	9th	-	-	-	10.10am
"		-	-	-	4.10pm
6	10th	10.00am	1.89	1.45	10.10am
"		4.00pm	3.61	1.41	4.10pm
7	11th	-	-	-	10.10am
"		-	-	-	4.10pm
"		10.00pm	4.28	-	-
8	12th	-	-	-	10.10am
"		-	-	-	4.10pm
"		10.00pm	-	3.28	-
"	13th	-	-	-	10.10am
"		-	-	-	4.10pm
10	14th	10.00am	1.80	1.74	10.10am
"		-	-	-	4.10pm
11	15th	-	-	-	10.10am
"		4.00pm	2.71	3.01	4.10pm
"		10.00pm	-	3.17	-
12	16th	10.00am	1.26	-	-
"		4.00pm	-	1.85	-
	21st			Aborted (no litter)	

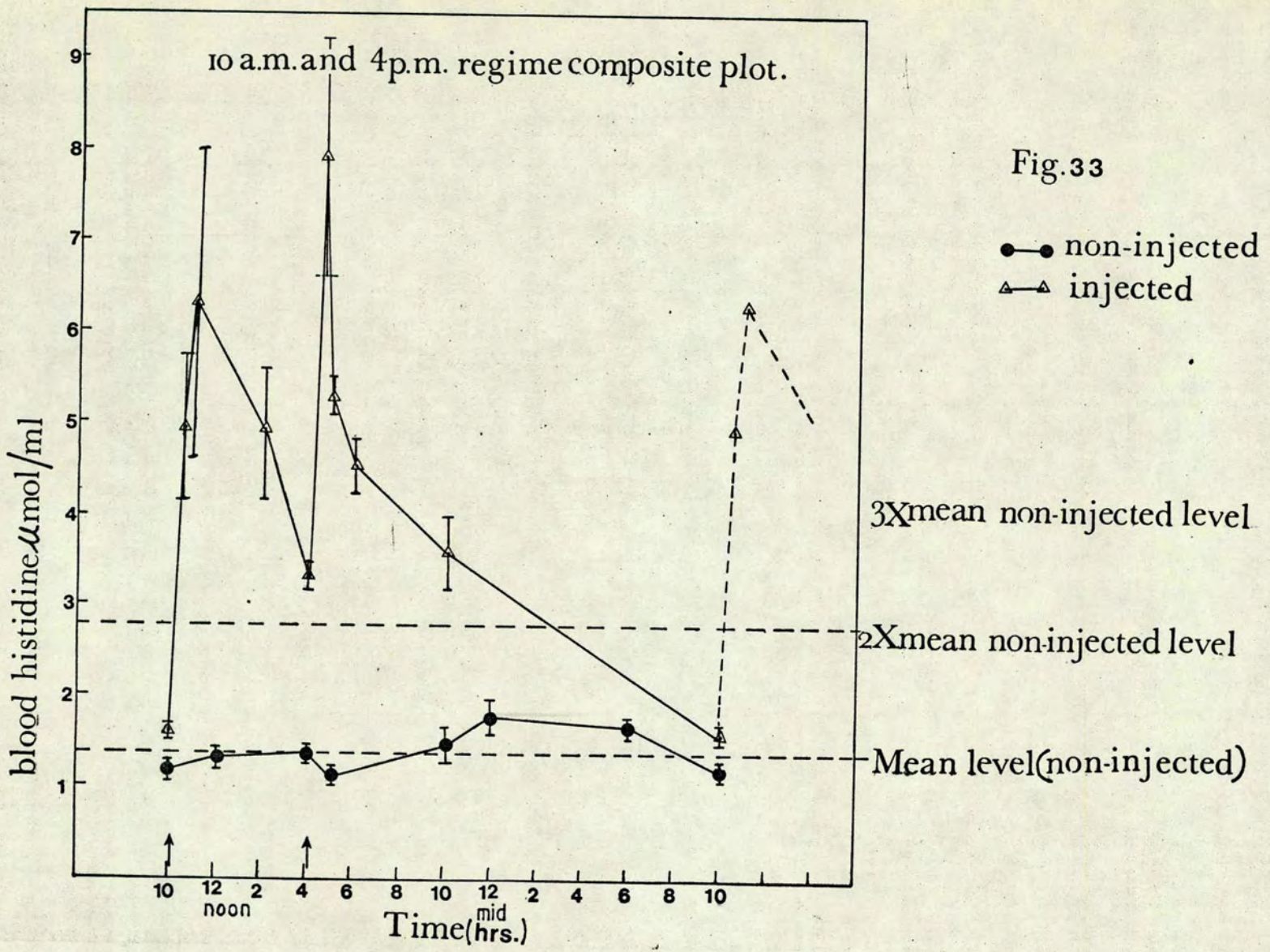


Fig.33

●—● non-injected

△—△ injected

six days. 3931 was bled before each injection and 3930 was bled at the same time as 3931. This experiment was set up to investigate how high the histidine in blood levels went up and how long they stay at that level and whether the animals survive (Table 9 , Fig. 31).

From the above experiment it was found, as predicted, that 0.5 ml of 5% histidine injection elevated the blood histidine level up to two to three fold six hours after injection. But the histidine level did not remain constant during the period of treatment. After 18 hours the histidine level in the blood declined to the values found in the non-injected histidinaemics.

In order to get a more detailed histidine profile for this injection regime two further animals (3950) and (3956) were used taking frequent blood samples during a 24 hr. period (Table 10, Fig. 32). It is seen that the levels rise very rapidly in the first hour after injection but decline at about the same rate. This injection regime would therefore expose the embryos, via the maternal blood, to something like 4 to 8 fold levels for about half of every 24 hour period (Fig. 32).

A 'composite plot' (Fig. 33 using the data from Tables 9 and 10 and 4) gives an idea of the exposure profile.

Injection of pregnant females

A further experiment was set up using pregnant females. Matings were set up with 4 his/his female mice and plugs observed to determine start of pregnancy. Four or five blood samples were taken from each mouse before the experiment started. Injections were given every day at 10 am and 4 pm starting from $7\frac{1}{2}$ days of pregnancy until $15\frac{1}{2}$ days of pregnancy. Blood samples were not taken every day (Table 11). One animal died during the course of the experiment (3951).

TABLE 12

EFFECT OF HISTIDINE INJECTION DURING THE SECOND WEEK OF PREGNANCY

a) Inner ear sections of exposed embryos at 17½ days and day of birth. All matings his/his ♀ and his/his ♂.

	LEFT					RIGHT				
	Utriculus		Sacculus		Cochlea	Utriculus		Sacculus		Cochlea
No.	Otoliths	Lumen	Otoliths	Lumen		Otoliths	Lumen	Otoliths	Lumen	
Embryos from 3931 at 17½ days (10.00am. & 4.00pm. regime)										
1.	Big	+	Big	+	+	Big	+	Big	+	+
2.	Big	+	+	+	+	Big	+	+	+	+
3.	Big	+	+	+	+	Big	+	+	+	+
4.	-	+	+	+	+	Big	+	+	+	+
5.	-	+	+	+	+	Big	+	+	+	+
6.	-	+	+	+	+	Big	+	+	+	+
7.	Big	+	+	+	+	Big	+	+	+	+
8.	Big	+	+	+	+	-	+	Big	+	+
9.	-	+	Big	+	+	-	+	Big	+	+
Embryos from 3932 at 17½ days (10.00am. & 10.00pm. regime)										
1.	+	+	+	+	+	Big	+	+	+	+
2.	+	+	+	+	+	Big	+	+	+	+
3.	Big	+	+	+	+	Big	+	Big	+	+
4.	Big	+	+	+	+	Big	+	+	+	+
5.	-	+	-	+	-	Big	+	+	+	+
6.	Big	+	+	+	+	-	+	+	+	+
7.	Big	+	Big	+	+	Big	+	+	+	+
8.	Big	+	Big	+	+	Big	+	Big	+	+
Litter from 3975 at day of birth (10.00am. & 10.00pm. regime)										
1.	Big	+	+	+	+	Big	+	+	+	+
2.	Big	+	+	+	+	Big	+	+	+	+
3.	Big	+	Big	+	+	Big	+	+	+	+
4.	Big	+	+	+	+	Damaged				
5.	Big	+	+	+	+	Big	+	+	+	+
6.	Big	+	+	+	+	Damaged				
7.	+	+	+	+	+	Big	+	+	+	+
Litter from 3973 at day of birth (10.00am & 10.00pm. regime)										
1.	Big	+	+	+	+	Damaged				
2.	Big	+	+	+	+	Damaged				
3.	Big	+	+	+	+	Big	+	+	+	+
4.	Big	+	+	+	+	-	+	-	-	-

TABLE 12

EFFECT OF HISTIDINE INJECTION DURING THE SECOND WEEK OF PREGNANCY

b) Inner ear sections of non-injected controls.
All ♀♀ his/his. Some ♂♂ +/+.

	LEFT					RIGHT				
	Utriculus		Sacculus		Cochlea	Utriculus		Sacculus		Cochlea
No	Otoliths	Lumen	Otoliths	Lumen		Otoliths	Lumen	Otoliths	Lumen	
Embryos from 3955 at 17½ days										
1.	-	+	-	+	-	-	+	-	+	-
2.	-	+	-	+	+	-	+	-	+	-
3.	-	+	-	+	+	Big	+	Big	+	+
4.	-	+	-	+	-	-	+	-	+	-
5.	Big	+	Big	+	+	-	+	-	+	-
6.	Big	+	+	+	+	Big	+	+	+	+
7.	-	+	-	+	-	Big	+	Big	+	+
8.	Big	+	Big	+	+	Big	+	Big	+	+
Embryos from 93 (++ ♂) at 17½ days										
1.	Big	+	Big	+	+	Damaged				
2.	-	-	-	-	-	+	+	+	+	+
3.	+	+	+	+	+	+	+	+	+	+
4.	+	+	+	+	+	+	+	+	+	+
5.	+	+	+	+	+	+	+	+	+	+
6.	Big	+	+	+	+	Big	+	+	+	+
7.	+	+	+	+	+	+	+	+	+	+
Embryos from 96 (++ ♂) at 17½ days										
1.	Big	+	+	+	+	Big	+	+	+	+
2.	-	-	-	-	-	-	+	-	-	-
3.	Big	+	Big	+	+	Big	+	Big	+	+
4.	Big	+	+	+	+	-	+	Big	+	+
5.	-	+	Big	+	-	-	-	-	-	-
6.	-	+	+	+	+	Big	+	Big	+	+
7.	+	+	+	+	+	+	+	+	+	+
8.	Not Sectioned									
9.	Not Sectioned									
Litter from 3949 Day of Birth										
1.	Big	+	+	+	+	Big	+	Big	+	+
2.	Big	+	+	+	+	Big	+	Big	+	+
3.	Big	+	+	+	+	Big	+	+	+	+

Fig.34

Blood histidine of pregnant injected females. 10 a.m and 4 p.m. regime.

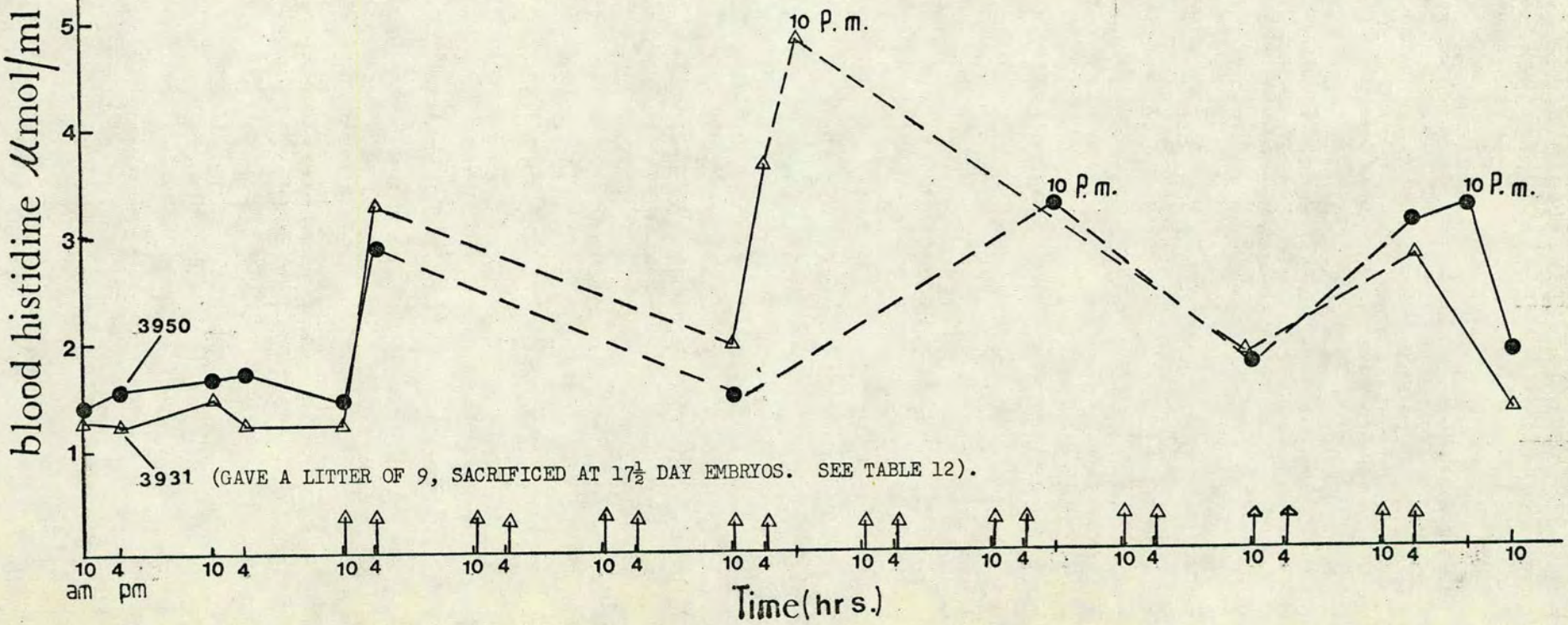


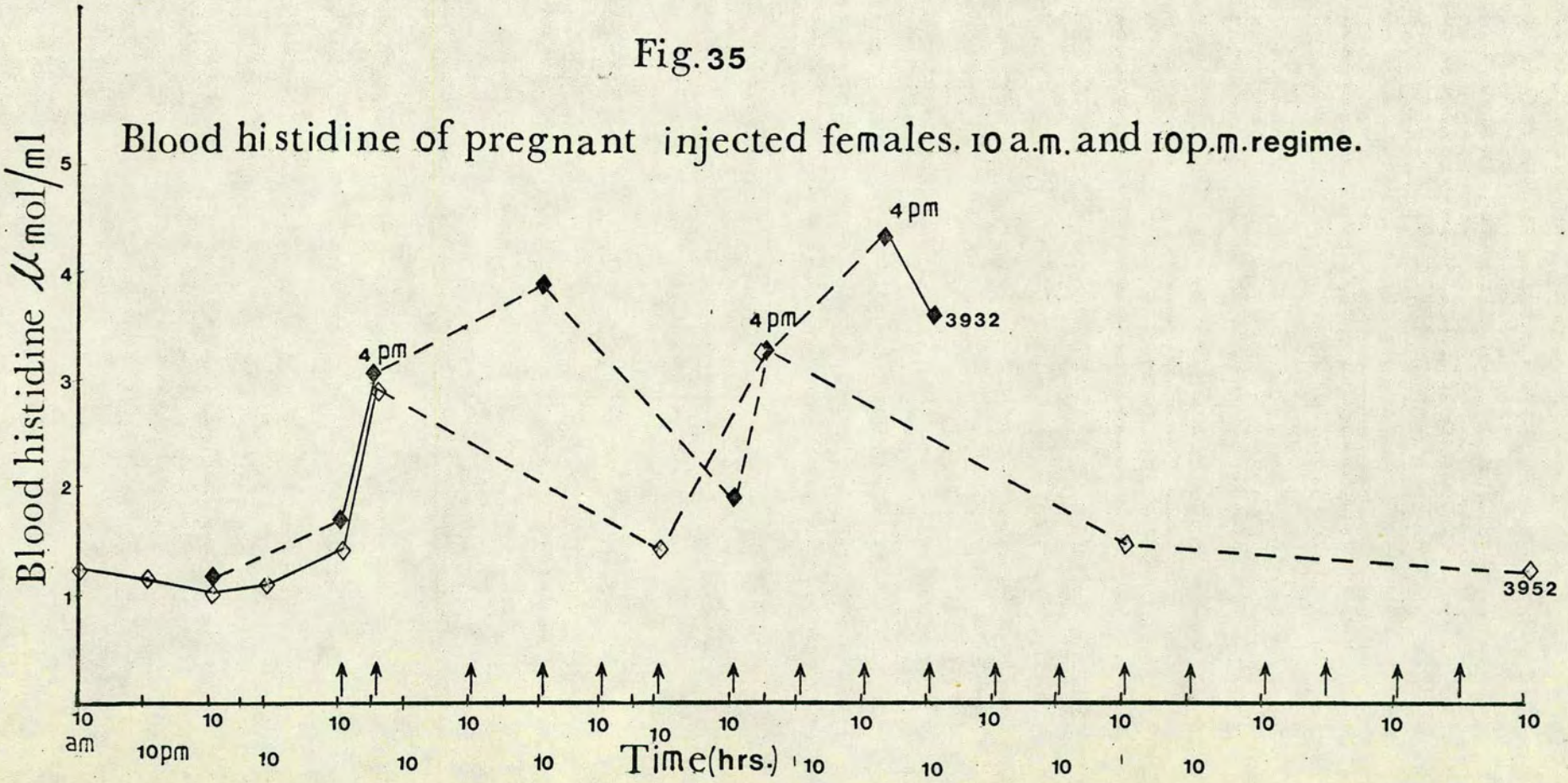
TABLE 13

PREGNANT FEMALES INJECTED WITH 0.5ML OF 5% HISTIDINE AT 10.00AM AND 10.00PM DURING THE SECOND WEEK OF PREGNANCY

Day	Day of Gestation	Time of Bleeding	Histidine µmol/ml		Time of Injection
			3952 (his/his)	3932 (his/his)	
1		10.10am	1.25	-	-
"		10.00pm	1.17	-	-
2		10.00am	1.02	1.17	-
"		10.00pm	1.13	-	-
3	7th	10.00am	1.48	1.73	10.10am
"		4.00pm	2.95	3.09	4.10pm
4	8th	-	-	-	10.10am
"		10.00pm.	-	3.88	10.10pm
5	9th	-	-	-	10.10am
"		10.00pm	1.45	-	10.10pm
6	10th	10.00am	-	1.89	10.10am
"		4.00pm	3.30	3.26	-
"		-	-	-	10.10pm
7	11th	-	-	-	10.10am
"		4.00pm	-	4.36	-
"		10.00pm	-	3.56	10.10pm
8	12th	-	-	-	10.10am
"		-	-	-	10.10pm
9	13th	10.00am	1.50	-	10.10am
"		-	-	-	10.10pm
10	14th	-	-	-	10.10am
"		-	-	-	10.10pm
11	15th	-	-	-	10.10am
"		-	-	-	10.10pm
12	16th	10.10am	1.21	-	-
"		-	-	-	-
13	17th	-	-	-	-
	21st		Aborted	Aborted	

Fig. 35

Blood histidine of pregnant injected females. 10 a.m. and 10p.m. regime.



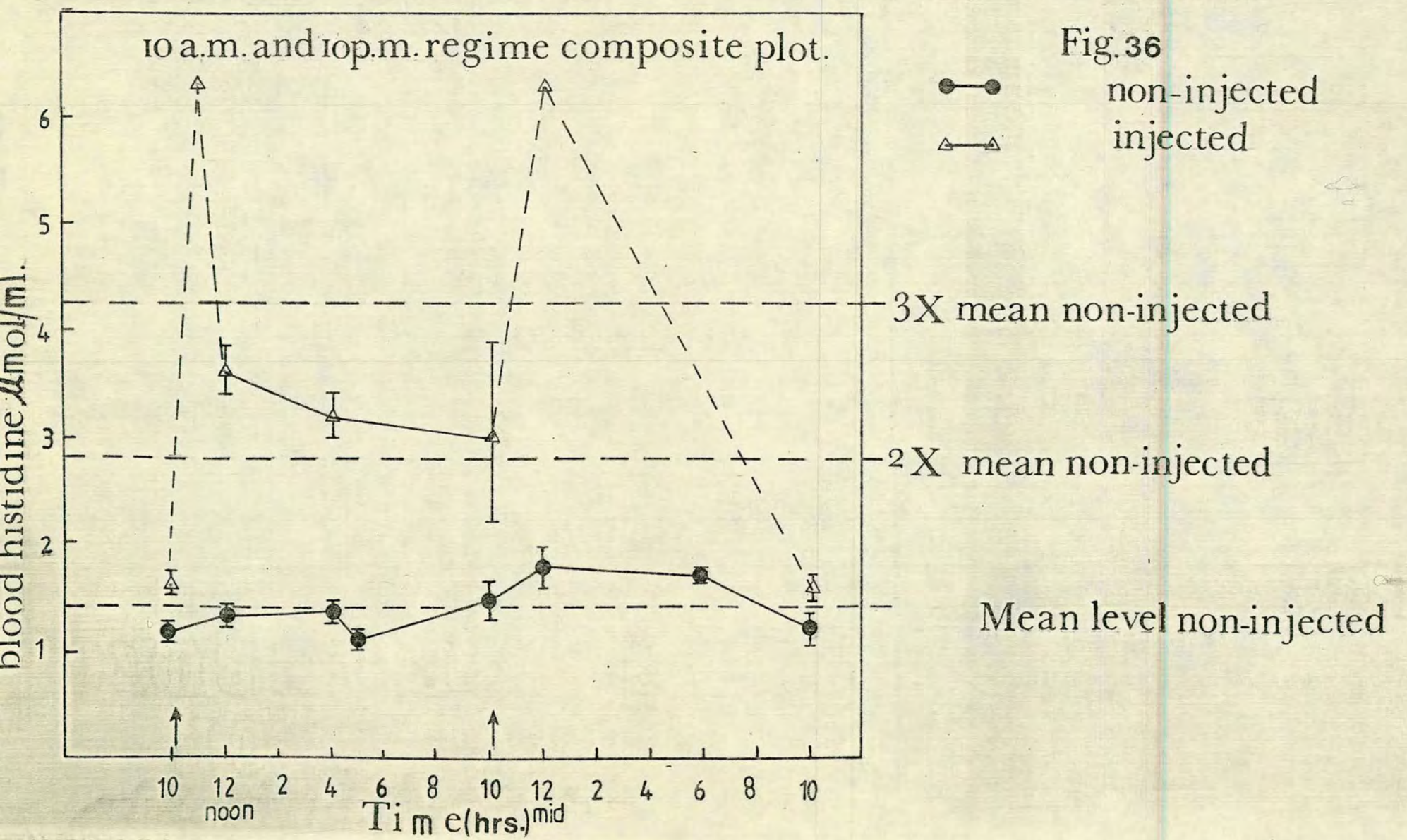


Fig. 36

non-injected

injected

TABLE 14

PREGNANT FEMALES INJECTED WITH 0.5ML OF 5% HISTIDINE AT 10.00AM AND 10.00PM

Day of Gestation	Day	Time of Bleeding	Histidine µmol/ml			Time of Injection
			3932 (his/his)	3973 (his/his)	3975 (his/his)	
	1	10.00am	-	1.37	-	-
7th	2	10.00am	1.91	1.10	1.46	10.10am
	"	12.00noon	-	3.91	-	-
	"	-	-	-	-	10.10pm
8th	3	10.00am	1.50	-	1.96	10.10am
	"	-	-	-	-	10.10pm
9th	4	10.00am	-	1.34	-	10.10am
	"	12.00noon	3.65	-	3.40	-
	"	4.00pm	-	3.29	2.55	10.10pm
10th	5	-	-	-	-	10.10am
	"	12.00noon	-	4.08	-	-
	"	4.00pm	3.89	-	-	10.10pm
11th	6	10.00am	2.17	-	2.03	10.10am
	"	-	-	-	-	10.10pm
12th	7	-	-	-	-	10.10am
	"	4.00pm	2.64	-	2.08	10.10pm
13th	8	-	-	-	-	10.10am
	"	-	-	-	-	10.10pm
14th	9	-	-	-	-	10.10am
	"	-	-	-	-	10.10pm
15th	10	10.00am	-	-	1.54	10.10am.
	"	12.00noon	3.57	-	2.98	
	"	-	-	-	-	10.00pm
16th	11	10.00am	1.34	1.42	-	-
17th	12	10.00am	1.10	-	-	-

One animal was not injected (3953). The injected animals were 3931 and 3950. The histidine levels during the course of injection of these animals are shown in Table 11 and Fig. 34. Only one animal (3931) appeared to be pregnant, 3950 and 3953 having aborted. The animal was killed at 17½ days of pregnancy, 2 days after termination of the injection. The embryos were embedded and sectioned as described in Materials and Methods. None out of 9 embryos of No. 3931 that were sectioned and examined was abnormal in both left and right ears. Four of them had no otoliths in the left ear and two had none in the right ear (Table 12a). Apart from the big crystals and absence of otoliths in some of the utriculi, the rest of the inner ear seemed to be normal. There was no distension of the utriculus, sacculus or cochlea. These animals did not appear to differ significantly from non-injected ones (Table 12a).

In view of the decline of histidine levels over 24 hours, a different regime was started for the next experiments. The 4 pm injection time was changed to 10 pm i.e. injected twice at 10 am and 10 pm. Animals 3932 and 3952 were injected at 10 am and 4 pm once and then injected at 10 am and 10 pm for eight days (Table 13 and Fig. 35).

A 'composite plot' of the 10 am and 10 pm regime is shown in Fig. 36 using the data from Tables 13 and 14. It can be seen that the peak levels are lower than in the previous regime but the mean is maintained at about 2 times the base level for longer periods.

The third experiment used 8 plugged animals, 3 of which were controls. 5 were injected at 10 am and 10 pm daily from 7½ days until 15½ days of gestation. 3 females out of 5 were later found to be pregnant and their histidine values are shown in Table 14. The

embryos from one of the females (3932) was examined at 17½ days and two litters were looked at on the day of birth. Apart from big crystals in the utriculus and sacculus none of the 17½ or newborn had both left and right inner ears abnormal. One 17½ day embryo had no otoliths in both utriculus and sacculus and the sacculus and cochlea were found to be distended, but the right ear, apart from big crystals in the utriculus, was normal (Table 12a).

Out of 3 females that were used as controls, the embryos from one were examined at 17½ days gestation and from one as newborn. One had aborted. Out of 8, 17½ day embryos from 3955, which was a control animal, 4 embryos had both left and right ear with no otoliths in utriculus and sacculus and the cochlea was distended. One foetus had the left ear abnormal but the right normal and one had the left ear normal and the right abnormal. All 3 newborn of control animal No. 93 had both left and right utriculus with big crystals. Apart from that there was no distension of the utriculus, sacculus or cochlea.

Discussion

The results of the blood sampling for both injection regimes showed that a substantial increased level of histidine was maintained over significant periods of each day. Any threshold effects of histidine concentrations should therefore be well exceeded.

A comparison of the ears of whole litters of such exposed embryos with non-injected controls showed no significant difference. This is in agreement with the previous findings, on dietary supplements, where the penetrance values of balance affected offspring were the same as for animals on normal diet. (Table 12a and b).

The conclusions from the diet experiments (Kacser et al. 1977) are therefore reinforced by these more rigorous and fuller data.

"The histidine in untreated his/his mothers therefore, must lie above a 'saturation level' beyond which histidine levels are no longer relevant in determining the proportion of affected offspring. This conclusion is reinforced by the observations, previously described (Bulfield and Kacser, 1974) that in any one litter there may be affected and unaffected offspring although all will have been exposed to the same maternal metabolites. In these circumstances other factors must be involved in the variation within and between litters. The low histidine diet, however, decreased the proportion of balance-defective offspring from 26% to 3%. In the lower range maternal histidine levels are therefore critical in determining the frequency and severity of balance-defective offspring".

The results presented here show that the effects of maternal histidinaemia are determined in early embryogenesis and that behaviourally normal offspring may have subclinical lesions as seen in the adult as well as the embryonic ear. Increased histidine concentrations of the mother did not affect the histological lesions. The 'biochemical hypothesis' of a penetrance threshold must therefore be rejected.

All the injection experiments (as well as most of the embryological analysis in previous Sections) were carried out on the Edinburgh stock (designated SEV) which, by this time had a fairly low and variable penetrance. In view of this the Cambridge stock was re-introduced (August, 1976) since Dr. Wallace had reported a continuing high incidence of balance-defective offspring. The purpose was to make comparisons, and investigate any possible genetic difference.

(2) The developmental hypothesis "The histidine levels in his homozygotes of the strains with different penetrances are not significantly different and above any threshold. The differences are caused by genes at other loci, and are manifested (a) either through the maternal intrauterine environment or (b) through the susceptibility of the offspring".

Certain crosses between the high penetrance strain from Cambridge (CAM) and the low penetrance strain at Edinburgh (SEV) would distinguish between (a) and (b). Hopefully the crosses might also indicate whether the difference was due to a single allelic difference or was polygenic.

If reciprocal crosses between CAM and SEV were set up, the genotype of the offspring of both crosses would be identical. Two possible outcomes can be envisaged. Designating CAM = H (high) and SEV = L (low).

1st possibility

H♀ X L♂	L♀ X H♂
Offspring have high incidence	Offspring have low incidence

Conclusion: The genetic background of H mother determines the number of affected offspring in the litter. The L allele(s) have the reverse effect.

2nd possibility

Offspring of both crosses have the same incidence.

Conclusion: The genetic constitution of the offspring (identical in both crosses) determines the incidence.

(a) The incidence is high: H (one or more genes) is dominant over L.

TABLE 15

CAM X CAM CROSSES

Mating No.	♀	♂	Litter No.	N	Ab
159	51.2 c	50.1 c	1	0	4
			2	0	5
			3	0	4
			4	dead	
160	51.2 d	43.2 c	1	dead	
			2	0	7
			3	7	1
			4	4	1
			5	0	2
199	51.9 b(30)	58.1 c(11)	1	dead	
			2	1	2
			3	0	3
202	51.10 c(1)	57.3 h(3)	1	0	2
			2	dead	
201	51.10 d(3)	56.2 c(11)	1	dead	
			2	0	5
			3	2	0
			4	dead	
			5	2	2
			6	0	8
199	51.9 b(30)	58.1 c(10)	1	dead	
			2	1	2
			3	0	4
			4	0	1
255	51.11 b(0)	58.4 f(0)	1	0	3
252	181.1 d(30)	159.3 g(0)	1	dead	
			2	3	0
			3	4	2
253	252.3 c(1)	160.4 p(1)	1	dead	
			2	2	5
258	181.1 b(3)	159.1 p(0)	1	0	3
			2	3	4
			3	dead	
259	258.2 a(0)	160.4 p(11)	1	0	7
24 litters				29	77

TABLE 16

SEV x SEV CROSSES

Mating No.	♀	♂	Litter No.	N	Ab
193	119.11 c(0)	146.10 s(0)	1	7	0
			2	8	0
194	162.1 b(0)	146.1 r(0)	1	7	0
			2	10	0
			3	dead	
			4	10	1
			5	dead	
195	162.1 c(0)	171.2 p (0)	1	5	0
			2	dead	
			3	5	0
196	151.6 c(0)	140.6 q (0)	1	7	0
			2	8	0
			3	6	0
			4	dead	
205	193.1 a(1)	196.2 p(0)	1	7	0
			2	dead	
215	194.2 c(30)	193.1 q(11)	1	2	0
			2	11	0
216	152.1 a(1)	152.1 t(10)	1	3	2
			2	6	1
			3	5	0
			4	4	2
			5	3	2
18 litters		-		114	8

TABLE 17

INTERCROSSES SEV/CAM AND CAM/SEV

179	119.7 a(33) SEV	159 B 50.1 a CAM	1	4	0
182	146.6 b(11) SEV	B 43.2 e CAM	1	6	0
			2	8	0
			3	8	1
			4	9	2
180	B 51.2 c CAM	146.6 p(0) SEV	1	dead	
			2	5	1
			3	dead	
181	B 51.2 d CAM	146.6 q(1) SEV	1	6	0
			2	4	0
			3	dead	
	CAM x SEV (5 litters) SEV x CAM (3 litters) Total 8 litters	-		35 15 50	3 1 4

(b) The incidence is low: L is dominant over H.

(c) The incidence is intermediate: one pair of alleles, H/L, has co-dominant effects or several loci show random dominance relations between the alleles.

The results are shown in Tables 15 - 21 and will be discussed in the light of these hypotheses.

RESULTS

Crosses between CAM X CAM

These animals were obtained from Cambridge in 1976. Crosses were made between a behaviourally normal female (since severely abnormal females cannot nurse their young) and a severely affected male. Even then there was difficulty in breeding these CAM animals because some females, even though they were behaviourally normal, had the tendency to eat the litter soon after birth.

The behavioural scoring of the surviving animals was done twice during the second and/or third week after birth as described in Materials and Methods, see Tables 15 & 21. The penetrance of the affected animals was found to be 73%.

Crosses between SEV X SEV

These animals were from our Edinburgh (SEV) stock. Males and females that were used for the crosses were behaviourally normal. The penetrance of the affected animals was found to be 6.6% (Table 16).

Reciprocal crosses between CAM and SEV

(Intercross = INT)

2 females from CAM were crossed with 2 males from SEV and 2 females from SEV were crossed with 2 males from CAM. The offspring were scored twice during the second and third week after birth. The results (Table 17) showed a low penetrance for both types of crosses.

TABLE 18

BACKCROSSES TO CAM

Mating No.	♀	♂	Litter No.	N	Ab
B.C. 1	182.1 b(3)	43.2 q(1)	1	3	6
B.C. 5	182.2 a(1)	43.2 q(1)	1	2	4
B.C.14	182.1 a(1)	43.2 q(1)	1	3	3
B.C.18	182.2 c(33)	43.2 q(1)	1	1	8
B.C.16	182.4 c(11)	160.2 q(1)	1	0	8
			2	0	6
			3	0	5
B.C. 2	182.1 c(11)	43.2 u(1)	1	3	4
			2	3	3
B.C. 4	182.1 f(33)	43.2 v(1)	1	dead	
			2	2	2
B.C. 8	182.3 a(0)	43.2 v(1)	1	2	3
B.C. 6	182.4 c(30)	159.3 p(1)	1	2	4
			2	6	1
			3	7	4
B.C. 7	182.4 f(0)	159.3 p(1)	1	4	4
			2	6	1
16 litters -				44	66

TABLE 19

BACKCROSSES TO SEV

Mating No.	♀	♂	Litter No.	N	Ab
B.C. 7 S	182.4 f(0)	213.3 p(0)	3	3	0
			4	8	0
			5	3	0
			6	dead	
B.C. 8 S	182.3 a(0)	206.2 p(1)	2	3	0
B.C.14 S	182.1 a(1)	215.4 p(33)	2	4	1
			3	2	1
			4	5	1
B.C.16 S	182.4 c(11)	213.3 p(0)	4	5	0
			5	5	3
	9 litters -			38	6

TABLE 20

SECOND BACKCROSS TO CAM

2 B.C.20	B.C. 7.2 f(13)	58.4 f(0)	1	1	4
2 B.C.21	B.C 5.1 a(1)	159.3 q(0)	1	3	1
2 B.C.22	B.C. 1.1 d(0)	159.3 q(0)	1	3	1
2 B.C.23	B.C. 7.1 d(31)	56.2 c(11)	1	2	7
			2	0	2
2 B.C.24	B.C. 7.2 b(33)	58.4 f(0)	1	1	2
2 B.C.25	B.C. 6.1 d(30)	56.2 c(11)	1	1	3
2 B.C.26	B.C. 14.1 a(0)	56.2 c(11)	1	0	4
	8 litters -			11	24

TABLE 21: Calculation of the between litter variation in different crosses

Mean fraction of abnormal = $\bar{r} = \frac{\Sigma Ab}{\Sigma(Ab+No)}$ Ab = abnormal, No = normal

$\Sigma(Ab+No) = N$ = total no. of animals

$\sigma^2 = \frac{\Sigma n_i (r_i - \bar{r})^2}{N-1}$ where n_i = litter size

r_i = fraction of abnormal in litter i

$\bar{r}$ = mean fraction of abnormal

Standard deviation = s.d. = $\sqrt{\sigma^2}$

Standard error = $\frac{s.d.}{\sqrt{l}}$ l = total no. of litters

Behavioural penetrance of CAM, SEV and their crosses

Cross	♀ ♂	N	No. of litters	(3 wks) Average Litter size	Mean $\bar{r}$	σ^2	s.d.	s.e.
CAM (H)	CAM x CAM	106	24	4.42	0.726	0.118	0.343	0.070
SEV (L)	SEV x SEV	122	18	6.78	0.066	0.016	0.128	0.030
Inter Cross INT	CAM x SEV	38	5		0.079			
	SEV x CAM	16	3		0.063			
	Overall = INT	54	8	6.75	0.074	0.067	0.080	0.028
B.C. to CAM	INT x CAM	105	16	6.26	0.581	0.073	0.270	0.068
B.C. to SEV	INT x SEV	44	9	4.89	0.136	0.022	0.148	0.049
2nd B.C. to CAM	B.C. x CAM	35	8	4.38	0.686	0.086	0.293	0.037

The combined penetrance was found to be 7.4%. The results show that 'high penetrance' is recessive to low penetrance and that the difference in penetrance is due to susceptibility of the offspring rather than maternal intrauterine environment.

To test the possibilities of whether the difference was due to a single gene or polygenes, backcrosses were set up.

(a) Backcross to CAM

(INT) X CAM (Table 18)

As already mentioned, there was great difficulty in breeding the CAM stock because of the mothers rejecting their young. It was decided to use the males of CAM stock for the backcrosses. The non-affected females from the F_1 (CAM/SEV) were crossed with males of the 'high line', CAM. All the F_1 non-affected females that were used in these crosses came from mating 182 of CAM/SEV (Table 17).

(b) Backcross to SEV

(INT) X SEV (Table 19)

Some of the females that had been used in the backcrosses to CAM were used again for these crosses. 4 females were used. They were crossed with 4 males from Edinburgh SEV stock. The penetrance was found to be 13.6%.

(c) 2nd Backcrosses to CAM (Table 20)

5 females of the 1st backcross which were non-affected, (and therefore had a high probability of being H/L) were used for these crosses. They were crossed with affected CAM high penetrance males.

Discussion

If the penetrance difference is due to a single gene, the F_1 animals would be heterozygous at this locus and hence the offspring of a backcross to the recessive (H) should segregate 1:1. Denoting the putative alleles in the CAM stock by H/H and in the SEV stock by L/L:

Backcross to CAM

H/L X H/H

50% H/L 50% H/H

Penetrance 7% 73%

Therefore the expected penetrance is (3.5 + 36.5%) i.e. 40%.
But the observed result showed a value 58.1% $\pm$ 6.8 s.e.

Backcross to SEV

H/L X L/L

50% H/L + 50% L/L

Penetrance 7% 7%

The expected penetrance of the backcross to SEV is 7%, but the observed result was found to be 13.6% $\pm$ 4.9 s.e.

While the difference of the backcross to SEV result to the expected is less than two standard errors, 13.6 - 7 = 6.6 (s.e. = 4.9) the backcross to CAM shows almost three times the standard error difference to the expected.

58.1 18.1
~~60.6~~ - 40.0 = ~~20.6~~ (s.e. = 6.9).

This must throw some doubt on the single gene hypothesis. Since the observed penetrance is higher than the expected (mean of parentals) any model containing two or more additively acting loci would only increase this deviation. There is, however, a two locus model which, in a formal way, is a better fit to the data. This assumes dominance

of the alleles in the SEV stock and postulates epistatic interactions between the two loci:

CAM = a/a, b/b = 73% penetrance.

SEV = A/A, B/B = 7% penetrance.

INT = A/a, B/b = 7% penetrance.

The backcross to CAM expectation would be

A/a, B/b X a/a, b/b.

Offspring classes	Genotype		Penetrance	Expected fraction of each class		% affected
1	A/a,B/b		7%	0.25	=	1.75
2	A/a,b/b	assume	73%	0.25		
3	a/a,B/b	assume	73%	0.25	=	54.75
4	a/a,b/b		73%	0.25		
		Expected % affected			=	56.5

This is much closer to the observed 58.1 and well within one standard error of 6.8. 58.1 - 56.5 = 1.6 (s.e. = 6.8).

As for the second backcross, since only unaffected females from the 1st backcross were taken we must calculate the probabilities of selecting females of the different genotypic classes.

Backcrosses with class (1), i.e. A/a, B/b females, will give 56% of affected offspring as before. Crosses 2, 3 and 4 will give 73% of affected offspring.

2nd Backcross to CAM

Expected fraction of unaffected ♀♀ of each genotype from 1st backcross		penetrance	% affected offspring
93/174 = 0.534	Class 1	56	29.9
81/174 = 0.466	Class 2,3,4	73	34.0
	Expected % affected	=	63.9

Since the second backcross was found to be 68%, the difference 68 - 64 = 4.0 (s.e. = 8.6) is again a not unreasonable figure. This two-locus hypothesis is a better fit, than the one gene hypothesis.

While the particular epistatic assumption does generate the observed penetrances, it would be unwise to put too much weight on this consistency. What is however clear is:

(1) The difference between CAM and SEV is genetic.

(2) It is not due to differences in maternal histidine levels.

(3) The difference is operating through the susceptibilities of the offspring.

(4) Alleles at not more than 2 loci are responsible for the difference. (To extend the special epistatic assumption, as well as that of dominance, to a large number of loci would stretch the credibility of the model. Polygenic differences are therefore rejected).

Morphology and Function

The experiments reported above on the genetic basis of penetrance differences were solely concerned with the character "behaviour" i.e. the functional result of the maternal condition. It had, however, been noted (Section I) that the morphological lesions, although correlated with behavioural abnormalities, were not invariably displayed in the same animal. In particular, a considerable number of animals with apparently normal functions were found to have sub-clinical lesions of the inner ear, occasionally quite severe. In the experiments on pre-natal embryos both in Section II and in the present Section on histidine injections, no behavioural correlate could, of course, be obtained.

TABLE 22

Age and Litter No.	No.	Ear Morphology								Function		Concordance Function/Morph.
		Otoliths		Canals		Cochlea		Pigments		Behaviour	Hearing	
		L	R	L	R	L	R	L	R			
6 days 1	S01	+	+	+	+	+	+	+	+	Unable to score	Unable to score	
	S02	–	–	–	–			–	–			
	S03	+	+	+	+	+	+	+	+			
	S04	+	+	+	+	+	+	+	+			
	S05	–	–	–	D		+	–	–			
	S06	+	+	+	+	+	+	+	+			
2	1	+	+	+	+	+	+	+	+			
	2	–	–	–	+	+	+	–	–			
	3	–	+	–	+	+	+	+	+	"	"	
	4	+	–	+	–	+	+	+	+			
	5	D	–	D	+	D	+	D	+			
	6	D	–	D	+	D	+	D	+			
3	S33	–	–	D	–	D	+	+	+			
	S34	+	+	+	+	+	+	+	+			
	S35	+	+	+	+	+	+	+	+			
	S36	+	+	+	+	+	+	+	+			
	S37	+	+	+	+	+	+	+	+	"	"	
	S38	+	+	+	+	+	+	+	+			
	S39	+	+	+	+	+	+	+	+			
	S40	+	+	+	+	+	+	+	+			
	S41	–	–	+	+	+	+	+	+			
4	S42	–	–	+	+	+	+	+	+	"	"	
	S43	–	–	+	+	+	+	+	+			
15 days 1	S77	+	+	+	+	+	+	+	+	Normal	+	N/N
	S78	+	–	+	+	+	+	+	–	Normal	+	N/A
	S79	–	–	+	+	+	+	+	–	Normal	+	N/A
	S80	+	–	+	+	+	+	+	+	Normal	–	N/A ? A/A
2	S109	+	+	+	+	+	+	+	+	Normal	+	N/N
	S110	+	+	+	+	+	+	+	+	Normal	+	N/N
	S111	+	+	+	+	+	+	+	+	Normal	+	N/N
	S112	+	+	+	+	+	+	+	+	Normal	+	N/N
	S113	+	+	+	+	+	+	+	+	Normal	+	N/N
	S114	+	+	+	+	+	+	+	+	bla+–	–	A/N
24 days 1	S54	+	+	+	+	+	+	+	+	Normal	+	N/N
	S55	–	–	–	–	–	–	–	–	bla+	+	A/A
	S56	–	–	+	+	+	+	+	+	Normal	+	N/A
	S57	+	+	+	+	+	+	+	+	Normal	+	N/N
	S58	+	+	+	+	+	+	+	+	Normal	+	N/N
2	S81	+	+	+	+	+	+	+	+	Normal	+	N/N
	S82	–	–	–	–	–	–	–	–	bla+	+	A/A
	S83	+	+	+	+	+	+	+	+	Normal	+	N/N
	S84	–	–	–	–	–	–	–	–	bla+–	+	A/A
	S85	+	–	+	+	+	+	+	+	Normal	+	N/A
	S86	–	–	+	+	+	+	–	–	Normal	+	N/A

TABLE 22 - cont'd

Age and Litter No.	No	Ear Morphology								Function		Concordance Function/Morph
		Otoliths		Canals		Cochlea		Pigments		Behaviour	Hearing	
		L	R	L	R	L	R	L	R			
35 days 1	S93	-	-	+	+	+	+	-	-	Normal	+	N/A
	S94	-	-	+	+	+	+	-	-	Normal	+	N/A
	S95	-	-	+	+	+	+	-	-	Normal	+	N/A
	S96	+	+	+	+	+	+	+	+	Normal	+	N/N
	S97	-	-	+	-	+	-	+	-	Normal	+	N/A
	S98	-	-	-	-	-	-	-	-	bla^{+}	-	A/A
	S99	+	+	+	+	+	+	+	+	Normal	+	N/N
	S100	-	+	+	+	+	+	+	+	Normal	+	N/A
	S101	-	-	+	+	+	+	+	+	Normal	+	N/A
2	S137	-	-	-	-	-	-	-	-	Normal	+	N/A
	S138	-	-	+	+	+	+	-	-	Normal	+	N/A
	S139	-	-	+	+	+	+	-	-	Normal	+	N/A
	S140	-	-	+	+	+	+	-	-	Normal	+	N/A
	S141	-	-	+	+	+	+	+	+	Normal	+	N/A
	S142	-	-	+	+	+	+	-	-	Normal	+	N/A
	S143	+	-	+	+	+	+	-	-	Normal	+	N/A

Penetrances (P_F = Function; P_M = Morphology)

15 days	P_F =	20% ?	;	P_M =	30%
24 days	P_F =	27%	;	P_M =	55%
35 days	P_F =	6%	;	P_M =	87%
Mean(24+35)	P_F =	15%	;	P_M =	71%

It was therefore desirable to have some quantitative data on the relative incidence of "behaviour" and "ear mporphology" as separate characters affected by the same condition. Table 22 gives the results of 10 litters, scored after birth, both for behaviour and ear morhpology of whole mounts. (The latter method does not give as much detailed information as would sections but the additional work involved was not considered justified).

The 6 day animals could not be tested for behavioural phenotype but the 15, 24 and 35 day old animals were so scored (although there may be some doubt of the 15 day results). The last column in the Table shows the concordance between the two characters (Function/ Morphology). A summary of the % affected animals is given in Table 22. The obvious result is that the penetrance (P_M) for the ear morphology is consistently higher than that for the functional (P_F) abnormality. In view of the small numbers (and the possible unreliability of the 15 day results, the 'adult' values (24 and 35 days) have been combined giving:

penetrance (behaviour) = 15%

penetrance (ear lesions) = 74%.

These litters were from the Edinburgh stock raised in 1975. For behaviour we therefore have a time course of penetrance:

	CAM 1971	1973	1974	1975	1977	CAM 1976
P	80%	59%	37%	15%	7%	73%

These results are consistent with the hypothesis, already mentioned, that relaxation of selection for behavioural defect in the Edinburgh stock has taken place.

Unfortunately no comparable data for the incidence of inner ear lesions are available but the earliest observations on the ears (1973) had indicated the presence of behaviourly normal animals which showed sub-clinical damage of the vestibule. Data bearing on this are, however, available from the pre-natal values of sections in Table 5. The phenotypes without designations ((L) or (H)) were all raised between May and December 1975. (Litters 14, 21, 36, 27, 28, 29, 35, 40). From these we find 22 normal and 13 abnormal animals. The re-introduced Cambridge stock at the end of 1976 (designated (H)) which showed a penetrance (of behaviour of 73%) is also represented in Table 5 (Crosses (H) X (H), 16, 21, 36, 41). We find 3 normal and 12 abnormal animals. These can be regarded as the equivalent of the 1971 stock when it arrived in Edinburgh.

We therefore find for pre-natally detected defects

1975	CAM 1976
28%	75%

Although numbers are small, and do not represent whole litters, the conclusions point in the same direction.

Penetrance and Expressivity

As has been evident from the work presented, the effect of abnormal histidine metabolism on the development of the hind brain and the inner ear is to cause certain morphological abnormalities as well as functional disturbances particularly of balance and of hearing. On the whole the evidence supported the view of Deol that these two aspects (morphology and function) are not necessarily causally related but should be regarded as two separate consequences of some common cause. This would not necessarily exclude correlations between the two such as severe inner ear defects associated with

extreme behavioural defects.

While the wide variation observed (even in animals of the same litter) made it possible to draw these conclusions, the variability constitutes a problem in itself. (See Fig. 21B).

Histidine Levels and Inner Ear Lesions at $16\frac{1}{2}$, $17\frac{1}{2}$ and $18\frac{1}{2}$ Gestation Days

As described in the previous Section the incidence of abnormality of the inner ear at 3 weeks after birth as well as during embryonic stage varied from animal to animal. The affected numbers of animals were also found to vary from litter to litter. In this experiment it was decided to look at the histidine levels in blood and liver in individual embryos of whole litters and at the same time examine the brain and the inner ear to see whether the embryos with inner ear abnormality have brain abnormality and what kind of histidine levels in blood and liver at $16\frac{1}{2}$, $17\frac{1}{2}$, $18\frac{1}{2}$ day gestation stages were found. From the histological study of the embryonic stages it is known that at these stages any abnormality of the inner ear is clearly seen, such as absence of otoliths, distension of endolymphatic system, etc. +/+ females mated with his/his(SEV) males were used as controls.

Blood and livers from mother and embryos were taken out and estimated for levels of histidine and other amino acids as described in Materials and Methods. All the heads from the whole litter (except the occasional embryo, which was found dead in the uterus) were fixed as described in Table 25. Some of the whole heads were sectioned, some in transverse sections and some in sagittal sections.

In those heads which had been fixed in Bouin, it was not possible to score the otoliths. Otoliths are preserved in acid fixation only 2 days after birth (Lyon, 1955, Veenhof, 1969). The results from

TABLE 23a

4072 SEV x SEV 16½ days

	GLU	GLY	ALA	ORN	LYS	HIS
1L						
2L						
3L	4.967	4.175	3.704	0.431	2.572	6.326
4L	3.813	3.296	2.874	0.395	2.092	4.846
5L	3.375	3.213	2.806	0.312	2.016	4.200
6L						
ML	1.632	2.294	5.681	0.181	1.042	11.402
Mean Embryonic L.	4.052	3.561	3.128	0.397	2.227	5.124
MB	0.389	0.485	0.992	-	0.871	1.338
1B	1.379	1.418	1.461	0.158	1.114	2.644
2B	0.854	1.623	1.352	0.073	1.003	2.636
3B	0.722	1.212	1.270	0.142	1.107	2.516
4B	0.719	1.260	1.215	0.104	1.080	2.630
5B	1.219	1.337	1.551	0.094	1.096	2.561
6B	0.947	1.417	1.315	0.127	1.167	2.419
Mean Embryonic blood	0.973	1.378	1.361	0.116	1.095	2.568
2A	0.913	0.858	1.154	0.321	0.914	2.310
3A	-	-	-	-	-	-
4A	0.135	0.202	0.212	0.110	0.156	0.403
5A						
6A						
Mean Embryonic A.	0.524	0.530	0.683	0.216	0.535	1.352

4015 CAM x CAM 16½ days

ML	1.685	2.655	6.056	0.477	1.355	11.088
1L						
2L	2.684	2.449	1.953	0.208	1.371	4.456
MB	0.327	0.597	0.662	0.125	0.582	1.118
1B						
2B	0.913	1.120	1.226	0.085	1.238	3.023

TABLE 23b

4092 (+/+ x SEV) 16½ days

	GLU	GLY	ALA	ORN	LYS	HIS
ML	0.940	1.743	4.406	0.405	1.145	0.514
1L	1.648	1.560	1.305	0.165	1.019	0.165
2L	5.916	4.664	3.920	0.689	3.081	0.583
3L	6.237	4.867	4.164	0.583	3.175	0.397
4L	4.615	3.987	4.137	0.529	3.163	0.572
Mean Embryonic L.	4.604	3.770	3.382	0.492	2.610	0.429
MB	0.157	0.278	0.601	0.063	0.814	0.0712
1B	0.923	0.648	1.101	0.114	1.097	0.125
2B	0.577	0.707	0.803	0.119	1.513	0.129
3B	0.607	0.712	0.916	0.082	1.550	0.150
4B	0.761	0.865	1.065	0.119	1.570	0.141
Mean Emb. blood	0.717	0.733	0.971	0.109	1.433	0.136
1A	0.169	0.315	0.669	0.070	1.103	0.178
2A	0.185	0.221	0.607	0.061	1.061	0.163
3A	0.300	-	-	0.084	0.721	0.195
4A	0.157	0.178	0.461	0.049	0.741	0.095
Mean Embryonic A	0.203	0.238	0.579	0.066	0.907	0.158

4073 (SEV x SEV) 18½ days

ML	1.669	2.431	5.006	0.182	1.156	11.021
MB	0.288	0.218	0.544	0.067	0.506	0.930
1B	0.659	0.952	1.011	0.202	1.957	2.909
2B	0.753	1.347	1.233	0.124	1.817	2.641
3B	0.829	0.779	0.953	0.113	1.326	1.888
Mean Emb. blood	0.747	1.026	1.066	0.146	1.700	2.479
1A	-	-	-	-	-	-
2A	0.655	1.136	2.940	0.723	2.571	5.874
3A	-	-	-	-	-	-

TABLE 24

Maternal and Embryonic Amino Acids in Blood of +/+ and his/his Mothers

	GLU	GLY	ALA	ORN	LYS	HIS
his/his (16½-18½d)						
E.B. (N=10)	0.899	1.246	1.259	0.121	1.291	2.587
M.B. (N=3)	0.335	0.433	0.733	0.096	0.653	1.129
R	2.7	2.9	1.7	1.3	1.9	2.3
+/+ (16½d)						
E.B. (N=4)	0.717	0.733	0.971	0.109	1.433	0.136
M.B. (N=1)	0.157	0.278	0.601	0.063	0.814	0.071
R	4.6	2.6	1.6	1.7	1.8	1.9

Previous reports: Rat: R = > 1
Mouse: R = 1.5 - 2.5

GLU = glutamate; GLY = glycine; ALA = alanine; ORN = ornithine;
LYS = lysine; HIS = histidine;

E.B. = Embryonic blood; M.B. = Maternal blood; R = E.B./M.B.

TABLE 25. Histidine levels and histological lesions in individual embryos at 16½, 17½ and 18½ days gestation.

Cross ♀ ♂	No.	Blood µmol/ml.	Liver µmol/ml	LEFT Utriculus Oto.	LEFT Utriculus Lum.	LEFT Sacculus Oto.	LEFT Sacculus Lum.	LEFT Cochlea	RIGHT Utriculus Oto.	RIGHT Utriculus Lum.	RIGHT Sacculus Oto.	RIGHT Sacculus Lum.	RIGHT Cochlea	Brain
+/+ x SEV	Mother 4092	0.07	0.51											
16½ days	Embryos 1	0.13	0.17	+		+			+		+			
(whole	2	0.13	0.58	+		+			+		+			
mounts)	3	0.15	0.40	+		+			+		+			
	4	0.14	0.57	+		+			+		+			
+/+ x SEV	Mother 4004	0.10	0.55											
17½ days	Embryos 1	0.11	0.25		+		+	+		+		+	+	
(Sections	2	0.10	0.19		+		+	+		+		+	+	
Acid	3	0.14	----		+		+	+		+		+	+	
Fixed)	4	0.08	----		+		+	+		+		+	+	
SEV x SEV	Mother 4072	1.34	11.40											
16½ days	Embryos 1	2.64	----	-		-			+		+			
	2	2.64	----	-		-			-		-			
(whole	3	2.52	6.33	+		+			+		+			
mounts)	4	2.63	4.85	Big		+			+		+			
	5	2.56	4.2	+		+			+		+			
	6	2.42	----	Big		Big			Big		Big			
CAM x CAM	Mother 4015	1.12	11.09											
16½ days	1	2.45	----	-	-	-	-	-	-	-	-	-	-	
(Sections	2	3.02	4.46	-	-	-	-	-	-	-	-	-	-	
Alchohol fixed)														
INT x CAM	Mother 4003	0.75	9.51											
	Embryos 1	3.11	1.77		-		-	-		-		-	-	+
	2	1.83	2.14		-		-	-		-		-	-	+
17½ days	3	2.81	6.11		-		-	-		-		-	-	+
(Sections	5	3.59	4.51		-		-	-		-		-	-	+
Acid	6	2.87	5.92		-		-	-		-		-	-	+
Fixed)	7	1.92	4.27		-		-	-		-		-	-	+
	8	3.02	4.62		+		-	-		+		-	-	+
	9	4.09	3.88		-		-	-		-		-	-	+
	10	2.73	4.42		+		-	-		+		-	-	+
SEV x SEV	Mother 4073	0.93	11.02											
18½ days	Embryos 1	2.91	----	+		+			+		+			
(Whole	2	2.64	----	+		+			+		+			
mounts)	3	1.89	----	Big		Big			Big		Big			

The embryos were dissected from the uterus at the various stages indicated. Blood and liver were taken for analysis. The heads were treated in three different ways:

a) Whole mounts (fixed in alcohol and cleared in benzyl alcohol). This reveals the otoliths but not details of the vestibule.

b) Alcohol fixed and sectioned. Otoliths and vestibule can be scored.

c) Bouin fixed and sectioned. Otoliths are dissolved by the acid, but vestibule and brain can be scored.

the +/+ embryos were in agreement with their findings because no otoliths could be seen in the four animals that were sectioned. All the other parts of the inner ear such as diameter of the endolymphatic sac, utriculus, sacculus and cochlea, however, could be seen.

Table 23 shows a number of litters of various genotypes and pre-natal ages with a selected number of amino acids. It was known that as for man (Butterfield and O'Brien, 1963), the rat (Boggs and Waisman 1969) and the mouse (Lajtha and Toth, 1973) show a concentration effect in the embryos compared to the maternal amino acid levels. This is fully confirmed for the litters examined here. Table 23 is summarised in Table 24. It is seen that the ratio R (embryonic blood/maternal blood) is consistently > 1.

This is true equally for the histidine value of +/+ and his/his mothers in spite of a 20 fold difference in absolute concentration. A similar effect has been reported for the phenylalanine values in man when normal and PKU subjects were compared. The ratio was R = 1.7 for PKU (about 30 fold higher absolute values) compared to the normal range R = 1.5 - 2.4, (Thomas et al. 1971). The same effect is observed in rats fed on diets supplemented with phenylalanine. R = 2.2 for controls compared to R = 2.4 for the 6-fold higher values on 7% supplemented diet.

A possible correlation of the individual histidine values and the morphological scoring of the head region of the embryos was then sought. Table 25 shows the results. It can be seen that there are no obvious correlations either within or between litters. This is consistent with the evidence, already presented, on the effect of histidine injections. The level of histidine in histidinaemic animals is not

related to the incidence or severity of histologically detectable lesions. The conclusion is therefore, again, that no consistent significant biochemical variation can explain the histological differences between animals of the same litter, or the differences between the CAM and SEV stocks.

GENERAL DISCUSSION

Having presented the evidence and discussed some of the results in the various sections it now remains to attempt to give a unified explanation of the syndrome in all its aspects. This will be discussed in relation to a number of theories which have been advanced in other similar cases and in particular to Deol's hypothesis based on a study of many mutants.

Before proceeding to the discussion it is important to note that comparisons, both of facts and theories, will be with mutants i.e. where the animal's own genotype is responsible for the observed abnormalities. In no case is the gene action known in molecular terms. (Some reference will be made to human inner ear abnormalities where neither the genetic basis nor any possible environmental effects can be distinguished). Although therefore our case differs in having a known biochemical basis it is, of course, not suggested that the, over 50 mouse mutants are connected with abnormal histidine metabolism. (Some of these have been screened for disturbances in amino acid metabolism but no such effect has been found. Kacser, unpublished). Two apparently opposing, facts will have to be borne in mind. On the one hand the behavioural phenotype is remarkably similar in almost all mutants and in our maternal effect. On the other hand the detailed morphological abnormalities where they are known, show distinct differences, either in the organ, type or onset of the lesions. This argues that either an important functional abnormality, e.g. in the nervous system, is identical in all the cases investigated or that the behavioural consequences

of a variety of abnormalities result in a limited change of behavioural repertoire - or both. The other general conclusion which emerges from these studies is that the organs responsible for the syndrome are either developmentally or functionally, subject to many independent effects which argues that this area is particularly sensitive to disturbances and has rather less "canalisation" than other functions. (It must, however, be borne in mind that we are dealing with a highly selected sample which, because of its ease of detection, appears to occur more frequently than other classes of abnormalities).

It is therefore reasonable to place our maternal effect into the context of all the other effects even though the immediate gene products are quite unknown. Many of the problems of detailed mechanism as well as more general relations between behaviour and morphology are common to the whole field.

It is convenient to sum up the important results which require to be related to one another and which must be accounted for in any hypothesis of the syndrome.

(1) The syndrome is produced by a maternal effect. The metabolic state of the mother, whether genetically or nutritionally caused, is a necessary condition. High concentrations of histidine or its imidazole derivatives are responsible.

(2) The interference with normal development occurs during the second week of pregnancy.

(3) Abnormalities are detectable from the 9th day of pregnancy. It is therefore a morphogenetic type.

(4) The behavioural abnormality shown by the animals from his/his mothers are very similar to the mutants with shaker-waltzer syndrome but in those various mutants, the offspring's own genotype is the cause of the abnormal behaviour whereas in our case it is caused by a maternal effect of histidinaemic mothers.

(5) The affected animals showed some or all of the following: circling behaviour, head tilting, deafness, reduced ability to swim, lack of disorientation after spinning, poor maze learning and hyperactivity. There are lesions in the inner ears, such as shortening or narrowing of the posterior vertical canals, narrowing of the anterior vertical canals and horizontal canals, shortening of the crus commune. Some otoliths are lacking from $14\frac{1}{2}$ day embryos and from $16\frac{1}{2}$ days gestation onwards, the endolymphatic duct and sac, ampullae, utriculus, sacculus and cochlea could be distended. In some adult animals, the saccular membrane has loops and in the cochlea the Reissner's membrane is either very stretched due to the distension of the scala media (cochlear duct) or sometimes part of the Reissner's membrane lie very close to the tectorial membrane.

(6) Other structures than the inner ear could be affected. Open neural tube at $9\frac{1}{2}$ days gestation, narrowed hind brain between $9\frac{1}{2}$ to $11\frac{1}{2}$ days gestation, reduced pigments in the inner ear, distension of some blood vessels at $17\frac{1}{2}$ days, nerves and ganglion cells disorganised in some adult ears.

(7) The correlation of behavioural defect and the inner ear is not complete, but whenever there was a severely affected animal, the inner ear was found to be affected as well.

(8) Some of the behaviourally normal animals were found to have inner ear abnormalities.

(9) There is variable expressivity of both behaviour and inner ear defects. They range from frequent tight circling to very slight head tilting on the one hand and severe distensions of both inner ears to only big otolithic crystals in one ear or the other.

(10) There is variability of penetrance as well as variable expressivity within litters at the behavioural and morphological level.

(11) The variability is found between ears of the same animal and even between structures of one ear.

(12) The genetic background of the offspring play a role in the defect. The difference between the Edinburgh stock and Cambridge stock exists even though they have the same histidine levels.

(13) Elevation of histidine levels (by injection) produced no apparent difference. Thus differences in expressivity and penetrance are not related to histidine levels at these high values.

In view of the known high concentrations of histidine and imidazoles in the maternal blood and the demonstration that these are reflected in even higher concentrations in the embryos, it is attractive to suggest that there is some direct effect of these substances on the development of the various structures. That this cannot be correct is principally shown by the evidence summarized in (10), (11), (12) and (13). Variation within a litter which is exposed to the same maternal condition, variation between ears of the same animal, variation between stocks of the same maternal histidine concentration all argue that this condition

can be the 'precipitating' agent but not the direct cause.

It is unreasonable to assume that enormous concentration heterogeneities can exist within one embryo whose width at e.g. 10 days is about 0.5 mm. The finding of one normal and one abnormal ear would require something like a 20 fold difference in concentration between the two halves. Similarly it seems unreasonable to envisage a 20 fold difference in blood supply caused by e.g. differences in histamine, a vasodilator, which, if it existed, would affect far more than the development of the otic vesicle. Some other process which can be subject to 'stochastic' variation is likely to be influenced by the metabolic environment. Such a process is likely to be a morphological event or series of events which are of the inductive chain type. If these events are at a stage of development particularly sensitive to disturbances such as neural tube closure and its subsequent interactions, the effects of the metabolic conditions can be viewed as pushing the system into a variety of morphological abnormalities which are detectable later on at various histological and functional levels.

A view not dissimilar to this has been expressed by Deol based on a study of many mutants. Many genes affect the behaviour as well as the inner ear in the mouse. Since the inner ear is the seat of equilibrium, and this sense appears to be disturbed in these mutants, behavioural defects have been assumed to be due to morphological defects of the inner ear such as in pallid, twirler, zig zag (Lyon, 1953, 1958, 1960) and waltzer type (Stein and Huber, 1960). On the other hand Deol (1966) proposed a different theory. His theory is based on

4 points. "First, extirpation of the whole or part of the inner ear whether, unilateral or bilateral, does not lead to circling (Lowenstein, 1936; Prosser, 1950). (Evidence from amphibians, fish and birds). Secondly in artificial waltzers, produced by means of drugs, no abnormalities of the inner ear could be detected, although widespread lesions were observed in the cerebellum and brain stem (Goldin, 1947; Goldin, Noe, Landing, Shapiro and Goldberg, 1948). Thirdly, fundamental anatomical differences may be observed in mutants with indistinguishable behaviour. Fourthly, with the exception of the abnormalities of the cochlea and deafness, no particular defect of the inner ear is invariably associated with any specific pecularity of behaviour".

Deol investigated the relationship of the inner ear defect and the behavioural defect by observing the behavioural defect of kinky mice (Fu^{ki}) and then looked at the inner ear in sections (histologically). He found that the inner ear abnormalities and the behaviour defect are not completely correlated, even though the inner ears are severely affected in animals with severely affected behaviour, moderate in animals with moderately affected behaviour and light or absent in animals with slightly affected behaviour. So he concluded that behaviour and inner ear defects must have a common origin or at least their causes are closely related. There was no anatomical investigation of the brain in kinky mice. But Deol pointed out that (Fu), an allele of kinky, is known to act on the nervous system. He assumed that the manner of action of the two genes might not be different.

In some waltzer-shaker mutants such as kreisler, dreher, splotch and looptail, the inner ears are affected as well as the neural tube, and since there is evidence in amphibians and in birds that the differentiation of the otic vesicle into a normal labyrinth depends on the influence of the neural tube, Deol suggested that the same kind of phenomenon exists in mammals as well. (See also Deol 1974 and 1976)

This theory is parallel with the theory he proposed for the relationship between pigments, inner ear, behaviour and the neural tube defect.

Deol investigated 16 mutants (spotting genes of coat colour) such as s^{l}/s^{l}, mi/mi, Mi^{wh}/Mi^{wh}, $Mi^{wh}/+$, W^{v}/W^{v}, $Sl^{d}/+$; rs/rs, Rw/+; $W^{v}/+$, Ph/+; $W^{v}/+$, Ph/+; Rw/+, Ph/+; $Sl^{d}/+$, $W^{v}/+$, Ph/+, Rw/+, $Sl^{d}/+$, +/mi, rs/rs, which have pigmentary abnormalities for the relationship of the inner ear and pigments. He found that 12 mutants showed some inner ear defects and all had pigment defects in the inner ear. (Deol, 1970).

From the 16 mutants he found two mutants (piebald-lethal and dominant spotting in which the neural crest was abnormal. The inner ears of those mutants are affected. In the other mutants there is no evidence of neural crest defect but the abnormalities of the inner ear are very similar to the piebald-lethal and dominant-spotting. He assumed that the neural crest might well be affected in those mutants.

As for the inner ear defect he suggested that it could be direct or indirect consequences of the abnormalities of the acoustic ganglion, which partly originates in the neural crest. He put forward this suggestion because in Dc/+ (dancer) he found that the acoustic ganglion

is affected and the inner ear is affected and the abnormalities of the inner ear appears two days later than the abnormalities of the acoustic ganglion.

He put forward two hypotheses to relate the inner ear and pigments defect.

(a) The gene primarily affected the neural crest. Since the neural crest affects melanoblasts (which are derived from neural crest) pigments may be affected. The acoustic ganglion (which partly originates in the neural crest) may also be affected and this in turn affects the inner ear.

(b) An alternative explanation is that either the pigment itself performs some unknown but essential function in the inner ear or the melanocytes do so. This theory could not explain some findings in the 10 albino mice he observed (Deol 1970) which have melanocytes but no pigments but the inner ear is normal. Furthermore the saccule, organ of Corti and the spiral ganglion of some of the 16 spotting genes were severely affected although these organs have no pigment even in normal animals. This explanation is therefore rejected.

There are some remaining difficulties in the 'neural crest' hypothesis. E.g. ($\underline{W^v/W^v}$, $\underline{sl^d/+}$, $\underline{rs/rs}$). There is pigment in the inner ear even when the coat is entirely white. Conversely there is 'spotting' in the inner ear when the head is fully pigmented ($\underline{Ph/+}$; $\underline{Rw/+}$). Such observations argue that either the recipient organ (ear or skin) has some regional specificity also affected by the gene or that the only effect is on the neural crest but that the genes have very precise effects on a mosaic neural crest. This view appears to be at variance with the conclusions of Lyon (1955) based on 6 mutants that "there was no correlation between lack of ear pigment and presence of behaviour defects". Apart from including albino pink-eye and brown which would not be expected to be affected, the other three showed the correlation.

In summary, then, Deol views the primary effect of the various genes as impinging on the neural crest when it is in the process of forming the tube which subsequently affects melanoblasts, acoustic ganglion and otic vesicle development.

None of the observations summarized in points (1) to (13) are at variance with this interpretation. It is useful to review in some detail the observations on various mutants and compare them with our results.

Behaviour and semi-circular ducts

Deol (1966, 1974 and 1976) reviews the evidence.

For example in the mutant shaker-1, the abnormal behaviour was detected at one week after birth but the inner lesion was detected only three weeks after birth. Moreover shaker-1 mice showed degeneration of the macula of the sacculus and the organ of Corti whereas in kreisler the inner ear was so grossly malformed, it was unrecognisable. In those two mutants the behaviour is almost identical. In the degenerative types such as: jerker, shaker-1, shaker-2, pirouette, spinner, varitint-waddler and waltzer which all showed waltzer-shaker syndrome there were differences in the anatomical abnormalities, the only common pathological factor is the degeneration of the macula of the saccule and the organ of Corti. In fidget, twirler, waltzer-type and zig-zag which are also waltzer-shaker type the macula of the saccule and the organ of Corti are normal but they showed one or more semicircular ducts affected. The former group has no defect in the semi-circular canals. He also pointed out that in waltzer type (Stein and Huber, 1960), a dominant with variable penetrance showed no circling or abnormality of behaviour but some mice showed defects of the semi-circular ducts. (Stein and Filosa, 1969).

His suggestion was reinforced by the histological evidence of the nerves on the development of the inner ear in dancer (Deol and Lane, 1966). The heterozygotes (Dc/+) showed waltzing syndrome, and the inner ear and the vestibular ganglion were found to be affected. At 12 and 13 day embryonic stages the nerves supplying the utriculus, anterior semicircular ducts and horizontal semicircular ducts were missing. He suggested that "the absence of the macula of the utricle and the cristae of the anterior and horizontal semicircular ducts is obviously the direct result of the missing nerves, for the macula and cristae arise from modification of the epithelium of the otic vesicle in response to contact with nerve endings". He suggested that the vestibular ganglion also has a direct inductive influence in the formation of the utricle and saccule.

Other evidence came from the mutant Nijmegan waltzer which showed incomplete penetrance for circling behaviour and/or variable expressivity (van Abeelen and van der Kroon, 1967). The inner ear of the affected animals have the horizontal canal either constricted in the middle or incomplete or missing completely. The crista of the horizontal canal is also affected in that it was reduced in size, Deol examined homozygotes nv/nv both normal and behaviourally affected animals to find out whether there is a correlation between behaviour and abnormalities in the horizontal semicircular canal. He found that they were like kinky mice in that abnormalities of the horizontal canal were severe in animals with circling behaviour but he also observed abnormal horizontal canals in a number of mice with normal behaviour. This observations led him to the same conclusions as that observed from kinky mice.

Observations on rotating (rg/rg) mice appear to support his view further. Rotating mice (Deol and Dickie, 1967) which showed waltzer-shaker syndrome have two types of inner ear abnormalities, both morphogenetic and degenerative type. Morphogenetic abnormalities were confined to the semicircular ducts and canals. The abnormality consists in a constriction in the central part of either the horizontal or the posterior vertical duct. The degenerative abnormality is confined to the macula of the saccule. The macula appears to develop up till the age of 40 days and then the epithelium becomes thinner and hair cells become reduced in numbers. Deol observed that the degeneration of the macula of the sacculus shows only after 40 days of age whereas the abnormal behaviour can be seen at 10 days of age. The constriction in the semicircular ducts should have no great effect because he argued that the lumen of the duct is continuous even though it is constricted and he thought it was possible for a pressure wave to pass through the otic fluid. His suggestion was supported by the finding that rg/rg animals with normal behaviour have severe inner ear defects.

In the present study some behaviourally normal animals have subclinical inner ear lesions (See Table 3A and B), but these abnormalities were not as severe as the affected animals where gross abnormalities were found in one or both inner ears. All the affected animals have inner ear defects whether it is severe or mild. These findings seemed to support Deol's hypothesis that the effect of the circling behaviour must have a common cause other than the effect in the inner ears.

In the sections of the affected adult animals only the inner ears were embedded and sectioned. Unfortunately Bouin's fixative did not

fix the nerves very well in the adult and only part of the utricular and saccular nerves were present in the sections. But in the developmental stages (embryos) the nerves and tissues were well fixed and there was no apparent difference in the utricular and saccular nerves of animals taken from his/his mothers compared with the controls.

Neural tube and hind brain

The development of the ear acts as a chain of inductions. The presumptive chordo-mesoderm causes the development of the hind brain and this as a secondary inducer stimulates the development of the ear vesicle. This kind of inductive action of the hind brain on the ear vesicle has been shown by transplantation experiments in amphibians and birds (Waddington, 1937, Jacobson 1966; Yntema, 1950; Detwiler and van Dyke, 1950). Due to technical difficulties there have been no such experiments done on mammals. However, Deol (1964a, 1964b) reinvestigated dreher and kreisler mouse mutants and suggested that in mammals too the inner ear development depends on induction by the hind brain. In kreisler the neural tube in the region of the inner ear is abnormal from the beginning. The normal neuromeres failed to form. Instead of the normal 6 neuromeres in kreisler the first three neuromeres are normal but the fourth was very big and twice the size of the third. The last two neuromeres are either completely absent or very poorly developed. The facial acoustic and glossopharyngeal vagal ganglion complexes which normally lie against the fourth and sixth neuromeres fail to form. They merge into each other and their cells spread out irregularly into the surrounding tissue and along the neural tube,

separating the otic vesicle from the neural tube. Later the inner ear was found to be abnormal. He suggested that the abnormality of the inner ear is due to the consequence of the separation of the otic vesicle from the neural tube and of the abnormal position and the lack of organization of the ganglia. In dreher the abnormalities of the rhombencephalon preceded those of the inner ear by almost 2 days. The thin, kite-shaped roof of the rhombencephalon does not extend far enough posteriorly. He argued that the malformations of the inner ear are consequent on the abnormality of the neural tube.

This suggestion was further supported by an observation of the mutant loop tail and splotch in which the neural tube was known to be affected from the earliest stages, and when he looked at the inner ear it was found that the differentiation of the otic vesicle was abnormal.

In the present case, a few embryos that were taken from his/his mothers at $9\frac{1}{2}$ days showed open neural tubes and at $10\frac{1}{2}$ and $11\frac{1}{2}$ days the embryos showed narrowed hind brains. The sections of the $11\frac{1}{2}$ day embryos showed that the fore brain, midbrain and hind brain all were narrowed, but the inner ear was found to be normal. Unfortunately there were no sections available at $10\frac{1}{2}$ days. Only one morphologically abnormal head and one morphologically normal head were sectioned at $9\frac{1}{2}$ days, and there is no histological evidence that the otic vesicle differs noticeably from the control.

If the hind brain abnormality was the invariable initial event reponsible for all subsequently observable phenotypes, the existence of two stocks with variable penetrance should give certain expectations as has been shown in section IV.

From Table 4 we find that the abnormal hindbrains detected between $9\frac{1}{2}$ and $11\frac{1}{2}$ days have somewhat different values.

	% hindbrain abnormality	% adult behaviour
L X H	21	7
I X H	28	58
H X H	33	73

The numbers are, of course, small and could account for the observed deviation. Furthermore, more subtle than gross morphological abnormalities may occur which could equally lead to behavioural changes. Although these data do not strongly support the hypothesis, they can be accommodated with it. A major remaining problem, however, is the fact that these lesions apparently disappear at later stages.

Otoliths

The normal fully formed otoliths of the mouse consist of a flat mass of small prismatic calcareous crystals in an organic matrix consisting of mucopolysaccharide. The calcium salts of otoliths of all vertebrates are present as calcium carbonate the so called otoconia (Lyon, 1955; Veenhof, 1969; Lim, 1973).

Our observations on the development of the otoliths raises two separate questions. The first is the cause of absence or modification of the calcite crystals, the second is the relation of such absence to behavioural defects.

In pallid mice Lyon (1955) found the otoliths of the inner ear were missing, and, in addition the pigment normally present in the inner ears of mice was found to be absent. She suggested that it was possible that the pigment cells of pallid mice produce some abnormal substance which actively inhibits otolith formation, but she also pointed out that did not seem to be a probable explanation because in the normal mouse the pigmentation of the secretory region was only just beginning at 15½ days when the calcium salts of the otoliths appeared. Nutritionally manganese deficient mice produced a phenocopy of the pa otoliths defect (Hurley, Everson and Geiger, 1958; Hurley et al., 1960). Erway, Hurley and Fraser (1966) fed pallid females with a high manganese content diet during pregnancy and they found that the abnormality of behaviour and the otolith disappeared in the offspring but did not alter the pigmentary defect.

In mocha mouse (Lane, 1967), muted mouse (Lyon and Meredith, 1965, 1969) tilted-head mouse (Kelly, 1958), ocular albino rabbit (Erway, 1968), grey-loco chukar partridge (Erway, 1968) and pastel mink (Erway and Mitchell, 1973)the utricular and saccular otoliths are missing as well as absence or marked reduction of the pigment cells and they also show behavioural anomalies. These animals have pigment mutant genes which interfere with the development of melanocytes within the inner ear. Mn supplementation of pallid mice (Erway et al. 1971) and pastel mink (Erway and Mitchell, 1973) prevents the otolithic defect. Erway et al. suggested that there may be a causal relationship between the presence of pigmented cells in the inner ear and the availability of Mn for otolith development. This hypothesis is supported by analyses which indicate that various pigmented tissue contain more

Mn than tissues lacking melanocytes (Cotzias et al. 1964, Van Woert et al. 1965).

Lim and Erway (1974) suggest that the manganese effect on otolith development is connected with the Mn requirement of enzymes involved in mucopolysaccharide synthesis. Since calcite crystal deposition appears to depend on the otolithic membrane (PAS positive and hence containing mucopolysaccharide) interference in this synthesis could cause a failure or alteration in the crystal formation. The reduction in Mn containing pigment cells would then be regarded as the prior event leading to the failure to produce normal otoliths. The period during which the supplementation is effective is restricted to 11 - 15 days of gestation, the latter part of which is the time of otoconia formation. There is evidence that this period is preceded by a rapid production of sulphated mucopolysaccharides (Shrader et al. 1973).

In the present case the first appearance of detectable calcite crystals was $14\frac{1}{2}$ days and absence of these tiny crystals was taken as evidence of abnormality. They "grow" up to $17\frac{1}{2}$ days when the otolith is fully developed. We also found pigmentary deficiency (although it was not looked for at early stages). As in pallid the otolithic membrane without crystals was clearly seen in affected mice. There is, of course, no evidence that manganese is involved in the present syndrome. No Mn-supplementation has been tried. But even if such experiments are negative, this does not argue that the histidinaemic condition could not result in the same kind of interference as a genetically determined manganese deficiency. If therefore the Erway model is accepted the general similarity of our observations would

make them consistent with such a mechanism. Alternatively, the pigment and otolith effects could be parallel effects of some common disturbance which, at some level has similar results in the two cases. The question of whether the otolith defects are responsible for the behavioural syndrome raises the same problem as the association of general inner ear abnormality and behaviour already discussed.

Experimental work on the labyrinth has suggested that the utriculus is concerned with the maintenance of muscle tone and with responses to tilting, unusual position and linear acceleration (Tait and McNally, 1934).

There seems to be considerable uncertainty about the function of the saccule (Loewenstein, 1936, 1950) who argued from extirpation experiments (not in the mouse) that the role in balance responses is doubtful. In pallid Lyon claimed that the sacculus has a definite effect on the response to a position change (Lyon 1951). She gave this suggestion for pallid mice that in all animals which lacked otoliths failed to respond, but the presence of the otoliths in the sacculus of one ear resulted in a normal response. Erway, similarly, assumes a causal relation between the morphological and functional phenomena, although he is cautious in asserting this. Such correlation as is found is equally explicable by the 'common cause' argument. Very scanty information on the possible involvement of nerve abnormalities in pallid is available.

In our present case too in the behaviourally affected animals, otoliths are completely absent or sometimes present in sacculus but

absent in utriculus or present in one ear but absent in another. Big crystals were frequently found instead of normal small crystals in embryos as well as in adult. They are found also in behaviourally normal animals but born from his/his mothers. So it seems that the change to big crystals does not have any effect on the function. In the present case, it is difficult to say whether the defect of the behaviour is due to defect in otoliths or in semicircular canals for in the affected animals the semicircular canal as well as the otoliths are affected.

The "melanocyte-manganese-otolith" hypothesis of Erway is in no way inconsistent with Deol's views, being a hypothesis of a particular part of the syndrome. The melanocyte abnormality could arise from a disturbance of the neural tube even though no evidence for gross abnormality has been reported.

Mechanism of distension

The adult and embryological study showed that the inner ear defect could be detected as early as the 14½ day embryos where the minute pinpoints of calcium salts were found to be missing and from 16½ days onwards the whole of the endolymphatic system was found to be dilated. Similar observations were obtained in mouse mutants such as kreisler, dreher, pallid and late adult in humans such as in Meniere's disease. While the observations establish clearly the sequence of events, the mechanism by which these abnormalities are brought about must now be discussed.

Lyon's osmotic theory

In pallid, the dilation of the endolymphatic system was found at two stages, namely at 16½ and 17½ day embryonic stages and not at

earlier than $16\frac{1}{2}$ or later than $17\frac{1}{2}$. Lyon (1955) claimed that this effect was temporary and this could be due to osmotic pressure. She stated that possibly the absence of crystals and the distension had a common cause. This present study of maternal effect of histidinaemia suggests that, if either of these mechanisms operate, the latter is more likely, since as described above, big crystals were found in the distended lumen of the sacculus, showing that the distension could not be the result of absence of the otoliths in the present case. She gave two suggestions. One possibility she put forward was that the secretion of calcium ions was normal but failed to deposit and thus free calcium ions would raise the osmotic pressure thus leading to hydrops. The alternative assumption she suggested was that other substances which remain in solution must have increased the osmotic pressure. She found that there was debris in the sacculus and endolymphatic sac and she suggested that this debris was exerting some osmotic effect. In the present case too, this kind of debris was found in the distended utriculus, sacculus, cochlea and endolymphatic sac, but the distension was found to be persistent up till the adult stage. In pallid, the cochlea, ductus reuniens, sacculus and endolymphatic sac were found to be distended but not the utriculus. The same kind of hydrops, excepting the utriculus, is also found in the Meniere's disease of man (Lempert, Wolff, Rambo, Wever and Lawrence, 1952). In the present study, the whole of the endolymphatic system including the utriculus was found to be distended. Pallid therefore differs from the present case in being (a) temporary and (b) restricted distension. It is nevertheless possible that the osmotic hypothesis applies to our condition, although

its duration and extent may be greater. In a later section we shall discuss an alternative mechanism.

The longitudinal flow theory

The explanations of dilation of the endolymphatic system suggested by many authors are conflicting. Guild (1927) who made experiments on normal guinea pigs trying to find the circulation of the endolymph concluded that "endolymph is formed by the stria vascularis, flows toward the basal end of the cochlear duct and through the canalis reuniens into the sacculus and from this through the ductus endolymphaticus into the saccus endolymphaticus, and leaves the membranous labyrinth by passing through the wall of the pars intermedia of the saccus into the numerous small blood vessels of this region". He also suggested from his observation that the endolymphatic sac had a definite function and served as the principal place of outflow of the endolymph. In that paper he also suggested that if the endolymphatic sac was destroyed there would be accumulation of endolymphatic fluid and distension would result. This Guild's theory of the flow of endolymph is known as the longitudinal flow theory.

Guild's theory was contradicted by Lindsay, Schuknecht, Neff and Kimura (1952) who did experiments on adult monkeys and cats.

In monkeys they destroyed the endolymphatic sac and part of the endolymphatic duct but they found no changes in the membranous labyrinth. In the cat they completely destroyed the endolymphatic sac and the duct but there was no histological or functional injury to the inner ear, so they suggested that "these should not be interpreted to indicate that these structures perform no function. Some service may be performed

which can be assumed by other structures in their absence. But destruction of the endolymphatic sac in cats resulted in mild endolymphatic hydrops which was discounted as a possible preparation artefact or manifestation of mild labyrinths (Schuknecht and Kimura, 1953). The studies carried out by Kimura (1967) on guinea pigs by destruction of the endolymphatic sac showed severe endolymphatic hydrops. It is likely that species difference may account for these varying responses and dysfunctions of the endolymphatic sac. Such experimental destruction of the endolymphatic sac which results in mild hydrops in the cat and severe hydrops in the guinea pig supports Guild's (1927) suggestion that the endolymphatic sac has a resorptive function. Moreover genetic absence of the endolymphatic sac in two mouse mutants, kreisler and dreher is associated with gross distension of the endolymphatic system.

In the present study, the distension of the endolymphatic system could not be explained as in kreisler and dreher where the endolymphatic hydrops could be due to absence of endolymphatic duct. For in the affected animals during embryonic stages the whole of the endolymphatic system was distended including the endolymphatic duct and sac and cochlea and the endolymphatic duct seems to be of normal length. Unfortunately in the adult animals, as only the inner ears were embedded and sectioned, the endolymphatic sac was not observed. But up till the birth the endolymphatic sac appears in the section and the enormous distension of the endolymphatic duct and sac could be found.

In Meniere's disease the cochlear duct, saccule, endolymphatic duct are swollen (Lempert, Wolff, Rambo, Wever and Lawrence, 1952). Meniere's disease hydrops and experimental hydrops are similar. There

was good correlation between pathologic changes of the endolymphatic sac and Meniere's disease. The endolymphatic sac is considered, therefore abnormal in patients with Meniere's disease and apparently plays a significant role in the pathogenesis of clinical endolymphatic hydrops in man (Kaufman, Marovitz and Sharnbaugh, 1969). They also pointed out that in patients diagnosed as having Meniere's, the perecentage abnormalities of the endolymphatic sac or duct found was 95%.

The radial flow theory

Naftalin and Harrison (1958) proposed a radial flow theory as compared to the longitudinal flow theory of Guild. These authors suggest that the fluid flow proceeds from perilymph through Reissner's membrane to endolymph with the stria vascularis acting as a selective absorbing site. This theory was supported by Lawrence et al. (1961) who examined in guinea pigs Reissner's membrane and basilar membrane with electron microscope. They found the basilar membrane consisted of four layers of cells. They suggested that the membrane appeared to be so constructed that it could serve as a selectively diffusing membrane. If this theory is accepted (and it appears to be founded on more recent and firmer evidence) the cause of any distension must be attributed to changes or faults in Reissner's membrane. It is perhaps significant that we observe distensions, thinning and other abnormalities in the Reissner's membrane (See Fig. 21C). Dohlman (1965) and Lawrence et al. (1961) suggest that Meniere's disease may be due to the consequences of the rupture of Reissner's membrane.

The excess tissue theory

Hertwig (1951, 1956) who studied the inner ear of the shaker with syndactylism mutant of the mouse found that the sacculus, utriculus,

the ampullae and the semicircular canals were collapsed. In the cochlea, Reissner's membrane was found to be collapsed as well and she suggested that these abnormalities could be caused by an insufficient production of the endolymph. Deol (1963) reinvestigated this problem and he found the first visible abnormality in the mutant labyrinth was the excessive amount of mesodermal condensation between the saccule and the utricle at 13 days of development. He also observed the slackening of Reissner's membrane in the cochlea and the swelling in the sacculus, his explanation for these abnormalities was that they were due to the excessive amount of tissue which was either concentrated in the centre of the membrane or evenly spread out. He also observed that the reduced membranous labyrinth was first apparent at 2 days after birth. At that time the stria vascularis which is thought to be the source of the otic fluid was in the earliest stage of its differentiation and he suggested that it had not started to function. This observation is contrary to Hertwig's explanation. Another of his observations was that the shrinkage of the membranous labyrinth was not uniform. The cochlear duct and semicircular ducts were closed at one place and wide open at another and the endolymphatic duct was dilated instead of collapsing as Hertwig suggested and the otic labyrinth kept on growing smaller even when the endolymphatic sac and parts of its duct had degenerated.

In the present study, the infoldings and outpocketings of the Reissner's membrane, saccular membrane and utricular membrane were found to be similar to the sy/sy case. It is, however, different to this case since in sy/sy mice, as Deol described, only part of the endolymphatic system

was dilated while in the present strain the whole of the endolymphatic system was dilated. Moreover there is no excessive mesodermal tissue observed between the saccule and the utricle at the early stages as in sy/sy mice. The main evidence that, in our case, 'excessive tissue' is not the explanation for the increased length of the membrane comes from the observation of the distances of nuclei in the cell layer of the membrane. These are further apart in the abnormal (long) membrane than in the normal. (Fig. 21C).

It is therefore concluded that the abnormalities observed are due to distension forces of the fluids during development. The precise cause of this is however, not apparent from the observations discussed.

Deafness

In discussing deafness three separate questions must be considered. (1) What lesions in the auditory apparatus (inner ear, outer ear auditory and other nerves) results in functional deafness? (2) What is the relation of the observed morphological abnormalities with deafness? (3) What are the correlations or causal relationships between these as well as with other abnormalities?

There are many possible mechanisms by which deafness can be caused. Ormerod (1960) in his paper on "The pathology of congenital deafness" pointed out that human deafness may be caused by:

"A. Failure to develop:

1. of the bony cochlea;
2. of the membraneous cochlea;
3. of the organ of Corti and the tectorial membrane;
4. of the conducting mechanism;
   (a) the middle ear and ossicles;
   (b) the external auditory meatus.

B. Interruption in development:

1. of the organ of Corti and the tectorial membrane;
2. of the ossicles of the middle ear;
3. of the external canal.

C. Degeneration of parts of the auditory apparatus which have already developed in some degree or have reached maturity.

1. of the canal of the cochlea or scala media.
2. of the sensory endorgan including the tectorial membrane.
3. of the nerve elements including the spiral ganglion and the basal nuclei."

All of these could be the result of inherited factors or due to toxic influences caused by maternal illness or adverse events in later life. In reviewing the evidence it should be remembered that in the mouse, although cochlear development starts at about 13 days of gestation, it continues until about the 12th day after birth. In view of this a precise distinction between 'degenerative' and 'morphogenetic' types may be difficult to make.

Most of the degenerative inner ear types that had been reviewed in the introduction were deaf. In all the deaf mutants the cochlear part of the inner ear was either affected before birth or showed degeneration later in life. In pirouette, shaker-1, and waltzer mice (Deol, 1956) the pathology is very similar. The organ of Corti developed normally for some time after birth in all three mutants, except that the tectorial membrane is a little thicker than normal. In all three mutants, and in varitint-waddler, jerker and shaker-2 (Deol 1954), the hair cells of the cochlea gradually shrink, the supporting cells lose their characteristic shape and form a mass of dedifferentiated tissue.

The spinner mouse (Deol and Robbins, 1962) is said to be deaf from birth, but the first sign of abnormality was found in the cochlea at the 15 days after birth. The hair cells in the organ of Corti are reduced in size and as they become older the hair cells reduced still further and gradually the hair cells disappeared completely. The tectorial membrane lost its connection with Corti's organ and it is free and floats in the scala media. The spiral ganglion and stria vascularis are also degenerated. In the mutant deafness, the earliest abnormalities of the organ of Corti and the stria vascularis are seen at the age of 10 days. The tectorial membrane does not lie over the hair cells but curls up. In mocha the abnormalities of cochlea were detected only at 27 days. Degenerative changes were observed in the organ of Corti, the stria vascularis and the spiral ganglion. The tectorial membranes too lost contact with the hair cells. In Snell's waltzer the first abnormality found was in the organ of Corti. The hair cells begin to degenerate at 12 days old. The tectorial membrane lost contact with hair cells. The animals were said to be totally deaf from birth.

In the above stated degenerative types of mouse mutants, the deafness sets in, in the scala media or the sensory end organ including the tectorial membrane or the nerve elements including the spiral ganglion and the basal nuclei. From this evidence it is by no means certain whether degeneration of some parts of the cochlea precedes changes in the spiral ganglion and other central nervous system changes since many of the structures probably require an intact nerve supply for their maintenance.

There are some mutants where there is clear evidence of early developmental abnormalities in the cochlea. These are, dreher, kreisler and shaker with syndactylism. There is one mouse mutant, briefly mentioned by Deol (1976), namely sightless (sig/sig) which, like the present case, appears to have early morphogenetic changes in the cochlea (14 - 16 days). (Khaze 'I. PhD Thesis London University 1974).

In the present case the abnormalities of cochlea can be seen by the distension of the cochlear duct from $15\frac{1}{2}$ days of embryonic stage and become more severe from $16\frac{1}{2}$ days up to the adult stage. But in the adult the abnormal structures of the organ of Corti and tectorial membrane are similar to the anomalies of the cochleas of the degenerative type. Associated with the abnormal cochlea of the adult there was disorganisation of ganglion cells, acoustic nerve and blood vessels. It is likely, though not certain, that these are secondary to the morphogenetic disturbance of the cochlea.

In summary, then, the deafness in our case is likely to be caused by the early distensions of the developing cochlea. Whether the disorganisation of the nerves supplying the cochlea is parallel with or a consequence of these changes is uncertain.

The development of the abnormality is fully described in Section II. Human cases of congenital deafness which are due to the failure of the internal ear to reach mature development have been grouped into six types and named after the first investigators who gave a full description of the changes in the ear. (Ormerod, 1960).

1. Michel type - Complete lack of development of the internal ear.

2. Mondini-Alexander type - Development only of a single curved tube representing the cochlea and similar immaturity of the vestibule and canals.

3. Bing-Siebenmann type - Bony labyrinth well formed but membranous part and particularly the sense organ is under-developed.

4. Scheibe type - Cochleo-saccular type. Vestibular part of ear developed and functioning. - Malformation restricted to membranous cochlea and saccule.

5. Siebenmann type - Changes mainly in middle ear and often due to hormone deficiency.

6. Microtia and Atresia of Meatus - Abnormalities mainly in external ear.

Deol (1968) in his review article stated that there is no mutant with complete absence of the internal ear. The most affected inner ear was found in kreisler where the inner ear was unrecognizable. Shaker with syndactylism seemed to fall in the group (4), Scheibe type, where the malformation of the inner ear was found in the membranous cochlea and saccule. The abnormalities of the cochlea found in animals born from his/his mothers are fairly similar, although they do not fall exactly into any of the above 6 groups classified by Ormerod. In Scheibe-type the tectorial membrane is formed but is distorted and flattened down over the organ of Corti and its supporting cells. The stria vascularis is degenerate. In the present case, too, the tectorial membrane is distorted but not flattened down on the organ of Corti and the stria vascularis is degenerated as well. In some cases of the Scheibe-type there is dilation in the cochlea but in the present

case the dilation is not found only in the cochlea but also in the sacculus, utriculus and ampullae as well. The abnormalities are not restricted to the cochlea and sacculus only as in the Scheibe type but extend to the utriculus and ampullae of the semicircular canals. The origin of these dilations has already been discussed under 'The mechanism of distension'.

The association of pigmentary deficiency with balance defects and with otolith abnormalities has already been discussed. In view of the close correlation of the various aspects of the syndrome there appears to be a relation between deafness and pigmentation. Such association has been noted in e.g. the white English Bullterrier; the Dalmation, some Persian cats and Angora rabbits (Ormerod 1960). Concerning man, LaFerriere et al. (1974) state:

"The biological relation between sensorineural deafness and pigmentary disorders is a fascinating area for speculation and research. Brown et al. (1971) estimate that pigmentary disorders may be involved in as much as 10% of the childhood deafness due to major genes. Certainly the association of patchy or total pigmentary disturbances affecting the hair, eyes, and skin with sensorineural deafness is characteristic of syndromes occurring in many mammalian species (Johnsson et al., 1973). In man, one of the best known examples is the Waardenburg syndrome. Fisch (1959) offers a provocative explanation for the seemingly unrelated pigmentary disorder and sensorineural deafness that comprise this syndrome, based on a theory of maldevelopment involving the neural crest. According to his hypothesis the developmental origin is a genetic fault in the neural crest, which subsequently dictates pathology

in many of its derivatives, including the inner ear and the pigment cells in many other locations." This is remarkably similar to Deol's view (although this is not mentioned in the paper) concerning the relation of pigment and circling.

From Tables 3A, B and C we can prepare a summary of the concordances of the different characters affected (Table 26). It can be seen that there is a very close correlation between behaviour, hearing pigments and cochlea. Consequently no conclusions as to their possible causal-developmental relations can be drawn. They are as likely to be related by descent as by a common cause.

Table 26

Correlation between, behaviour, hearing, cochlea and pigments in adult animals.

B/H	B/P	H/C	H/P
75 +/+	74 +/+	73 +/+	75 +/+
	1 +/- (1)	2 +/- (1)	
		2 -/+	
	4 -/- (1)	2 -/- (1)	4 -/- (1)
47 -/-	43 -/-	39 -/-	43 -/-
122	122	122	122

122 animals were available for comparison. The four columns give the concordances between two different phenotypes . B = behaviour; H = hearing; P = pigment (vestibular); C = cochlea. The sign "+" indicates normal, "-" abnormal (in any way). (The (1) means that only one of the two ears was affected. Thus in e.g. column 3 "2 -/- (1)" means that 2 animals were found which had impaired hearing and showed cochlear abnormality in one ear.

It should be remembered that there are a number of well known cases (pallid, twirler, zig-zag, fidget) which have abnormal behaviour but are not deaf. The 'common cause' hypothesis must then assume that in these cases the initial action of the gene is not sufficient to extend (in time?) to the development of the auditory apparatus.

Penetrance and Expressivity

Differences in penetrance can be caused by variation at loci which do not affect histidine levels. These genetic background differences, however, cannot be responsible for the within litter variation. It could be argued that both the 'high' and 'low' stocks were segregating for these background genes (i.e. that they were not homozygous as is assumed in the analysis of the crosses) and that unaffected offspring were heterozygous for the (dominant) low penetrance allele (L). If that were so, then breeding from such individuals would be equivalent to a backcross. In fact, all the 'pure line' crosses were carried out using non-affected females. They generated, in the high line, continued high penetrance. They were therefore genetically 'high' and the cause of their being unaffected must therefore be 'environmental'.

Increase in histidine does not result in increased penetrance of behavioural or morphological abnormalities. The environmental difference cannot, therefore, be fluctuations in histidine concentrations. This is reinforced by the analysis of individual offspring from whole litters. Furthermore differences between ears of the same individual make a biochemical fluctuation hypothesis unlikely. The maternal

histidinaemic condition must therefore be viewed as one of the factors leading to a possible disturbance of early development. Genetic factors can increase or decrease this possibility. Fluctuations of some kind, acting on these two factors, can make them go over some 'threshold'. Although it has been convenient (and sometimes necessary) to classify (individuals, structures, ears, etc.) into 'abnormal' and 'normal', such classification conceals a considerable range of effects. Expressivity merges into Penetrance. A close examination of the evidence presented looks more like a continuous than a discontinuous range of variation. This is particularly evident from the sectioned material but even at the behavioural level the phenotypes vary from violent circling to barely perceptible head tossing. The classifications therefore reflect more the mean of the variation rather than define a true threshold.

Since we have argued that the biochemical disturbance is not likely to be related in any simple quantitative way to the resulting abnormality, we must speculate where the effect is likely to have its primary focus, where, in fact, the fluctuation observed can have their origin. If we accept that a disturbance at the neural tube is the primary event which, through the complex series of events which follow, may have morphological and behavioural consequences, it is this event which must be subject to the fluctuations. The first thing to note is that, because of the bilateral symmetry of development, disturbance on the 'left' need not be reflected by disturbance on the 'right', although one may expect a high correlation between the two. Secondly, the correlation of severity at all levels is also expected on this

'hierarchical' view of the syndrome. The interaction of neural tube, otic vesicle and acoustic ganglion then places the phenomenon in the morphogenetic class of phenomena where physical factors such as proximity, rates of movement, and inductive chains are variables of the processes. These normal variations, in the normal mouse, are not such as to result in abnormal development in the vast majority of cases. Subjected to the histidinaemic condition on the other hand, the normal buffering is disturbed or the mean of the fluctuations is increased. Viewed in this way we can consider the maternal effect as amplifying a 'normal' range of variation. The background genes, of which we have uncovered possibly one pair of alleles, could then be regarded as a 'mild' form of 'behavioural' genes of which so many 'strong' alleles have been detected in the past.

This work started with the notion that, unlike the previously investigated cases of genes with unknown products, a precisely defined metabolic condition had demonstrable morphological and functional consequences. There was therefore the hope that a detailed investigation would reveal the 'bridge' between molecule and morphology. While a good deal of insight into many aspects has been gained, we are still looking across the valley from both sides without knowing where this bridge is.

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DECLARATION

I declare that this thesis was composed by myself and that the work contained within it was my own.

Signed Khin Mya Mya .....

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