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Streptomycin in the chick embryo: post-hatching vestibular behavior and morphology

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Summary. Developing chick embryos were exposed to streptomycin injected on days 5 through 13 of the 21 day developmental period. Histological and behavioral abnormalities were found almost exclusively in chicks exposed after day 7. The nature of the behavioral deficits included abnormal head posture, head tremor, and inability to compensate for applied vestibular stimuli. Head movement measurements showed that the head tremor had frequencies of oscillations from 10–35 Hz. The amplitude of the tremor was a large as 10°. Histology showed damage to the secretory dark cells of the membranous labyrinth in those chicks that showed behavior changes. Even with increased dosages chicks exposed prior to day 7 rarely showed abnormal vestibular behavior but instead experienced increased mortality. Further tests examining tissue levels of streptomycin showed little or no streptomycin in embryos until day 10. These results are discussed in terms of their utility as an alternative model to surgical manipulation of the vestibular system in developing embryos. Behavioral consequences are compared to other work with drugs and to the effects of weightlessness and unusual environments on vestibular orientation and behavior.

Key words: Streptomycin – Vestibular – Development – Histology – Chicks

Introduction

The vestibular system plays an important role in spatial orientation. Its disruption by disease, injury or environmental changes can result in profound disorientation. Experiments that control vestibular stimuli or modify portions of the vestibular end organ have been instrumental in understanding how the vestibular system contributes to the perception of orientation (Howard and Templeton 1966; Howard 1982). Much of this past research has dealt with the consequences of a change in vestibular function after full development has been attained. Only a few animal studies have investigated the effects of vestibular disruption during development. In the chick, several studies have examined orientation behavior and morphologic consequences of unilateral and bilateral otocyst ablation in the embryo (Heaton 1972; Decker 1970; Levi-Montalcini 1949). Such modifications during development have resulted in abnormal orientation behavior and changes in viability as well as derangements of brainstem anatomy in the developing embryo.

Our goals in these experiments were to develop a non-surgical method of destroying vestibular function in the embryo and to study the behavioral and histological consequences of this destruction. Previous experiments in *hatched* chicks have shown that continuous exposure to streptomycin over 7 days will produce vestibular damage that can be observed both behaviorally and morphologically (Park and Cohen 1982). In our experiments, we used the otoxic antibiotic streptomycin to damage the embryo's developing vestibular system and studied the histological and behavioral consequences in the hatched chick. Only a single dose of streptomycin was necessary, probably since it is excreted unchanged by the kidney and this is recycled in the closed system of the egg.

Methods

Three experiments were performed: The first examined the effect of streptomycin when introduced at different stages of develop-

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ment on vestibular function. The second examined the effect on vestibular function of increasing dosage of streptomycin when introduced at early and late stages of development. The third examined the fate of streptomycin once it was injected into the egg.

Experiment 1. Varying day of injection

Procedures. To examine the relationship between streptomycin ototoxicity and embryonic development, 350 fertilized chicken eggs (Gallus domesticus, SPAFAS Inc. Norwich Ct.) were divided into 7 experimental groups of 48 eggs each and a control group of 14 eggs. Each experimental group was assigned to one of 7 separate injection days corresponding to 5, 6, 7, 8, 9, 11, or 13 days after incubation. Prior to incubation, eggs were stored at 9° C (Gottelieb 1962) for 1–5 days to facilitate handling of the chicks that hatched. The eggs were incubated in a moving air incubator (Humidier model 200) set to 37.5° C and turned through an angle of 90° every 8 h. Humidity was maintained at approximately 52%. On the day of injection, 2 to 5 embryos were separated from the group and staged by the Hamilton-Hamburger (1951) method to estimate the age of the embryos. Prior to injection, each egg of the group was candled to remove dead embryos (averaging 4 eggs per group) and to choose an injection site. A space near a large chorioallantoic vessel was identified and marked on the shell with a pencil. The egg was then weighed (range of 48–75 g) using a balance scale and a volume of 22% solution needed to produce a dose of 400 mg of streptomycin sulfate per kilogram egg weight (mg/kg) was calculated (averaging 0.1 ml/egg). A small hole (0.5–1.0 mm diameter) was produce in the shell with a hand held electric drill (Dremel Co.). The 1 cm long needle of a standard tuberculin syringe was inserted perpendicularly through the hole to its full length, and the piston of the syringe slowly depressed. Vigorous injection of the solution was avoided since previous X-ray experiments employing radiopaque dyes (unpublished data) demonstrated that too rapid injection of liquid into the egg by this technique could produce a jet capable of tearing the various delicate membranes present within the egg. The hole in the shell was then sealed with wax and the egg returned to the incubator.

After the expected 21 day period of incubation the chicks began hatching. At this time the embryos were transferred from the incubator to a hatcher where the temperature was held at 38° C with humidity of 88%. Hatching took anywhere from 5 to 48 h for the chicks that survived. After hatching, each animal was marked with a leg tag and placed in a brooder held at a temperature of 29° C.

Behavioral tests. Each animal's vestibular behavior was assessed between 60 and 90 min posthatching by observing head tremor, post-rotatory head nystagmus, and general motor reflexes. After these tests had been performed, the animals were returned to the brooder, where they were observed for at least 3 days and in most cases for a week or more.

Post-rotatory head nystagmus was induced by spinning the animal clockwise on a rotating disk at 1.3 rev/s for 35 s and then suddenly stopping the disk. Animals were tested with and without vision. An aluminum foil hood that completely covered the chick's head and eyes was used as a blindfold (Wallman, personal communication). Without a blindfold, the animal had a rich visual surround to view in the laboratory.

Head tremor was scored qualitatively on the four point scale used by Park and Cohen (1982) of: no tremor, slight tremor (characterized by induced, low amplitude, low frequency movements), moderate tremor, and severe tremor (characterized by spontaneous, large amplitude, high frequency movements). In addition, head movements from a select group of animals were recorded 1, 2 and 5 days after hatching using a magnetic search coil system as described below (Head Movement Measurement System).

Examination of general motor control was divided into 3 components: 1) head compensation to body tilt, 2) recovery of erect posture, and 3) reflex coordination. Test 1 was conducted by holding the animal in one hand and slowly rotating it in the sagittal plane until inverted. Test 2 consisted of rolling an animal onto its back and observing whether it was capable of restoring erect posture within 90 s of concerted effort. For test 3 (Kovach 1970), an animal was placed on its back in a stable position. The head was turned toward one side, and the experimenter observed if the chick extended the ipsilateral leg and contracted the contralateral leg.

Behavioral evaluation formula. Animals were classified as normal or abnormal by distilling the results from the behavioral test into a single boolean data point. It was felt that to compensate for the variability in any one test that a coincidence of abnormalities would be found in a vestibularly impaired animal. Thus for each chick, the data from all five test were condensed using the formula:

$$D = \frac{2(T + N) + 1.5(HR + PR + RR)}{5}$$

where T is tremor, N is nystagmus and HR, PR, and RR stand for head righting, postural righting, and the righting reflex, respectively. Each variable was given a score of 1 if positive or 0 if negative; an animal was described as abnormal when $D \geq 1$. In practical terms, this meant that three of the five tests must be positive and that one of the three positive tests was either head tremor (T) or post-rotatory nystagmus (N). These latter two tests have been shown to be highly correlated with vestibular damage produced by streptomycin (Park and Cohen 1982).

Head movement measurement. Head position in three chicks was measured 1, 2 and 5 days after hatching. The chicks were chosen for their vigorous and spontaneous head tremor. Head movements were measured using a magnetic search coil system (Robinson 1963; Rummel 1984). Briefly, a coil of wire attached to the head transforms an oscillating magnetic field into a time varying voltage. This signal is amplified and the subsequent detection results in a signal which varies as the sine of the angle between the axis of the coil and the magnetic field. However, for small angles the system is reasonably linear without the sine correction.

The search coil used to record head movements was composed of a forty-turn coil of no. 44 magnet wire and was affixed to the top of the chick's beak with paraffin wax. The search coil system had a bandwidth of 900 Hz, a sensitivity of 400 mV per 5°, and a linear range of ± 20° in both horizontal and vertical directions.

The animal was secured to a platform inside the field coils such that the search coil remained within the linear region of the oscillating magnetic field. This made the system insensitive to translational movements and therefore measurement of pure horizontal and vertical rotation of the head was possible. Recordings were made for approximately 10 min in which the chick was free to make natural head movements in horizontal and vertical planes.

The resulting analog signal was recorded on-line by a digital computer (DEC PDP 11/34). The sample rate was 400 samples/second using a twelve-bit A/D converter which gave a resolution of 0.012°. The resulting records were then analyzed by hand and with the aid of a computer. Hand analysis recorded amplitude of the movements while the computer was used to calculate the power spectrum of the head oscillations.

Histology. Animals were selected for histologic examination from 4 groups: 1) those receiving streptomycin on developmental day 12

or later (stage 38) showing vestibular behavioral abnormalities upon hatching; 2) those receiving streptomycin on developmental day 7 (stage 30) showing no abnormalities of vestibular behavior; 3) Animals receiving 400 mg/kg on day 12 and showing vestibular behavioral abnormalities at hatching, but which were allowed to recover normal behavior (1 week) before histological examination; and 4) control animals treated only with saline. In addition, tissue from group 1 was processed for electron microscopy.

Birds were anaesthetized with ether and fixed via cardiac perfusion through the apex of the left ventricle. Outflow was provided through a puncture made in the right atrium. For fixation, approximately 150 ml of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2–7.4) was introduced through an 18 gauge needle.

Immediately on completion of perfusion, the head was separated from the body and divided in the midsagittal plane. The brain was removed and additional fixative was introduced via needle directly into the porous temporal bone. The process was repeated through the external auditory canal. Trimmed temporal bones were stored in cacodylate buffer, gently decalcified in EDTA, and infiltrated and embedded whole in glycomethacrylate plastic. Sections 2.5 microns in thickness were cut using glass knives on a Serval JB-4 microtome, stained with toluidine blue and examined and photographed with a Zeiss microscope.

Experiment 2. Increasing drug dosage

Procedures. A total of 97 fertile chicken eggs were divided into two groups of 45 eggs with the remaining 7 eggs set aside as controls. One group was injected after 6 days (stage 27) of incubation and the other after 12 days (stage 38) of incubation. On the day of injection, groups of nine eggs were injected with streptomycin at either 400, 700, 1000, 1200, or 1600 mg/kg. The procedures used for injecting, weighing, incubating, and hatching of the eggs including evaluation of post-hatching behavior and histological procedures were the same as in experiment 1. No quantitative recordings of head movements were made on this population of chicks. Finally, a control experiment using quantities of sterile saline varying from 0.1–0.5 ml was performed to account for any effects due to the added fluid volume in the egg when delivering high dosages to the egg.

Experiment 3. Tissue drug levels

Procedures. A total of 60 viable incubating eggs were divided into six equal groups and treated with single injections of streptomycin at 400 mg/kg on days 5–10 after the start of incubation (stages 25, 27, 29, 32, 35, and 37). Each group of ten eggs was further divided into two groups of five each, and harvested either after 45 min or 48 h of further incubation with streptomycin. The eggs were opened into a petri dish and the embryos were rapidly dissected free of the surrounding strucures including the amnionic membrane. Each embryo was then rinsed with sterile saline and briefly held against filter paper to remove excess fluid. Embryos were sealed in small dry glass bottles and stored at –70° C. At the time of tissue drug level determination, the embryos were thawed and weighed. Then 5 or 10 ml of saline was added to each embryo, which was then reduced to a fine suspension by treatment with a Brinkman Polytron PCU-2 homogenizer with a 7 mm probe. The mixture was allowed to sit for 30 min and then centrifuged at 1500 rpm for 10 min, which resulted in a clear supernatant which was drawn off and refrozen for later testing. Supernatant streptomycin levels were measured by the method of fluorescence polarization immunoassay (Abott Diagnostics), and whole embryo streptomycin concentrations were calculated.

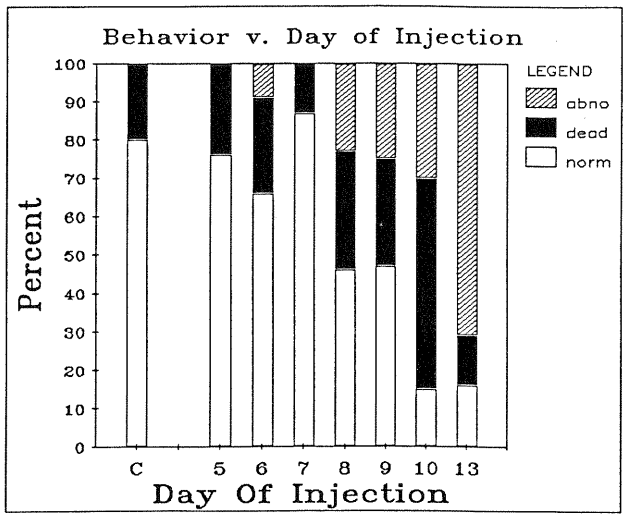

Fig. 1. The behavioral results from chicks dosed with 400 mg/kg between days 5–13 during development

Results

Experiment 1. Varying day of injection

A single injection of streptomycin at 400 mg/kg introduced after day 7, *in ovo*, resulted in vestibular abnormalities in newly hatched chicks. The proportion of affected chicks depended on the day of injection. Figure 1 shows that the percentage of chicks with abnormal behavior increased with stage of development, from a minimum of zero at day 5 (stage 26) to a maximum of 70% at day 13 (stage 39). Accordingly, the percentage of normal chicks steadily declines from 78% at day 5 (stage 26) to 15% at day 13 (stage 39). In the control population, 80% of the chicks hatched and all were normal. Therefore, except for days 5–7 (stages 26, 27, 29), all injected populations showed large differences in the proportion of normal chicks as compared to controls. At the same time, the percentage of chicks that failed to hatch remained generally constant over this period, except for day 10 (stage 35), and was similar to the percentage found in the control population.

The results shown in Fig. 1 were distilled from multiple behavioral scores generated by Eq. (1). The distribution of animals showing specific behavioral abnormalities for each day is shown in Fig. 2. The data from the control population shows several chicks with single positive tests but none with combined scores that exceeded the criterion defining abnormality. For the injected population, the rise in the percent abnormal animals shown in Fig. 1 is correlated with an overall increase in the number of positive responses in each test category. However,

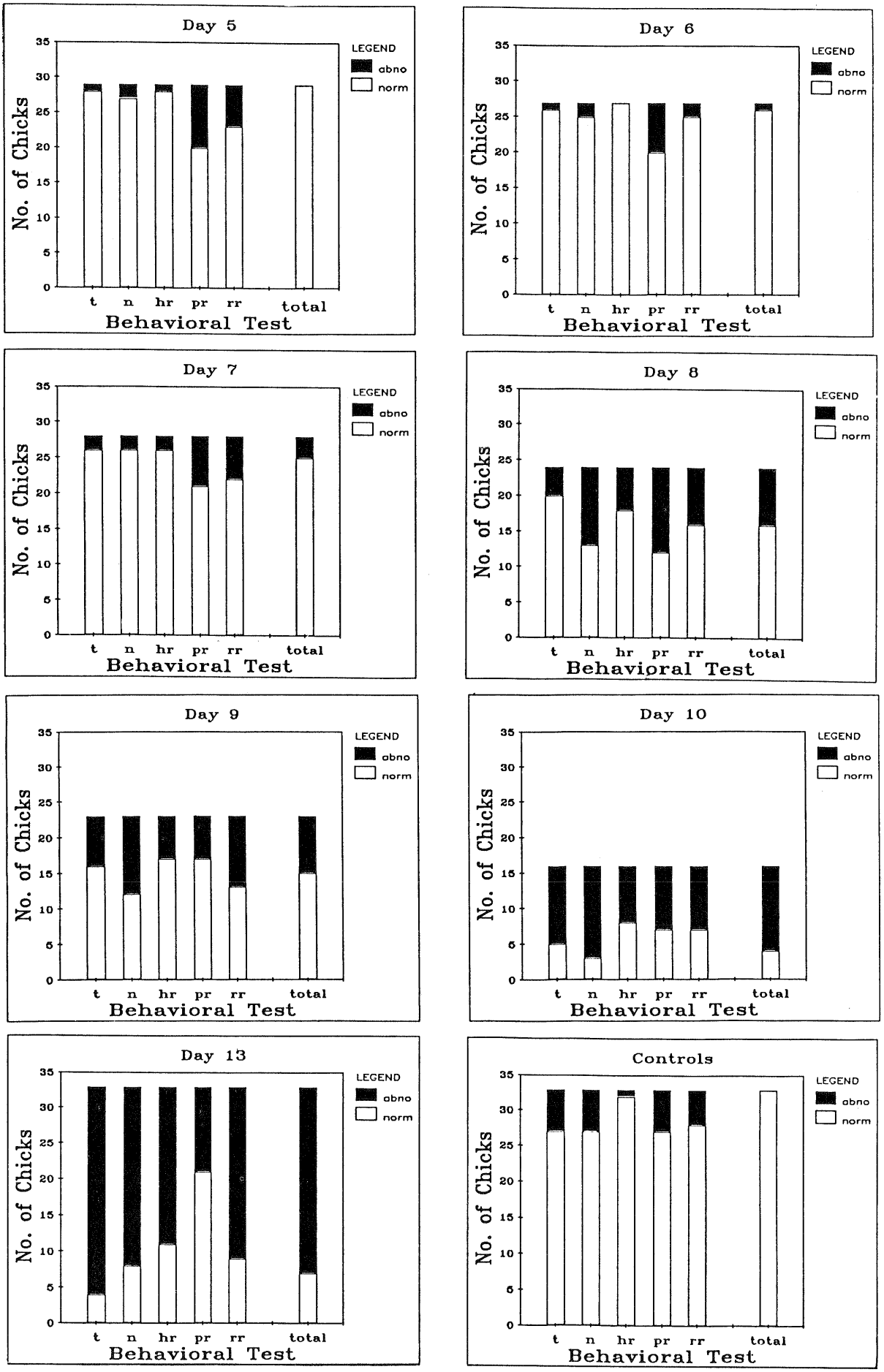

Fig. 2. The breakdown of the results in Fig. 1 into behavioral categories for each group of chicks receiving 400 mg/kg

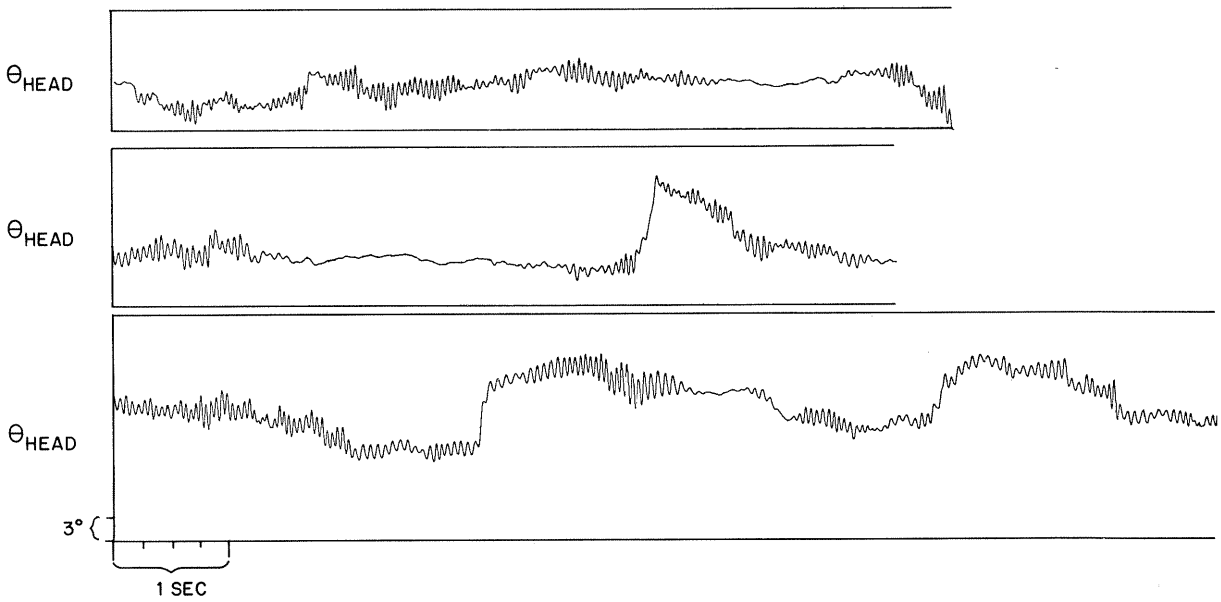

Fig. 3. Recordings of head tremor from 1 chick (horizontal only) showing the form and the frequency of the head tremor found in chicks with severe head tremor

only a small percentage of these abnormal chicks showed positive results on all tests. For example, in the group injected at day 13 (stage 39), almost all (97%) of the chicks showed a positive result on at least one test. Of these, 71% were characterized as vestibularly impaired, with only 20% of these chicks showing positive results in all five tests. On the other hand, the coincidence of head tremor and absences of nystagmus increased with stage of injection. At day 13 (stage 39), 82% of the chicks having nystagmus or tremor had both. At day 11 (stage 35), just over a third (34%) had both, while at day 8 (stage 33) this ratio is 41%.

Behavioral observations

Post-rotatory nystagmus. The expected post-rotatory response in normal chicks is characterized by a strong, right-beating, nystagmoid head and/or eye movement. The head nystagmus varied from large amplitude, high frequency right-beating of the head, to low amplitude, low frequency beating of the head. Occasionally, the head remained stationary and vestibularly driven nystagmus could only be observed in the eyes. Twenty seven of 33 control chicks showed a normal post-rotatory response. In addition, a certain percentage of the injected chick population also show normal post-rotatory responses. This percentage was a function of day of injection (Fig. 1 and 2).

Chicks receiving streptomycin and scored as having abnormal post-rotatory response showed a total lack of a detectable head or eye nystagmus response. Post-rotatory behavior in these cases consisted either of a retraction of the neck with no active head movement with eyes tightly closed or of a high frequency (> 20 Hz) head tremor that was inducible independent of a rotatory vestibular stimulus. These behaviors were seen in the streptomycin treated animals but not in the few controls showing no post-rotatory nystagmus.

Tremor. Tremor was found only in animals that received streptomycin. The tremor, apparent in horizontal and/or vertical planes, was either low frequency wobbling, or more often, a high frequency extremely vigorous sinusoidal oscillation, of the head. The tremor amplitude in both cases varied widely across the chick population as well as over time for an individual chick.

Either form of tremor could occur spontaneously or be induced by voluntary or externally induced movements of the chick. Voluntary movements of the chick such as pecking, walking and head movements were seen to initiate tremor. External disturbances of the head such as a light tap on the beak could induce tremor with high reproducibility. The more severe the tremor the easier it was to induce with an external stimulus. A quantitative analysis of these tremor head movements appears below.

General motor behavior. 1) Head compensation to body tilt: Normal chicks extended their necks to maintain a horizontal position of the head. Abnormal

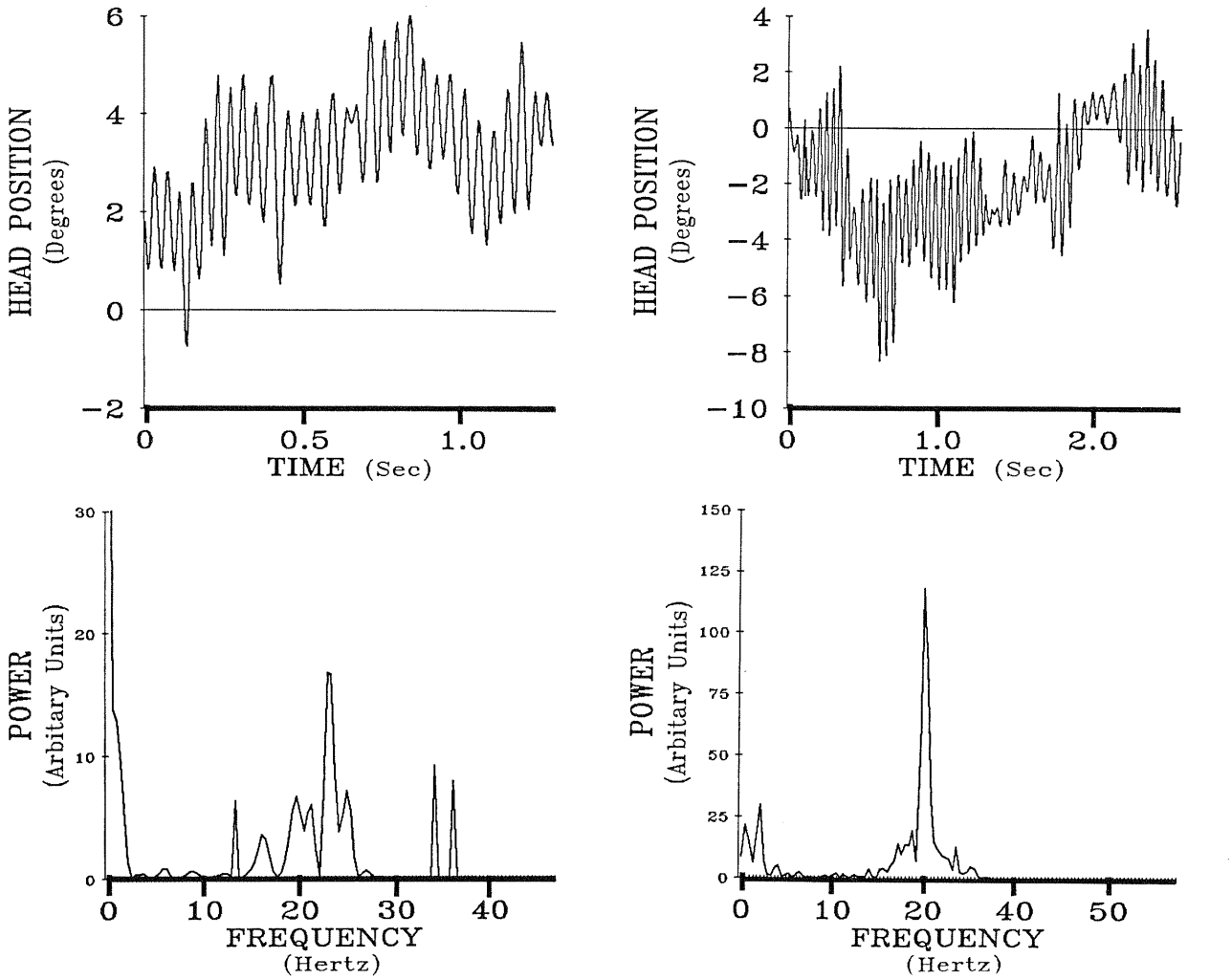

Fig. 4a. Head position recordings from chick with head tremor (above) and the corresponding frequency content of the displayed movements (below). **b** Head position taken from a different chick showing higher frequency tremor (above) and the frequency content of these movements (below)

chicks allowed their heads to be rolled in a fixed posture with their bodies, unable to sustain an upright head posture. During the test, abnormal chicks did not extend their necks as did the normal chicks instead they either did nothing or contracted their necks towards their body.

2) Recovery of erect posture: Normal chicks usually recovered erect posture almost immediately; a few chicks used random, uncoordinated activity to recover within the 90 s observation period. In the control population, only 6 of 33 chicks failed to meet to meet the criteria for righting. Responses from abnormal (treated) chicks consisted of poorly coordinated flailing movements; a few did not even make an attempt at righting but simply remained totally passive when turned on to their back and released, a behavior never seen in control chicks with posture problems.

3) Reflex coordination: The stable supine position necessary to perform the reflex coordination test was difficult to achieve in most chicks. When this position was obtained, negative tests were consistently composed of extensions of the leg on the side toward which the head was turned accompanied by a tight contraction of the opposite leg. Positive tests were characterized by a normal reflex toward one side, but none toward the other, or a lack of response towards either side. Rarely, chicks could not be placed in a stable position and these birds were also considered positive. Of the 33 controls chicks, 5 failed this test mainly due to the inability to obtain a stable position.

Postural effects. The standing posture of the chick was too difficult to quantify to include in the behavioral formula. However, some abnormal

chicks, all of whom received streptomycin, showed obvious postural abnormalities that deserve notice. Chicks had unstable standing posture with a tendency to stumble backwards and either fall or squat to prevent falling. Walking was tentative and erratic with the animal sometimes deviating off course. Some chicks with tremor were observed to squat down rapidly during a spontaneous attack of tremor. These postural abnormalities were found to subside over time and within 3 days were less obvious and usually gone in 1 week.

Recovery. The return to normal vestibular behavior was more difficult to assess than was the initial deficit. Consequently, the most obvious sign of abnormality, tremor, was used to assess the post hatching recovery. Like the initial deficit, recovery was subject to wide variability among different chicks of the same stage group. Head tremor generally decreased in severity over time until it became undetectable. Most of the recovery took place during the first 24 to 48 h with only few exceptions. Although all surviving chicks regained some motor stability with time, not all affected chicks appeared normal by the 14th day after hatching.

Head movement recordings. Only chicks with large amplitude high frequency head tremor were chosen for quantitative head movement recordings. The head tremor, shown in Fig. 3, consisted of a sinusoidal motion which could occur in either horizontal or vertical planes or in both (only horizontal shown). Although occasionally accentuated following a head movement, oscillations most often were ongoing, spontaneous and continued through head movements. The oscillations were sometimes modulated in amplitude, as shown in Fig. 3.

These bursts of oscillatory head movements were recorded in all three birds tested and appear representative of the remainder of the chicks showing severe tremor. No such oscillations were observed in normal chicks. A portion of the data from two chicks was selected for further analysis to examine its frequency content. Figure 4 shows both the head movements and the corresponding power spectrum. In each case, the frequency content of the head tremor falls in the range of 10–35 Hz. Figure 4a (lower) shows the peak almost exclusively at 20 Hz while in Fig. 4b (lower) frequencies are found both above and below 20 Hz with the main frequency lobe at 24 Hz.

Exceptional cases. Several chicks in the course of these and other identical experiments showed very bizarre head posture after hatching. The chick shown in Fig. 5 is representative of the small group of animals that hatched with severe orientation problems after having been injected with 400 mg/kg on day 13. The most striking observation was head posture. This chick walked around with its head completely tipped backward so that its beak pointed posteriorly, never holding its head in a normal position except when resting its head on the floor. The chick would take several steps then lose balance and tip over on its side. Even when the chick fell on its side, the head remained in extreme extension. This head posture did not change with eyes open or closed. Vestibular testing showed that the animal displayed no post-rotatory nystagmus either with eyes open or closed. During rotation, it would hold its head with its beak pointing towards the ceiling. Head tremor was present (1–5°) and the chick was unable to right itself when rolled on to its back. The chicks displaying these severe changes were euthanized after 24 h; the severity of the abnormality did not diminish over this time period.

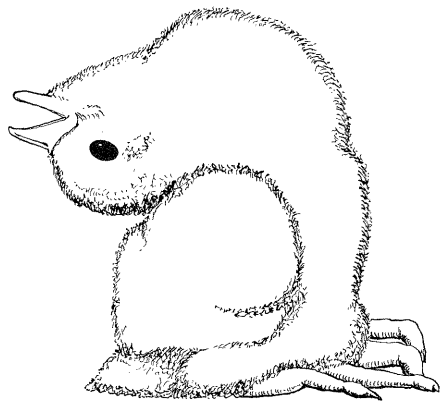

Fig. 5. Chick dosed with 400 mg/kg on development day 13 showing abnormal head posture

Histology

Chicks treated with streptomycin at 400 mg/kg on day 13 of development display distinct degenerative changes confined to specific cell groups of the membranous labyrinth. The cells affected are of the dark cell secretory type, involved in the production of endolymph (Dohlman 1964). These cells occupy a significant proportion of the surface area of the membranous labyrinth, and are concentrated near the three cristae ampullarae of the semicircular canals and near the utricular macula, but not the saccular macula of the otolith organs (Kimura Lundquist Wersall 1964).

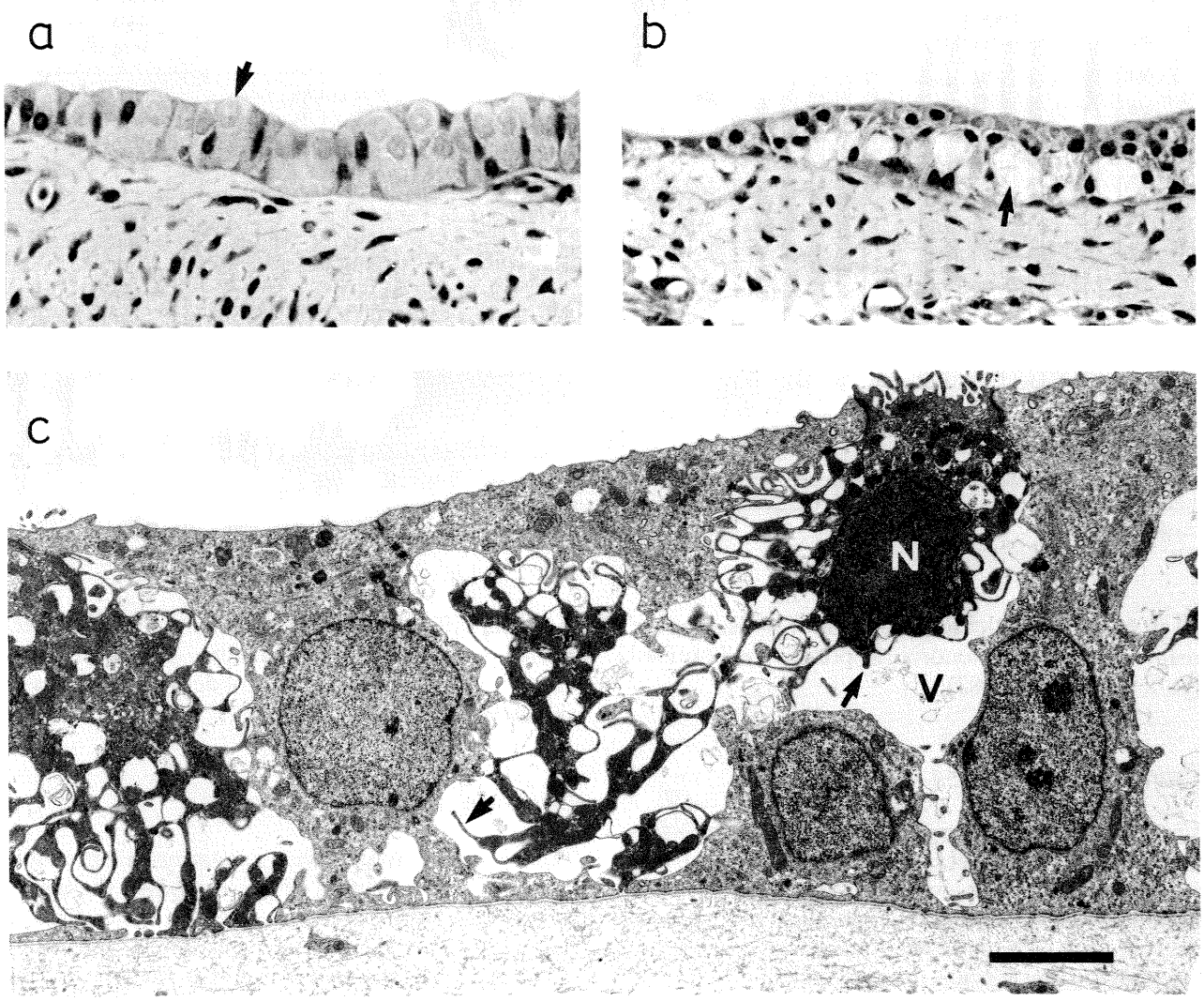

Fig. 6a–c. Damage to secretory dark cells at base of horizontal canal crista ampulla in newly hatched chick receiving an ototoxic dose (400 mg/kg egg weight) of streptomycin sulfate on day 12 of development. **a** Control ear showing larger secretory dark cells (arrow) intermingled with smaller "light" cells (staining darker here due to toluidine blue stain) (× 125). **b** Streptomycin damaged epithelium showing large "vacuoles" (arrow) forming in dark cells with nuclear displacement and shrinkage. **c** Electron micrograph of cells treated similarly to (**b**) showing that vacuoles are due to loss and retraction of dark cell processes (arrows), with resulting formation of large extracellular spaces. Remainder of cell shows cytoplasmic and nuclear shrinkage (nucleus, "N") (× 5200). Scale bars: **a, b** – 40 μm; **c** – 5 μm

The changes identified on light microscopy consist of striking vacuolization at the basilar aspect of the dark cells (Fig. 6b). This effect was quite prominent in the tallest dark cells located most proximal to the edges of the ampullary and utricular neuroepithelia (the "pyriform" subtype described by Park and Cohen 1982), and seen to a much lesser extent in the distal, more flattened cells ("cuboidal" subtype). The damage was focal, with severely damaged cells intermixed with only mildly or undamaged cells. Similar damage was seen to persist in tissues from animals allowed to survive for 1 week and which had recovered normal behavior.

At a dosage of 400 mg/kg there appeared to be no light microscopic level damage to sensory hair cells of either the cristae or maculae, nor to any other cell type other than the dark cells, including the tall secretory cells found in the planum semilunatum, which straddles the cristal mounds. Ears from animals receiving streptomycin on day 7 showed no changes when compared to controls (Fig. 6a). Electron microscopy of the dark cells from animals treated on day 13 (Fig. 6c) shows loss and retraction of the basal cell processes of the secretory dark cells. This loss of processes gives rise to large extracellular spaces resembling vacuoles on the light microscopic

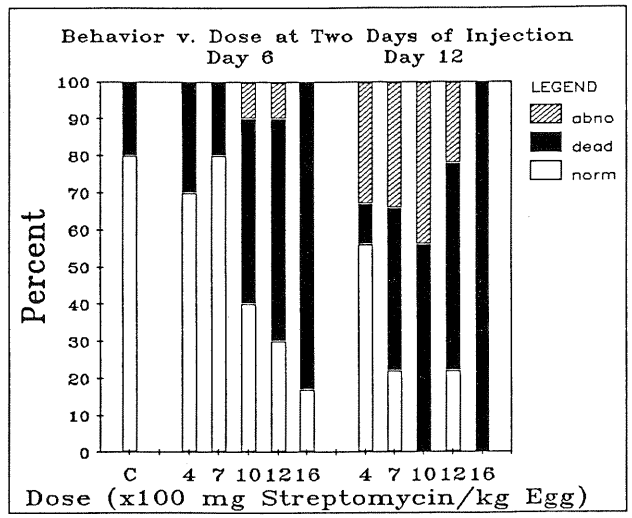

Fig. 7. Summary of the behavior in two populations of chicks at different days of development when given increasing dosages of streptomycin

Table 1. Streptomycin tissue levels (μ/mg of tissue)

Day (stage)	45 min Avg (SD)	48 h Avg (SD)
5 (25)	37.4 (54.8)	0 (0)
6 (27)	0.6 (1.2)	6.8 (8.5)
7 (29)	0 (0)	12.6 (10.2)
8 (32)	0 (0)	14.2 (4.5)
9[a] (35)	Chick A 0	Chick 1 0
	Chick B 0	Chick 2 5
	Chick C 0	Chick 3 13
	Chick D 9	Chick 4 14
	Chick E 1457*	Chick 5 729*
10[a] (37)	Chick F 0	Chick 6 14
	Chick G 9	Chick 7 18
	Chick H 11	Chick 8 358*
	Chick I 12	Chick 9 859*
	Chick J 1488*	Chick 10 1078*

[a] The variability of the data required individual listing of the data values. Consequently, no average and standard deviations were calculated. Asterisk is used to show high tissue levels

level. In addition, dense collections of ribosomes are seen between mitochondria, as previously described (Park and Cohen 1982).

Experiment 2. Increasing drug dosage

Varying the dose of streptomycin from 400 mg/kg to 1600 mg/kg at two stages of development, days 6 and 12, resulted in behavioral abnormalities in both groups (Fig. 7). However, regardless of the dose delivered the percent of abnormal chicks was clearly less with the day 6 group than with the day 12 group. The mortality rate rose sharply in both groups when more than 400 mg/kg was injected. In the day 6 group, the mortality increased from 25% to 85% with increasing dosages. In the day 12 group, the increase was from 10% to 100%,; no chicks hatched after 1600 mg/kg of streptomycin was injected.

Almost of all the day 6 chicks that did survive the increased dosage of streptomycin appeared totally normal in behavior as compared to control chicks. There were no abnormalities of head movement, tremor, or nystagmus. However, the higher dosed chicks that hatched often died within 2 days. The cause of this delayed mortality is uncertain. The severity of the behavioral abnormalities was greatly increased by the very high dosages injected in eggs at day 12 (stage 38). High dosages also had a negative effect on general chick health. Both vestibularly healthy and abnormal chicks showed a greater tendency to suffer a variety of problems if injected with high dosages of streptomycin. These problems included difficulties in internalizing the yolk sac, closing the abdomen post hatching, and lower levels of activity and feeding in the brooder (no measures of animal weight were made).

Experiment 3. Tissue drug levels

The amount of streptomycin appearing in embryonic tissue after introduction of the drug into the egg is related to the stage of development of the embryo at the time of drug administration as shown in Table 1. At 45 min after injection, most eggs treated on days 5–9 (stage 25–35) contained embryos with very low or absent levels of drug. The more developed embryos, especially at day 10 (stage 37), showed the most consistently measurable amounts of streptomycin.

At 48 h after introduction of the drug, day 5 (stage 25) treated embryos continued to show no measurable streptomycin, while most of the day 6–10 (stage 27–37) treated embryos had streptomycin levels showing a rough positive correlation with stage. A very wide variation in tissue streptomycin levels is evident from examination of the data, even within groups of eggs treated identically.

Discussion

Our behavioral and histological observations of the chick show that a single injection of streptomycin, given *in ovo,* damages the vestibular system. The behavioural consequences resemble those found:

1) when streptomycin is given during maturation, 2) after extirpation of the otocyst in the embryo, and 3) during exposure to weightless environments. The histologic findings confirmed work by others that streptomycin causes damage localized at the labyrinthine epithelium. The interference with sensory input during development offered by the streptomycin toxicity model can be an important tool used to extend previous work on sensory deprivation to the vestibular system, and to contrast it with similar studies on vision and audition.

Histology

The limited morphologic examination conducted on the vestibular end-organ showed degenerative changes after *in ovo* introduction of streptomycin sulfate in a single dose. In chickens (Park and Cohen 1982) as well as in guinea pigs (Lindemann 1969), the secretory dark cell appears to be the primary target of damage in streptomycin toxicity. Once damaged, these cells are unable to maintain optimal endolymph hemostasis, leading to dysfunction of the sensory hair cells, in particular the ampullarae type I units (Hawkins and Preston 1975). Cytologically, the changes we observed in the dark cells were similar to those seen by Park and Cohen (1982) after they exposed hatched chicks to serial doses of streptomycin. Our animals displayed dark cell alterations including striking vacuolization (Fig. 6b) which on ultrastructural examination corresponded to loss of basal processes (Fig. 6c). The lesions were highly focal in nature with cells displaying extreme changes located adjacent to normal appearing ones.

As previously reported (Park and Cohen 1982) we observed differential sensitivity of the various morphologic cell types that make up the extensive dark cell network (Kimura Lundquist Wersall 1964) distributed throughout the labyrinth. However, unlike those chicks subjected to a multiple dose schedule after hatching, *in ovo* exposure affected mainly the pyriform dark cells located at the base of the cristae ampullarae in all three semicircular canals. The smaller cuboidal cells and the intermediate size cells of the cruciate eminence showed less damage or were unaffected. This pattern of damage may be a consequence of the stage of development at the time of drug dosage with differential stage-specific sensitivity of the various dark cells types, or to differences in regenerative capacity, and may be related to the unusual pharmacokinetic environment within the egg as compared to that in the postpartum animal. The morphologic changes occurring during the period after streptomycin dosage and before hatching are currently under investigation and will be separately reported.

The chick's ability to repair the damage to the dark cells induced by streptomycin appears to be limited, at least during the first week after hatching. While considerable behavioral improvement occurred, vacuolization of dark cells persisted in birds examined one week postpartum. That such behavioral normalization can occur was shown previously by Heaton (1972) who performed unilateral otocyst ablations on day 2 chick embryos and observed marked vestibular deficits on hatching, which spontaneously corrected within the first 48 h.

Behavior

Our animals' intense head tremor, head righting behavior and walking difficulty is similar to that described by Park and Cohen (1982) using hatched chicks. In both studies, disturbed animals displayed an inability to make righting movements of the head when inverted, preferring rather to retract their heads. Also walking was tentative and erratic. Finally, although not quantified in their study, the head tremor they described appears similar to that exhibited by our animals. The tremor in our animals does not appear to be associated with vision since covering the animal's eyes did not change the tremor characteristics. Also, the tremor did not reappear in recovered animals when vision was removed. Similar tremor-like responses have been reported in bilateral labyrinthectomized monkeys (Igarashi Watanabe Maxian 1970) and pigeons (Oosterveld, personal communication). Our data and that of others indicates that these tremor movements are closely linked with changes in the vestibular system.

The high frequency character of the head tremor bears a striking resemblance to the oscillating eye movements found in the chick (Turkel and Wallman 1977) and pigeon (Nye 1969). The frequency components of the oscillatory eye movements are in the range of 28 to 35 Hz (Nye 1969). The chicks that we studied with severe head tremor, displayed oscillations in a broader frequency range 10–35 Hz (Fig. 4). However, both head tremor and eye oscillations can occur spontaneously, be triggered by a self-movement, and showed modulated amplitude behavior suggesting some control of the oscillations (Fig. 4). The reason for the eye oscillations has yet to be discovered. A recent report has shown that they are not due to an underdamped oculomotor effector system (ie. muscles, tendons, connective tissue, etc) (McVean and Stelling 1986). Quite likely, the head oscillations are also not due to an underdamped head

effector system. However, the close resemblance of head oscillations to those of the eye suggest a common mechanism. In that respect, it is interesting to note that eye movement signals have been found in the neck muscles of both cat and monkey. A possible explanation of the head tremor found in affected chicks is that such head oscillations are manifestions of signals from a common CNS controller that also produces the chick's eye oscillations. Further studies measuring simultaneous head and eye movements in affected chicks will be needed to identify the relationship of the two responses.

The extreme extension of the neck displayed by some chicks when lifting their heads suggests that otolithic function is impaired in these birds, and/or that the semicircular canals are providing misleading information. The nature of this information is suggested by the similarity of this head posture to some of the head postures adopted by pigeons when exposed to weightlessness Oosterveld and Greven (1975) interpreted this behavior as reflecting the birds sensing a constant vertical acceleration downward (i.e., falling) to which they componsate with a pitch up of the head and body. Since the sensations continued during the 20 s weightless period, the animal continued to pitch the head back and flew in a backward loop. In perhaps a similar manner these chicks are sensing a falling (otolith) or a constant pitch-down (semicircular canal) due to vestibular damage. Such continuous signals would cause the animal to react with a compensatory upward movement of the head.

Tissue streptomycin levels

The finding of very low tissue levels of streptomycin in a portion of the post day 8 embryos and in virtually all of the pre day 8 embryos may explain the lack of histological and behavioral changes in corresponding proportion of hatched chicks. In those cases after day 8 where streptomycin was demonstrated in the embryonic tissue, concentrations varied widely, most likely accounting for the spectrum of behavioral changes. The lag in the correlation between streptomycin levels on day 8 and corresponding behavioral changes may be due to the small sample size.

These findings are in contrast to the effects of kanamycin on the auditory system. Fermin and Igarashi (1983) showed that a single injection of kanamycin on development day 7 results in damage to the auditory end organ in hatched chicks, implying penetration of the drug into the embryo at this stage. We found vestibular changes only using higher dosages in more mature embryos. The reason for these differences is unclear, especially since the chemical properties of the two aminoglycosides, kanamycin and streptomycin, are similar (Goodman-Gillman Goodman Rall Mured 1985).

The mortality rate we found with higher dosages of streptomycin in the embryo is very different than that reported by Park and Cohen (1982). Even at dosages of 800 and 1200 mg/kg body weight, injected over many days, they found no change in weight gain or mortality for their chicks. In contrast, we found that most of the embryos injected once with 1000 mg/kg egg weight or greater, either failed to hatch or died within 48 h of hatching. This suggests that the lethal effect of streptomycin on the hatched chick is less than that in the embryos.

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