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A STUDY OF THE ELECTROCHEMISTRY AND OSMOTIC RELATIONSHIPS OF THE COCHLEAR FLUIDS IN THE NEONATAL RAT AT THE TIME OF THE DEVELOPMENT OF THE ENDOCOCHLEAR POTENTIAL

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SUMMARY

1. Changes in the endocochlear potential between the 8th and 18th days after birth were investigated in the rat. Initially the potential was low but its magnitude increased rapidly between the 11th and 16th day. During the 13th and 14th days the rate of increase was approximately 1 mV/hr.

2. The rapid potential increase arose virtually simultaneously in all three turns of the cochlea.

3. Histological examination revealed the cochlea, including the hair cells of Corti's organ and the stria vascularis, to be fully mature before the period of rapid change in the endocochlear potential, apart from the cells of Claudius, whose final development coincided with the latter part of this phase.

4. The endolymphatic sodium concentration (average 1·0 m-equiv/l.) had attained the very low adult level in the earliest period studied. The potassium and chloride concentrations were slightly below the normal adult levels, the result of some degree of general hypo-osmolality present at this time.

5. The endolymphatic ionic concentrations remained unchanged during the phase of rapid increase in the endocochlear potential.

6. The findings thus indicate that the distinctive endolymphatic ionic composition and the endocochlear potential arise largely independently and in succession during cochlear maturation.

7. No differences in osmotic pressure were demonstrated between endolymph, perilymph and serum. The problems concerning the homoeostasis

of the inner ear fluids do not consequently seem to be complicated by unusual hydrodynamic aspects.

8. Alterations in body fluid osmolality, produced by intraperitoneal injection of water or hypertonic glycerol, were accompanied by simultaneous changes in the osmotic pressures of the inner ear fluids. Some portion of the membranes bounding the endolymphatic space is therefore considered to be freely permeable to water.

9. The investigations provide no further information about the nature of the endocochlear potential, although an increase in the electrical resistance of the cochlear duct membranes is thought responsible for its appearance. The time relationships of this period support the concept that the potential is an essential feature of the mechano-electric transduction process.

INTRODUCTION

The characteristic feature uniquely distinguishing cochlear endolymph amongst the mammalian tissue fluids is the association of its high potassium and low sodium content, first described by Smith, Lowry & Wu in 1954, with the presence of a high positive potential, initially demonstrated by Békésy in 1952. Although these very unusual findings are now generally accepted as being of importance in the normal physiological mechanisms of hearing, there is still a good deal of obscurity concerning both the precise source of this potential and the nature of the processes determining the ionic constitution.

Bosher & Hallpike (1965) were led to argue, from their study of the genetically induced deafness in the cat, that the normal endolymphatic system probably passed through a critical phase in its maturation process shortly after birth. Direct supporting evidence for this concept was subsequently provided by the reports which revealed the cochlear microphonic potentials and other responses to sound stimulation to be absent before the early post-natal period in a variety of laboratory mammals (Schmidt & Fernandez, 1963; Änggård, 1965; Mikaelian & Ruben, 1965; Crowley & Hepp-Reymond, 1966). Of particular interest was the discovery that the endocochlear potential, also, appeared about this time (Schmidt & Fernandez, 1963; Änggård, 1965).

However, no information has, as yet, been available concerning the ionic constitution of the endolymph in early life. It therefore seemed worth while to investigate the changes occurring in the inner ear fluids during the neonatal period and attempt, if possible, to correlate the alterations in the endocochlear potential with the chemical composition. In addition, it also became necessary to investigate the osmotic relationships of the endolymph and perilymph.

The rat was chosen as the experimental animal because Bosher & Warren's (1968) findings would thus be available for comparison. The endolymphatic sodium, potassium and chloride concentrations, these authors have shown, are maintained in the adult animal by active transport mechanisms, which are highly oxygen dependent and almost certainly located in the cells of the stria vascularis. Subsequently, Kuijpers & Bonting (1969) have demonstrated the presence of a relatively high concentration of a sodium-/potassium-activated, ouabain-inhibited ATPase in the mature stria vascularis.

The initial results from the first series of experiments have been reported by one of us (S.K.B.) as part of a thesis presented to the University of London (Bosher, 1969).

METHODS

White Wistar rats were used for the experiments and their age was calculated from the date of conception, as preliminary experiments revealed excessive variation to occur in the results if the age of the animals was calculated from the time of parturition, presumably due to differences in the length of gestation. Females were examined for the presence of spermatozoa in vaginal smears and those found to have positive results were thereafter isolated. Proof of successful mating was taken as evidence of conception, which was considered to have taken place on the day of examination. Where it has been necessary, for comparison with the findings of other workers, the post-natal age of the animals has been calculated from the conceptual age assuming a gestation period of 22 days, the average length in the stock used.

Collection and manipulation of the inner ear fluids. The experimental procedure was essentially the same as that used by Bosher & Warren (1968) in adult rats. The animals were anaesthetized with urethane (0·01 g/10 g body wt.) administered by intraperitoneal injection and the tympanic cavity was exposed by means of a lateral approach. After identification of the cochlea by reflexion of the mucosa or, in the younger animals, removal of the mesenchymal tissue filling the middle ear, a small fenestra (80–100 μ) was made in the labyrinthine capsule over the stria vascularis of the middle turn by means of a hand-operated, spear-pointed micro-drill. Following satisfactory measurement of the endocochlear potential (see below) a Pyrex glass micropipette, tip diameter 5 μ, was inserted at the same site and to the same depth as the potential-recording electrode and a quantity of endolymph, limited to about 20 nl., was aspirated. Slight positive pressure was applied to the aspirating system, which was filled with mineral oil, during both insertion and withdrawal of the pipette, to prevent contamination with tissue fluid, and the endolymph samples were sealed from contact with the air by the subsequent aspiration of a small quantity of oil previously inserted into the middle ear.

Next, a further measurement of the endocochlear potential was made and if this was not within 10 mV of that originally obtained in animals between the ages of 37 and 40 days or 5 mV in animals between the ages of 30 and 36 days, undesirable structural damage was considered to have taken place and the endolymph sample was not submitted to analysis. Finally, a specimen of perilymph was obtained in a similar manner from the adjacent scala vestibuli of the basal turn. These manipulations were, in general, completed in about 30 min from the time of administration of the anaesthetic, thus avoiding deterioration in the animal's general condition.

A number of experimental difficulties were encountered, of which the marked

fragility of the labyrinthine capsule and the increased haemorrhage, particularly from the large sinusoidal vessels of the middle capsular layer, were anticipated. There were two further troublesome features, namely the invalidation of a significant number of electrical recordings due to the progressive decline of the potential value, presumably the result of inadequate sealing around the micro-electrode, and the relatively large number of occasions when the volume of endolymph which could be aspirated was very small (< 3 nl.). The impression obtained during such experiments was that some anatomical structure, such as the tectorial membrane, had come into apposition with the micropipette tip, thus preventing further ingress of endolymph. As a result, no analytical procedures could be undertaken on a number of samples and in others the volume was insufficient for the full range to be performed.

All endolymph and perilymph specimens were discharged immediately after collection into an oil-filled chamber, in which all further manipulations were conducted. Initially, they were examined under a magnification of × 500 and any found to contain cells or debris were rejected. Subsequently, successive sub-samples were aspirated for the various analytical procedures under the same magnification using measuring pipettes with attached silica markers. Each sub-sample used for the estimations of the potassium and sodium concentrations was then ejected into 1 μl. distilled water which was finally aspirated into a silica pipette, of tip diameter 100 μ, for storage. Samples of appropriate standard solutions were also prepared in a similar manner using the same measuring pipettes to ensure that the volumes of both the standard solutions and the inner ear fluids being examined were identical.

All the glassware used, including the aspirating pipettes, was coated with 0·6 % dimethyldichlorosilane, a silicone water repellent.

Measurement of the endocochlear potential. Conventional Pyrex glass microelectrodes, of tip diameter 1 μ, filled with a solution approximating in composition to adult endolymph (KCl 140 m-equiv/l., NaCl 1 m-equiv/l.) were used for the electrical recordings, in conjunction with a digital voltmeter (Solartron LM 1604) and an electrometer pre-amplifier (Analogue Devices 310). The reference electrode was a chlorided silver wire buried in the deep scapular muscles. The value of the endocochlear potential was taken as the magnitude of the recorded potential change between the tissue fluid overlying the spiral ligament and the interior of the cochlear duct, corrected for the electrode tip junction potential measured in 154 mM-NaCl solution. Only those recordings fulfilling the requirements specified by Bosher & Warren (1968) were regarded as satisfactory.

Determination of the sodium and potassium concentrations. The cation concentrations were estimated by means of the technique of total emission flame spectrophotometry (Ramsay, Falloon & Machin, 1951) using the method developed by Bosher & Warren (1968). In this the sample to be analysed, initially of the order of 2 nl. in volume, is placed on a platinum element and then dried in a hot air current, before being volatilized at a temperature of 1100° C into the gas stream supplying the burner. The excitation of the emission is achieved by subsequent ignition of these gases and a hydrogen + air flame, temperature 2000° C, is employed for this purpose. The total emission from the sample is integrated and compared with that from an identical volume of standard solution of known concentration. The spectral characteristics of the photomultiplier, E.M.I. type 6255A, limited its sensitivity at the potassium wavelength and, as contamination of the flame by extraneous potassium was not a serious hazard, the original Pyrex glass chimney and capping glass funnel were removed during the potassium analyses to increase the sensitivity of the apparatus in this respect. With a standard solution comparable in composition to endolymph, the standard deviations of replicate analyses were ± 6 % for sodium and ± 1·3 % for potassium. Repeated tests were also carried out to ensure no departure

from linearity in the relationship of the emission magnitude and the sample size of the element under analysis occurred over the concentration range covered by the samples and standards in any individual estimation.

Determination of the chloride concentration. For the estimation of the chloride concentrations an adaptation of the electrometric technique developed by Ramsay, Brown & Croghan (1955) was employed. In this method, the chloride ions are titrated with silver ions released by passing an electric current through a silver electrode and the end-point is determined potentiometrically. The current used to liberate the silver ions also charges a condenser (20 μF) placed in series with the silver electrode and the charge developed during the titration is thus a measure of the initial chloride concentration. The sample volume was 1 nl. and the S.D. of replicate estimations of chloride solution 108 m-equiv/l. was $\pm 0{\cdot}83$ %.

Determination of the osmotic pressure. The freezing point depression method of Ramsay & Brown (1955) was utilized for the determination of the osmotic pressures of the inner ear fluids. Sample volumes of 1–2 nl. were found to give the most accurate results and the standard deviation of replicate estimations of a standard solution, osmotic pressure 7·47 atm (at 38° C), was $\pm 0{\cdot}007$° C ($\pm 1{\cdot}3$ %). Although little difficulty was experienced with the endolymphatic estimations, those of the perilymph were often invalidated, when the protein normally found shortly after birth in this fluid was present in relatively high concentration. In this circumstance, its distribution in the small sample droplets (volume 50–100 μl.) did not appear to be uniform and reproducible results could not be obtained. In consequence, no attempt was made to measure the osmotic pressure on those specimens in which this phenomenon was observed as such determinations were considered unreliable.

Histological examination. Both ears of fifteen animals between the ages of 33 and 40 days, in whom successful collection of the inner ear fluids had been performed on the left ear, were examined histologically. Fixation was achieved by immersion in 10 % formaldehyde (Baker, 1965) and not intra-vitam injection, due to shortage of time during the experimental procedure, but the results in general appeared to be satisfactory. The material was embedded in Celloidin after decalcification in 1 % nitric acid, sectioned in the horizontal plane at 5 μ and every 5th section stained with Ehrlich's haematoxylin and eosin.

RESULTS

Changes in the endocochlear potential

Reliable electrical measurements were obtained from ninety-nine animals during the period investigated, the 30th to the 40th day post-conception (8th–18th day after birth) and the striking changes found in the magnitude of the endocochlear potential are shown in Fig. 1.

On the 30th day the potential was very low in comparison with the mature level, being on average only 14 mV, and during the following 3 days the rate of increase was slow so that the mean potential had only risen to 19 mV on the 33rd day. The next day the rate of change increased somewhat but this was then largely overshadowed by the rapid increase during the succeeding 2 days, an increase of approximately 50 mV in 48 hr. Thereafter, although the rate of change was greatly reduced, it was not inconsiderable and, in consequence, the average potential found on the 38th day (87 mV) had almost attained the average adult level of 92 mV

as determined by Bosher & Warren (1968). Subsequently, however, the rate of increase declined considerably and even on the 40th day this level had not quite been achieved.

As satisfactory samples of the inner ear fluids could not be obtained, the period immediately after birth was not investigated but extrapolation from the values obtained between the 30th and 33rd day suggests the endocochlear potential probably arises initially about the time of birth.

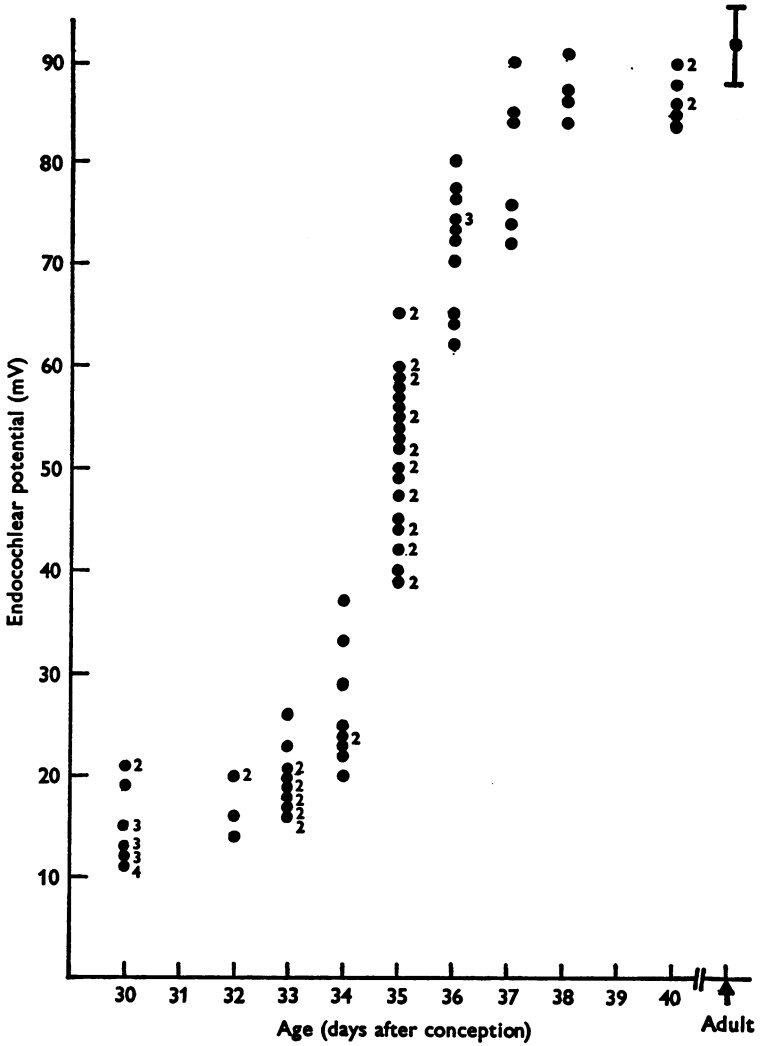

Fig. 1. The change in the endocochlear potential with age. The range and mean of the potential in adult rats (Bosher & Warren, 1968) is also shown. Numerals indicate the number of experiments giving the same individual result. The average length of gestation was 22 days.

The results substantially extend those of Schmidt & Fernandez (1963), who were able to show in their subsidiary investigation of five members of one litter of white rats that the endocochlear potential after birth was very low (1 mV at 1 day, 6 mV at 3 days, 11 mV at 6 days), while at 14 days (about 36 days post conception) it had reached 68 mV. These findings are clearly in accord with those of the present experiments, although the authors suggested the potential increased progressively from birth, similarly to the development of the endocochlear potential in the pouch young of the Virginia opossum (*Didelphis virginiana*). Here, differences undoubtedly exist due to the very prolonged time course, apparently common to all aspects of cochlear maturation in this particular species.

However, Ånggård (1965) in a detailed investigation of cochlear development in the rabbit found a somewhat similar period of rapid development of the endocochlear potential, although he placed little emphasis on its importance in relation to the mechanisms determining the endolymphatic ionic constitution. In this species the potential increased approximately 10 mV/day between the 6th and 13th days after birth, whereas before the 6th day only small and possibly insignificant potentials could be recorded and after the 13th day the rate of increase was much less marked.

Schmidt & Fernandez (1963) and Ånggård (1965) were concerned with the relationship between the endocochlear potential and the cochlear microphonic potentials and they were able to demonstrate a remarkably close correlation between the increase in the magnitudes of these potentials in both the opossum and the rabbit. While it was not possible, in the experiments here reported, to investigate the changes in the cochlear microphonic potentials this aspect of cochlear maturation in the albino rat has been carefully documented by Crowley & Hepp-Reymond (1966). According to these authors, the first very restricted potentials appeared on the 8th or 9th day after birth and subsequently attained the adult characteristics at about the 20th day, except for the magnitude of their maximum amplitude, which was still a little low. Of particular relevance in the present context was their report that the character of the frequency–response curve altered abruptly between the 12th and 14th days when the peak sensitivity changed from 3 kHz to the adult value of over 40 kHz, a change associated with a rapid increase in sensitivity.

The phase of greatest development of the cochlear microphonic potential thus seems to coincide with the period of rapid increase in the endocochlear potential. Furthermore, other similarities are also apparent, such as the presence of relatively small cochlear microphonic and endocochlear potentials before the 11th day (after birth) and the slight reduction in their maximum amplitude still noticeable at 18 days. It therefore seems not unreasonable to conclude that a close correlation probably also exists in

the rat between the development of the endocochlear potential and the cochlear microphonic potentials.

Variations in the development of the endocochlear potential

The extent of the inter-animal variation in the magnitude of the endocochlear potential in any one litter was determined in eleven litters between the ages of 33 and 37 days (inclusive), three to seven animals being examined from each litter. As might be expected from the relatively large differences observed in other features, such as the separation of the eyelids, the variation found, 2–11 mV (mean 7 mV), was often comparatively

TABLE 1. The change in magnitude of the endocochlear potential (mV) along the cochlear duct

Animal no.	Age (days)	Basal turn	Middle turn	Apical turn
D1	35	69	65	61
D2	35	80	73	64
D3	35	77	70	66
D4	35	83	76	69
D5	35	71	62	57
M2	Adult	100	95	89

large, although in each case it was less than the total range of the potential for the day concerned. In five instances animals from the same litter were examined on two or three different days and the results obtained with respect to each litter fell largely within the same portion of the total range for each of the days concerned but little emphasis can be placed upon this attempt to document individual variations in the rate of increase of the potential in view of such large inter-animal variations.

The differences in the magnitude of the endocochlear potential in the three turns of the cochlea were also studied in five animals aged 35 days (i.e. the middle of the period of rapid increase in the potential). The results, presented in Table 1, reveal a difference of about 7 mV to be present between the individual turns, a figure somewhat less than the value of approximately 12 mV found in neonatal rabbits by Änggård (1965). Previous workers have also investigated the possible occurrence of such differences in adult animals (Misrahy, Hildreth, Shinabarger & Gannon, 1958; Gisselsson, 1960; Suga, Morimitsu & Matsuo, 1964; Kuijpers, 1969*b*), but there has been a marked divergence both in the values obtained and in the conclusions advanced. In consequence, the magnitude of the endocochlear potential was recorded in all three turns of individual cochleae in three maternal animals and a representative set of results is included in Table 1. These subsidiary experiments established the differ-

ence between each of the three turns in the mature cochlea to be about 5 mV, which does not differ substantially from the figure obtained in the neonatal animals. Hence, the conclusion that the rapid increase in the endocochlear potential occurs virtually simultaneously throughout the whole cochlea seems inescapable.

Morphological development

The short period during which the sudden increase in the endocochlear potential takes place is clearly a very important phase in the functional development of the cochlea and it seemed of value, therefore, to determine the extent of the structural development at this time. On the 33rd day post-conception (11th day after birth), at the commencement of this period, the cochlea, as judged by light microscopic appearances, was fully developed apart from the rudimentary cells of Claudius lining the external sulcus. In particular, the scala media and the perilymphatic scalae were fully formed, while the stria vascularis, tectorial membrane and the organ of Corti, including the hair cells, supporting cells and tunnel of Corti, were completely differentiated, findings fully in accord with those of Wada (1923) and earlier workers. No subsequent morphological change was visible in the cochlear duct until the 36th day when the cells of Claudius began to enlarge and the cells of Böttcher became distinguishable for the first time. The maturation of these cells was rapid and appeared complete on the 38th day, their development thus coinciding with the latter part of the period of rapid change in the potential. This development appeared to occur more or less simultaneously throughout the whole length of the cochlea, only a slight lag being discernible in the apical as compared to the basal regions.

These results, it is interesting to note, reveal a striking similarity to those of Mikaelian & Ruben (1965) in their detailed study of the development of the cochlear microphonic potentials in the normal CBA-J mouse. On the 8th day, when these potentials were first recorded, the anatomical maturation of the cochlea was considered (by light microscopy) to be fully complete apart from the cells of Claudius, which were still completely rudimentary and, furthermore, the subsequent differentiation of these cells was found to coincide with the period of rapid change in the potentials commencing 2 days later. Furthermore, Kikuchi & Hilding (1965, 1966), in their electron microscopic investigation of normal mice, reported that the organ of Corti appeared fully mature at the time when cochlear microphonic potentials could be first elicited, while the stria vascularis also seemed completely or almost completely differentiated at this time. In consequence, as there seems good reason to believe maturation in the mouse differs only in detail from that in the rat, it appears not unreasonable

to suggest the organ of Corti and the stria vascularis may well be fully developed as judged by electron microscopy as well as light microscopic appearances at the beginning of the phase of rapid development of the endocochlear potential.

Endolymphatic ionic constitution

The full analytical results with respect to endolymph are shown in Fig. 2. Due to the technical difficulties described earlier, satisfactory samples of endolymph were obtained in only forty-three of the ninety-nine experiments in which the endocochlear potential was successfully recorded. Sodium estimations were performed on every specimen aspirated but potassium and chloride estimations were limited to twenty-five and twenty-three instances respectively.

Sodium. The endolymphatic sodium concentration, it was discovered, had already attained the very low adult level at the commencement of the period under investigation when the endocochlear potential was very small, apart from one 30-day animal. In this case the concentration (3·2 m-equiv/l.) was not substantially above the normal range and the significance of such an isolated observation must necessarily remain obscure at present. This mature concentration level, furthermore, was maintained apparently unchanged and virtually within the normal adult range of 0·41–1·44 m-equiv/l. (Bosher & Warren, 1968) throughout the period of rapid development of the potential.

Potassium. The analytical results with respect to the potassium concentration present a slightly different picture. In the earliest 3 days of the period studied, the findings, although few, are all significantly below the adult range. Thereafter, an increasing number of results come to lie within this range but, nevertheless, values in the upper part of the range are small in number and consequently the mean figures lie below the adult average of 154 m-equiv/l. (Bosher & Warren, 1968).

The initial impression therefore is one of slight immaturity in the potassium-regulating mechanisms in the majority of instances. However, closer examination reveals a number of discrepancies from this view. On the one hand, there is no associated increase in the sodium concentrations to maintain the endolymphatic osmotic pressure and, on the other, both the perilymphatic and endolymphatic chloride, together with the perilymphatic sodium concentrations (Figs. 2 and 3) are likewise comparatively low. The possibility thus arises that the low endolymphatic potassium concentrations result not from immaturity but from diminution in the osmotic pressure, and so dilution of the endolymph, secondary to some degree of general hypo-osmolality. This possibility seemed worth while investigating in more detail and will be considered further in the descrip-

tion of the osmotic relationships of the inner ear fluids described below.

Another feature, apparent in Fig. 2, is the considerable inter-animal variation present, a variation which appears to be greater than that seen in the chloride concentrations, and so the individual results for the animals

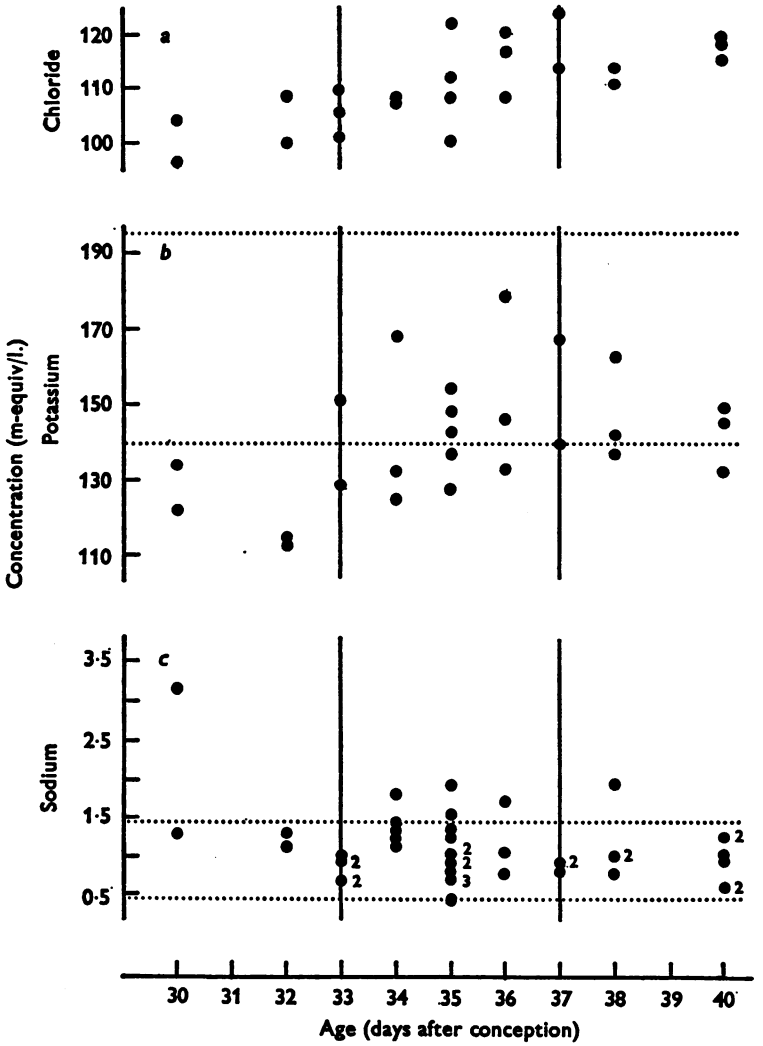

Fig. 2. The endolymphatic analytical results. The horizontal lines define the normal adult range for sodium and potassium (Bosher & Warren, 1968); the difference in scale will be noted. The vertical lines delimit the period of rapid development of the endocochlear potential. Numerals indicate the number of experiments giving the same individual result. The average length of gestation was 22 days.

in question are shown in Table 2. In eighteen instances the endolymphatic analytical estimations enable the amount of anion additional to chloride, presumably mainly although not entirely bicarbonate, to be calculated. In twelve of the animals the figures so obtained lie within the range 25–29 m-equiv/l., a range in good agreement with the endolymph pH range of 7·3–7·5 demonstrated in adult guinea-pigs by Misrahy, Hildreth, Clark & Shinabarger (1958). In five other animals an alteration of the order of

TABLE 2. The endocochlear potential (mV) and analytical results (m-equiv/l.) obtained in the experiments in which the endolymphatic potassium concentration was determined

		Endolymph				Perilymph		
Age (days)	Animal no.	Potential (mV)	Na	K	Cl	Na	K	Cl
30	72	11	1·3	122	96	117	6·0	100
	96	12	3·2	134	104	137	6·2	105
32	71	16	1·3	114	108	—	6·8	111
	75	20	1·1	115	100	121	6·7	105
33	46	16	0·7	129	105	125	8·7	108
	20	19	1·0	151	101	—	—	—
34	42	24	1·8	125	—	120	—	—
	41	33	1·1	132	107	155	4·0	—
	45	25	1·3	168	—	—	—	—
35	37	44	0·9	128	101	108	7·3	98
	28	47	0·9	137	111	124	—	116
	47	49	0·7	142	—	124	6·3	—
	29	53	1·9	148	121	138	4·7	114
	35	52	1·5	153	—	—	—	—
36	11	80	1·7	132	108	116	6·5	101
	90	64	1·1	146	120	121	5·3	115
	6	65	0·8	178	117	144	7·5	120
37	87	90	0·9	140	114	132	—	114
	54	85	0·9	168	124	150	7·6	125
38	93	84	1·0	137	111	132	7·4	113
	88	86	0·8	142	114	132	7·8	116
	12	87	1·0	162	—	142	8·4	116
40	19	90	0·9	131	118	120	5·3	115
	18	90	1·0	145	—	—	—	—
	25	86	1·2	148	—	129	5·9	119

10 % in the potassium concentration would give an 'additional' anion concentration within the same range, but some degree of acidosis or alkalosis may well have been present in these animals as a result of the known imperfections of acid–base regulation in neonatal animals (McCance, 1964). The remaining animal represents the only one of this group in whom the potassium concentration was exceptionally high. By a coincidence, the endolymph osmotic pressure was also determined in this animal (no. 6) in

a single pilot study. From the result, 7·36 atm at 38° C, the expected cation concentration can be calculated and this then gives a value for the 'additional' anion concentration of 27 m-equiv/l. In this experiment it therefore seems probable that some contamination of the endolymph occurred during its manipulation. In consequence, it would appear advisable to regard the three other instances (animals nos. 12, 45, 54) in which relatively high endolymphatic potassium concentrations were obtained as being possibly unreliable.

Examination of Fig. 2 also reveals the absence of any appreciable change in the potassium concentration at the time of the rapid development of the endocochlear potential.

Chloride. As mentioned above, there is a general trend for the endolymphatic chloride concentration values to be related to the age of the animal, the lower figures occurring in the younger individuals. Here, too, there is an apparent lack of change during the period of marked alteration in the endocochlear potential, an aspect common to all the ionic concentrations.

Thus the findings presented above clearly show that the distinctive character of the endolymph arises during the functional maturation of the cochlea by two processes occurring largely independently and in succession, the first being the establishment of the high potassium, low sodium concentrations and the second the development of the high positive potential.

Perilymphatic ionic constitution

The perilymphatic analytical results are presented in Fig. 3 and, in general, are consistent with the widely accepted view that the perilymph is essentially an ultrafiltrate of plasma, but the range of the sodium concentrations (102–155 m-equiv/l.) is significantly lower than the adult range of 115–180 m-equiv/l. (Bosher & Warren, 1968). Both this finding and the tendency for the lower values within the chloride concentration range to occur in the younger animals have been noted and briefly discussed earlier, while the range of the potassium concentrations is much the same as the adult one. The probable explanation of these observations is examined in more detail in the next series of experiments.

Osmotic pressure determinations

Since the problems in interpreting the analytical findings made further investigation of the osmotic relationships of the inner ear fluids desirable, an attempt was made in a small series of eight animals, aged 35 days post-conception, to measure the endolymphatic and perilymphatic osmotic pressures in addition to the usual range of chemical analyses. Satisfactory determinations, however, were obtained with respect to both fluids in only

three instances, although five endolymphatic and six perilymphatic results were obtained in all. These estimations gave a range for the osmotic pressure of the endolymph of 7·006–7·400 atm (at 38° C), while the corresponding figures for perilymph were 7·040–7·318 atm (at 38° C). In the

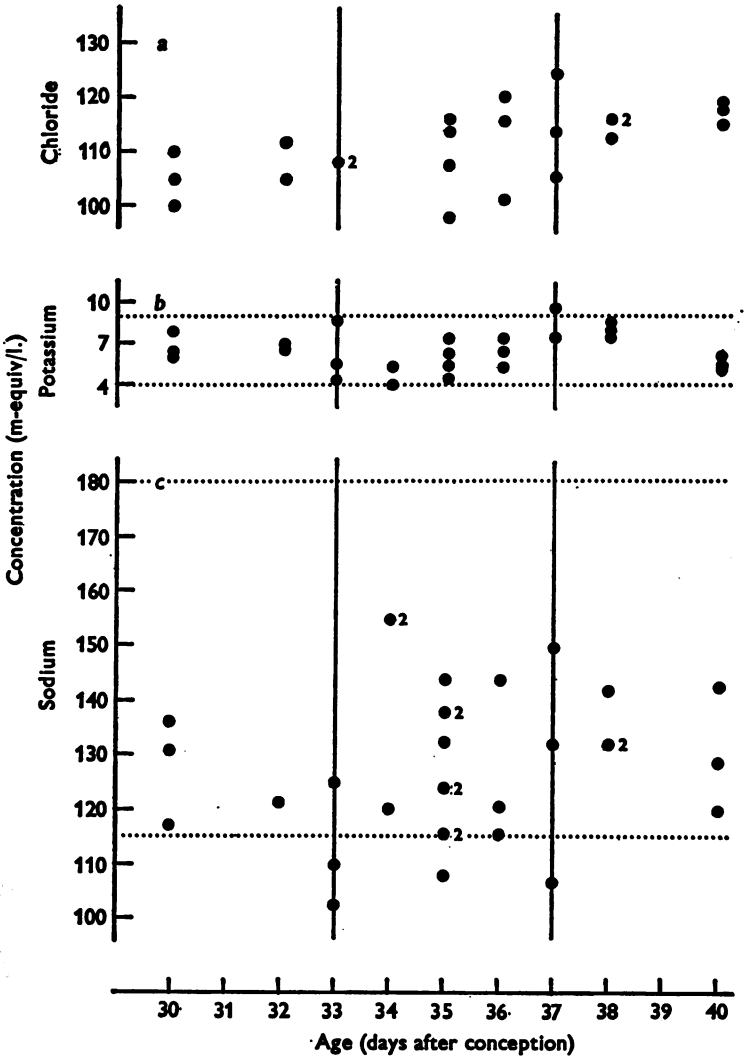

Fig. 3. The perilymphatic analytical results. The horizontal lines define the normal adult range for sodium and potassium (Bosher & Warren, 1968); the difference in scale will be noted. The vertical lines delimit the period of rapid development of the endocochlear potential. Numerals indicate the number of experiments giving the same individual result. The average length of gestation was 22 days.

three animals for whom values for both fluids were available the differences found between the endolymphatic and perilymphatic osmotic pressures were 0·042, 0·312 and 0·320 atm. The two latter figures are only slightly greater than the standard deviation of the replicate estimations of an appropriate standard solution ($\pm$ 0·1 atm at 38° C) and, in view of the technical difficulties encountered in connexion with the biological fluids described earlier, the differences between the two inner ear fluids are not considered to be significant.

It is of interest to correlate these findings with the osmotic pressures calculated from the average cation concentrations of the fluids, assuming an equivalent amount of anion to be also present. In the case of the endolymph (142 mM-K^+, 1 mM-Na^+) the value so calculated is 7·30 atm at 38° C, which indicates that the osmotic pressure of this fluid can be explained solely by its ionic constituents. The contribution of any non-electrolytes, such as glucose and urea, therefore appears to be very small at this age and hence their concentrations in the endolymph appear relatively low. Such a finding thus provides additional evidence against the possibility of the endolymph having a major role in the metabolic supply of Corti's organ. By contrast, the figure calculated for the perilymph (127 mM-Na^+, 6 mM-K^+), 6·81 atm at 38° C, is 0·38 atm below the observed mean, a difference which corresponds closely to the osmotic pressure calculated from the glucose and urea concentrations on the generally accepted basis that perilymph is an ultrafiltrate of plasma.

For comparison with these inner ear fluid measurements, the serum osmotic pressure was determined in a subsidiary series of ten 35-day animals and the range so ascertained was 6·906–7·438 (mean 7·228) atm at 38° C. These results therefore reveal there is no significant difference, in 35-day animals at least, between the osmotic pressures of the endolymph, perilymph and serum. In this context the recent demonstration (Beentjes, 1970), despite the considerable technical difficulties involved, of the absence of any difference in the hydrostatic pressures of the endolymph, perilymph and blood in the adult cat seems of especial relevance. These two studies together provide strong evidence against the possibility that the problems concerning the homoeostasis of the inner ear fluids are complicated by any unusual hydrodynamic features.

The question of the presence of a difference in general body fluid osmolality between neonatal and mature rats still remained. The serum osmotic pressures were accordingly determined in a small number of adult animals and the range found was 7·82–8·07 atm (at 38° C). These values are, indeed, significantly greater than those present in the 35-day animals and do, in fact, correspond closely to the adult endolymphatic ionic concentrations. There is, consequently, substantial support for the concept

that the low endolymphatic potassium and chloride concentrations are due to the hypo-osmolality of the body fluids and not to any immaturity of the homoeostatic mechanisms concerned. The hypo-osmolality, it seems reasonable to suggest, results from the renal immaturity known to be present during the neonatal period (McCance, 1964).

This concept has far-reaching consequences for it implies that some portion, at least, of the membranes bounding the endolymphatic space must be freely, or relatively freely, permeable to water. Furthermore, the active transport systems at this age do not seem able to maintain the endolymphatic ionic concentrations at a constant level in the face of alterations in the body fluids. These matters are of considerable importance and an attempt was therefore made to investigate them more directly.

Effect of hypo-osmolality of the body fluids

The experiments to be described were conducted, as indicated above, principally to provide some information concerning the permeability to water of the cochlear duct membranes. Distilled water at body temperature was injected intraperitoneally in a dose of 1 ml./10 g body wt. at varying intervals before the measurement of the endocochlear potential and the removal of samples of the inner ear fluids. The dose selected was found, by a preliminary study, to be the largest which could be employed without any risk of death or deterioration in the condition of the animals being examined. The possibility of the administration of the water producing any alterations in the inner ear fluids secondary to such deterioration was thus avoided. The age of the animals investigated was 35 days (post-conception).

Osmotic pressure. The changes produced in the osmotic pressures of the labyrinthine fluids are shown in Fig. 4 and it will be seen that the primary effect of the sudden increase in body water was dilution of both the endolymph and perilymph, presumably in common with the general extracellular fluid. An attempt was made to collect fluid specimens during the period when this dilution was occurring but this proved to be technically extremely difficult and, in addition, the precise timing of the process could not be ascertained because delays of up to 10 min after the injection of the water were found before any demonstrable effect on either the endolymphatic, perilymphatic or serum osmotic pressures could be demonstrated. In consequence, the process would seem to occur fairly rapidly and certainly the maximum effect appears to have been reached by 26 min, the shortest period for which satisfactory results are available.

Thereafter, there was relatively slow but progressive recovery and the osmotic pressure of the inner ear fluids had returned completely, or almost completely, to normal in the animal examined after an interval of 2 hr.

A finding of particular importance was that the osmotic pressures of the endolymph and perilymph, in each instance, were reduced to the same level and the differences apparent in Fig. 4, with the possible exception of the last animal, were not significantly greater than those found in the normal group of animals. The possibility of this feature being an artifact due to the admixture of the two fluids, resulting, for example, from rupture of Reissner's membrane, could be definitely excluded both from the analytical results and the endocochlear potential measurements.

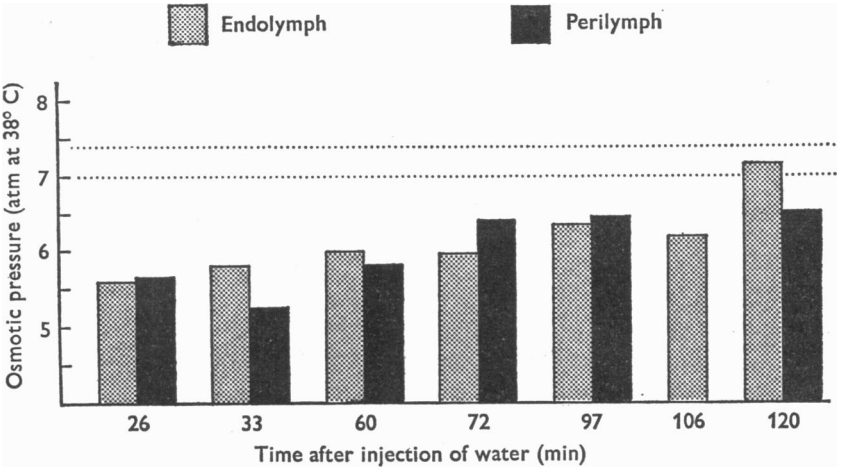

Fig. 4. The changes in the osmotic pressures of the inner ear fluids in 35 day animals after intraperitoneal injection of 1 ml. water/10 g body wt. (the time intervals are not drawn to scale). The dotted lines indicate the range found in control animals.

Consequently, the comparatively sudden and marked decrease in the osmotic pressure of the fluids surrounding the endolymphatic space must, almost certainly, have been associated with an equally rapid and extensive ingress of water into the endolymph. As the general body fluid changes were the maximum possible and the reduction in the extracellular fluid osmotic pressure was of the order of 1·5 atm, the difference in osmotic pressure between the two fluids, which would necessarily result in these circumstances from any appreciable impermeability of the delimiting membranes, should have been manifest. However, since the dilution process could not be fully documented, the possibility exists that a small lag in the fall of the endolymphatic osmotic pressure might have occurred initially due to the presence of some minor impermeability of these membranes. But, since full equilibration had been achieved at 26 min, such impermeability, if present, could only have been slight in degree and it thus seems reasonable to

conclude that some portion, at least, of the membranes bounding the endolymphatic space is freely, or almost freely, permeable to water.

Chemical composition. The analytical results not only revealed the cation concentrations in both endolymph and perilymph to be reduced but also showed this reduction to be on average 10% greater than the value expected from the osmotic pressure measurements in the case of the endolymph and 12% greater in the case of the perilymph, a difference not statistically different from the endolymph figure. These findings thus imply that some osmotically active substances, other than sodium or potassium, are mobilized with consequent maintenance of the osmotic pressure to some extent.

As the nature of these substances seemed of possible importance, an attempt was made to investigate the problem further in seven adult rats. Samples of blood were removed from these animals 30 min after the intraperitoneal injection of water in the same dose as before (1 ml./10 g body wt.), urethane anaesthesia again being employed. The average fall in the osmotic pressure was found to be 0·78 atm at 38° C and the diminution in the plasma sodium concentration was approximately 4% greater than the figure expected from the osmotic pressure measurements. The glucose concentrations, however, remained within normal limits, presumably due to the compensatory adjustments resulting from the induced hypoglycaemia, and the urea concentrations were also unchanged.

The divergence between the osmotic pressure and the cation concentrations in the inner ear fluids are probably attributable to similar changes. Such a conclusion may be of significance in case of the endolymph, unlike the perilymph, for it suggests these non-electrolytes only appear in appreciable concentrations in this fluid in conditions associated with induced water movements.

Examination of the results from the inner ear fluids revealed the cation concentration changes to be limited to the endolymphatic potassium and the perilymphatic sodium ions. In the case of the endolymphatic sodium concentration (range 0·46–1·47 m-equiv/l.), while the homoeostatic mechanisms, it could be argued, might be more efficient with respect to this ion, it must be emphasized that, because of the very low normal concentration, any dilution effects would be correspondingly small and so not discernible from the normal variation. The normal perilymphatic potassium concentration (range 4·3–9·4 m-equiv/l.) is almost certainly the result of the release of intracellular potassium now generally accepted as taking place in these circumstances.

With respect to the chloride ions, there was a decrease in concentration in both the endolymph and perilymph, which was comparable in degree

in each individual animal to the diminution in either the endolymphatic potassium or perilymphatic sodium concentrations.

Endocochlear potential. The results from a further eleven experiments are also available, in addition to the seven documented above, in regard to the potential measurements. Both the range, 41–67 mV, and the mean, 55 mV, do not differ substantially from those found in the normal group of animals (Fig. 1).

Histological examination. The inner ears which had not been subjected to fluid aspiration were examined in four animals but no abnormality was visible using light microscopy; in particular no evidence of any hydrops was found.

As the analytical results indicated that approximately 20 % dilution of the endolymph occurred at the time of maximum diminution in the osmotic pressure, it seemed reasonable to expect some increase in volume to have been present. Such an increase could have been accommodated by dilatation of the saccus endolymphaticus. Although this structure appeared fully distended in the animals examined, the presence of volume changes cannot be established with certainty by histological means because of the normal gross variation in its size, and direct experimental observation proved to be impossible.

Effect of hyperosmolality of the body fluids

Three subsidiary experiments were also performed in which concentration of the endolymph tended to occur, since the compensatory changes in the active fluxes in such a situation would be opposite in type to those occurring in the experiments described above. Unfortunately, the effects of dehydration in neonatal animals are extremely grave and it was impossible to achieve any gross change of this nature without affecting the general condition of the animal adversely and so invalidating the results. In consequence, only relatively small changes could be studied but these did provide the additional opportunity of documenting the effects of minimal changes in composition compared with the much more severe ones already achieved.

The maximum effect permissible in 35-day animals resulted, a preliminary series of experiments revealed, from the intraperitoneal injection of 0·15 ml. of a 33⅓ % (v/v) glycerol solution/10 g body wt. The endolymphatic osmotic pressure in the three animals studied was 7·54, 7·54 and 7·67 atm (at 38° C), 28 and 32 min after the glycerol injection, i.e. a little greater than the maximum value found in the control animals, revealing that slight but significant concentration of the endolymph was produced in each instance. The analytical results revealed the endolymphatic potassium (146–154 m-equiv/l.) and the chloride (120–125 m-

equiv/l.) concentrations to lie at the upper limit of the normal range (Fig. 2) as might be expected and, once more, no significant change was noted in the endolymphatic sodium concentrations, probably for the reason discussed above. The osmotic pressure of the perilymph, on the two occasions it could be measured with accuracy, was 7·61 and 8·11 atm (at 38° C), showing a comparable degree of concentration to be present.

Endocochlear potential measurements were obtained from twelve animals in all and in this case too both the range, 39–66 mV, and the mean, 51 mV, were normal. Histological examination also failed to reveal any abnormality, such as collapse of the cochlear duct.

The findings indicate, therefore, that, when the changes in the body fluids are slight or when hyperosmolality is produced, comparable alterations occur in the inner ear fluids. The situation in these experiments is thus essentially the same as in the previous series described above and the evidence again suggests the membranes surrounding the endolymphatic space to be permeable to water, at least over part of their area.

DISCUSSION

The origin of the endocochlear potential. One problem raised by the present investigation is the nature of the mechanisms responsible for the sudden increase in the magnitude of the potential observed between the 34th and 37th day (post-conception). On the whole, the most likely explanation seems to be that the full potential becomes manifest as a result of a rapid increase in the electrical resistance of some portion of the membranes bounding the endolymphatic space at this time. In such a situation, the processes underlying the production of the potential would be fully active in the younger animals studied, when it will be remembered the endolymphatic ionic concentrations have already attained their mature levels, but would be unable to achieve the adult levels due to the relatively high permeability of this portion of the boundary membrane in its immature state. While it is tempting to correlate such a change in resistance with the coincident development of the cells of Claudius, very little is known about the function of these cells and so it would be prudent to regard their relationship to the process as obscure.

However, due to our lack of knowledge about the electrical characteristics of the cochlear duct during this period, the alternative possibility that the increase in the endocochlear potential results directly from the final maturation of the underlying mechanisms cannot be disregarded. At the present time, the evidence which is available from the effects of perfusion of the perilymphatic scalae with solutions of varying ionic composition (Konishi & Kelsey, 1968*a*) and the effects of various inhibitor

agents, in particular tetrodotoxin (Konishi & Kelsey, 1968*b*), tetraethylammonium chloride (Katsuki, Yanagisawa & Kanzaki, 1966) and ouabain (Kuijpers, 1969*a*, *b*; Konishi & Mendelsohn, 1970), strongly suggests the endocochlear potential arises primarily from the activity of the transport mechanisms concerned in the maintenance of the normal endolymphatic ionic constitution. Since the increase in the electrical potential occurs after the production of the mature ionic constitution, this second alternative would imply the occurrence of a change in mode of these active transport systems from electro-neutral to electrogenic during the period under discussion, an alternative felt to be less likely.

Alternatively, if the endocochlear potential proves, despite the evidence quoted, to be substantially a modified sodium diffusion potential, then the phase of rapid increase in the potential could be the result of the development of selective permeability to sodium of some portion of the membranes bounding the endolymphatic space during the period in question.

The role of the endocochlear potential. As discussed earlier, development of the microphonic potential during cochlear maturation is probably dependent on the increase in magnitude of the endocochlear potential. This view receives strong support from the demonstration by Honrubia & Ward (1969) of a close correlation between the magnitude of these two potentials in mature guinea-pigs when changes in the d.c. potential of the cochlear endolymph were produced by means of a polarizing current, a study which confirmed and extended the results of earlier workers (Davis, Eldredge & Gannon, 1958). Certainly, the view previously advocated, that the appearance of the microphonic potential was solely related to the final structural maturation of the hair cells of the organ of Corti, appears to be no longer tenable in view of the reports revealing these cells to be fully mature morphologically at the time of the rapid increase in the frequency response range and sensitivity of this potential (Mikaelian & Ruben, 1965; Kikuchi & Hilding, 1965).

Since the exact relationship of the cochlear microphonic potential to the transduction process responsible for nerve fibre stimulation is obscure, the investigation of other aspects of the development of hearing are of interest. In the CBA-J mouse, Mikaelian & Ruben (1965) found action potentials could be recorded at the round window and the Preyer reflex elicited for the first time on the day after the appearance of the microphonic potential. Änggård (1965) reported difficulties in obtaining satisfactory recordings from the primary auditory nerve fibres in the rabbit, but was able to obtain reliable evidence showing that the initial appearance both of activity in the second-order auditory neurones and of acoustically evoked reflex responses coincided with the development of the endocochlear and microphonic potentials.

The inception of hearing thus seems to occur during the period of rapid increase in the endocochlear potential and after the characteristic ionic composition has been attained. Hence, the conclusion that the potential is an essential feature of the transduction mechanisms in the cochlea appears inescapable.

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