THE EFFECTS OF CALCIUM ON COCHLEAR POTENTIALS IN THE GUINEA-PIG

by

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I. GENERAL INTRODUCTION

This investigation was prompted by clinical reports of changes in hearing sensitivity in patients having recently undergone parathyroidectomy. It was thought that this might be associated with changes in the blood calcium concentration (Gannon, 1963).

A search of the literature did not disclose any investigations into the role of calcium in cochlear function. Furthermore, only one report of the determination of calcium concentration in the cochlea has been published to date (Citron and Exley, 1957). In normal guinea-pigs the concentration of calcium was found to be the same (3.0 mEq./L.) in perilymph, endolymph and cerebrospinal fluid.

There are marked chemical differences between cochlear endolymph and perilymph, the most striking being the differences in sodium and potassium ion concentrations (Smith, Lowry and Wu, 1954). The effects of changing the concentrations of these ions on the electrical potentials of the cochlea have been extensively investigated (Tasaki and Fernández, 1952; Tasaki, Davis and Eldredge, 1954). Therefore, as a primary objective, it was decided to describe the effects, if any, of calcium concentration changes on the electrical responses of the cochlea to sound stimulation which, hitherto, had not been done.

The electrical phenomena of the cochlea constitute four separate and characteristically different potentials (Davis, 1957) which originate in four different cell populations. While the action poten-

tial originating in the eighth nerve fibres within the modiolus of the cochlea (Davis, Tasaki and Goldstein, 1952) is explained by the ionic mechanism of the nerve impulse (Hodgkin and Huxley, 1952) opinions regarding the mechanism of production of the other potentials are still controversial. Whether or not the production of the cochlear microphonic and summating potential involves ionic membrane currents of sodium and potassium is not known.

The role of calcium in bio-electric phenomena has been extensively studied with respect to ionic mechanisms in myelinated an nonmyelinated nerve fibres, synaptic transmission, and neurohumor production and release. The considerable recent evidence that acetylcholine may participate in the efferent control of the cochlea via the olivo-cochlear bundle, and the well documented relation of calcium to acetylcholine release suggests at least one basis for a possible interaction between calcium dynamics and cochlear function.

Furthermore, control of membrane permeability to ions appears to be a property of the calcium content of the excitable membranes (Koketsu, Nishi and Soeda, 1963) which operate in the manner of an ion exchange mechanism and according to the law of mass action (Carvalho, Sanui and Pace, 1963). It is therefore possible that, based on the nature of interaction between calcium concentration changes with each of the cochlear potentials, some inferences could be drawn as to the possible mechanisms involved in the production of these potentials.

However, the meaning of 'positive' results is not always clear. In the case of the cochlear potentials the recorded voltage may be altered

through effects upon structures not directly responsible for the generation of the response, or upon metabolic processes (Wing, 1959). On the other hand, before 'negative' results can be interpreted as showing absence of interaction between calcium and the mechanism of generation of a given potential it must first be demonstrated that adequate amounts of the cation actually arrive where the supposed mechanism is operating.

Unfortunately, there is no known way of obtaining dependable quantitative information concerning actual concentrations of a substance at its sites of action within the cochlea. Nevertheless, a close approximation would be realized by completely perfusing the area with a solution of the substance under investigation in known concentration. The fluid spaces within the organ of Corti are not directly accessible. However, the ionic barrier-structures as located by Tasaki (Tasaki and Fernández, 1952; Tasaki, Davis and Eldredge, 1954) do not include the basilar membrane, which they have found to be highly permeable to solutions of electrolytes, as opposed to Reissner's membrane and the tectorial membrane which do appear to constitute ionic barriers.

It seemed plausible that total replacement of the perilymph in scala tympani of the cochlea by perfusion with solutions of various known concentrations of calcium should be effective in changing the calcium concentration of the fluids bathing the organ of Corti in a qualitatively predictable manner, even if not quantitatively known.

In view of the above considerations, three stages of the problem were formulated as the objectives of the present investigation:

1. to develop a technique for perfusing scala tympani of the guinea-pig cochlea with solutions of different concentrations of calcium while recording simultaneously the sound-evoked cochlear potentials,

2. to describe the changes in the sound-evoked potentials of the cochlea as a result of changes in calcium concentration in scala tympani, and

3. to correlate the effects of calcium concentration changes on cochlear potentials with the possible mechanisms believed to be involved in their production.

II. LITERATURE REVIEW

A) Morphology of the guinea-pig cochlea

1. Gross anatomy

a) The scalae

The guinea-pig cochlea is contained in a snail-shaped shell of thin bone situated within the air filled auditory bulla and is therefore freely accessible over a considerable part of its surface (Fig. 1). Internally the cochlea consists of a membranous canal spirally coiled around a central axis or modiolus for about four and a half turns. It is divided longitudinally into three parts: scala vestibuli, scala media and scala tympani.

Figure 2 shows a cross section of the second turn of the cochlea in greater detail. The endolymphatic space is delineated by the following tissues: the epithelial layer above the limbus, the inner layer of Reissner's membrane, the stria vascularis and the cells of Claudius and of Hensen. Opinion differs as to whether the boundary is completed by the reticular lamina and the cells of the inner sulcus (Tasaki, Davis and Eldredge, 1954) or by the tectorial membrane (Tonndorf, Duvall and Reneau, 1962). The remaining spaces are filled with perilymph.

b) The organ of Corti

The mammalian cochlea (Fig. 2) is provided with two kinds of hair cells; these cells are regarded as true sensory elements of the greatest importance in the transformation of sound waves into electrical

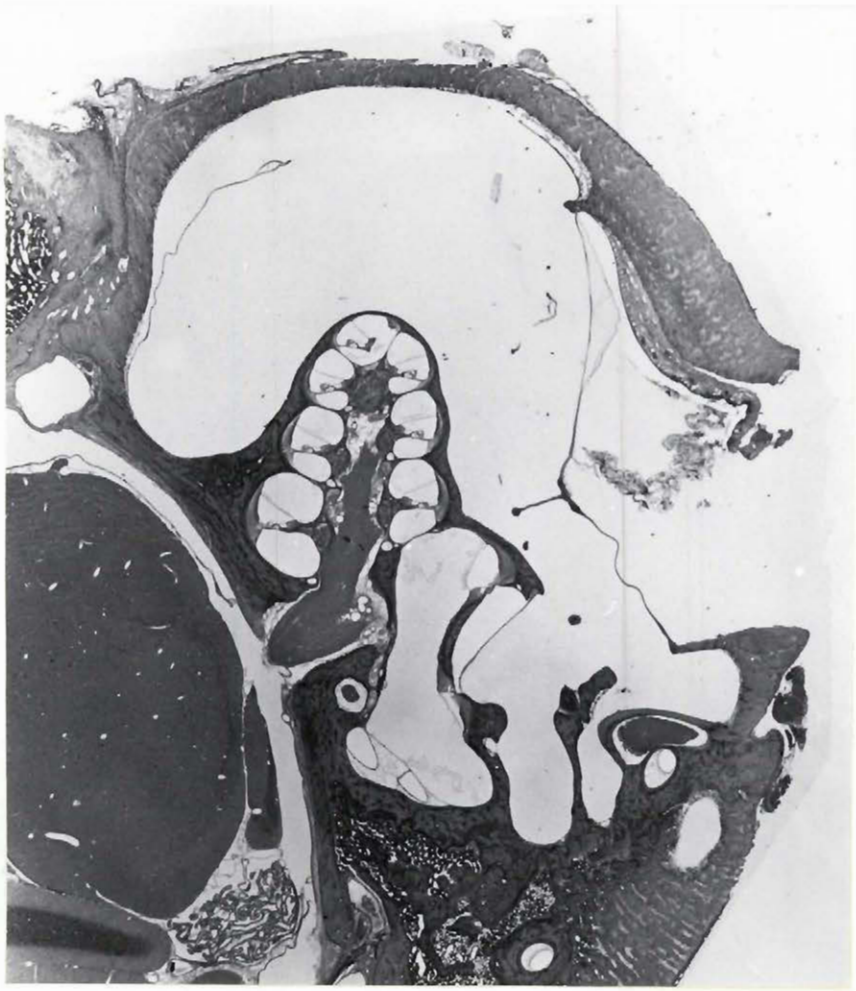

Fig. 1. GROSS ANATOMY OF THE COCHLEA IN THE GUINEA-PIG

Section of the guinea-pig bulla showing a mid-modiolar section of the cochlea. Note the air-filled space within the bulla and the thin wall of the cochlea (Magnification, 12X).

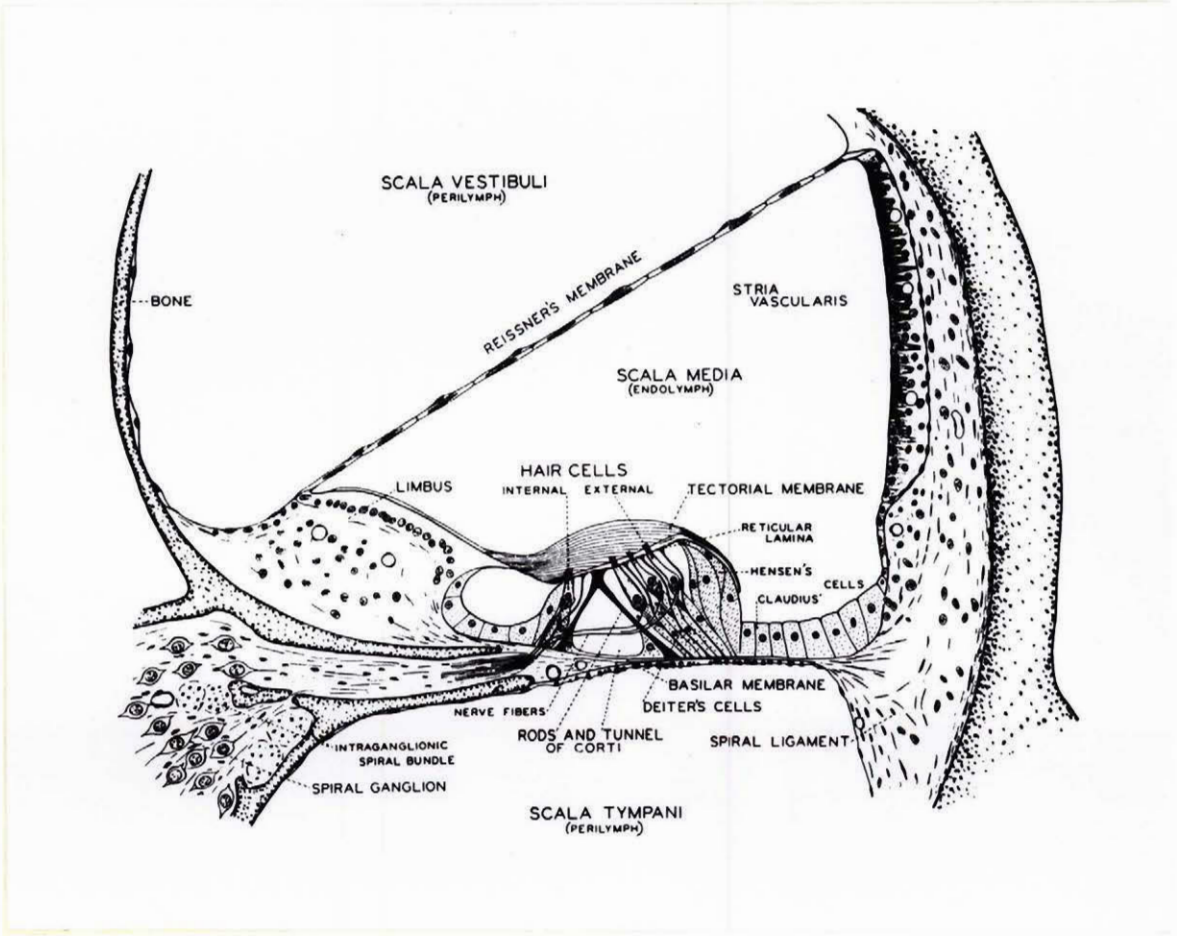

Fig. 2. CROSS SECTION OF THE COCHLEAR PARTITION OF THE GUINEA-PIG

Diagram of the structures of the cochlear partition, including the organ of Corti, based on fixed and stained sections of the second turn of the guinea-pig cochlea. Details of the tectorial membrane are based on descriptions of the microdissections of fresh specimens (Davis and Associates, 1953).

energy and nerve potentials. The external and internal hair cells are both provided with sensory hairs on their upper surface where they are surrounded by a ring of phalanges of the Deiter's cells and are embedded in the reticular lamina; they have a rich neural end-apparatus in a hollow of the Deiter's cells. But, they show at the same time pronounced differences in their structure, probably related to their bio-electric activity (Engström, 1960a).

2. The labyrinthine fluids

The electrolyte distribution within the endolymph and perilymph is known to play an important role in the genesis of the cochlear potentials (Tasaki and Fernández, 1952; Tasaki, Davis and Eldredge, 1954; Vosteen, 1961). Therefore, the composition of the fluids bathing the hair cells and the nerve endings of the cochlear nerve has been investigated by many workers.

The most important contribution has been made by Smith, Lowry and Wu (1954) who first used modern microchemical techniques to analyze the electrolyte content of guinea-pig labyrinthine fluids. Further refinements and the development of ultramicroanalytical methods were later used by Citron, Exley and Hallpike (1956) on guinea-pig and cat fluids, confirming the results of previous investigators. The most important data on the composition of the guinea-pig fluids is given in Table I in abbreviated form, based on the compilations of Vosteen (1961) and Smith (1961a). Citron and Exley (1957) appear to be the only authors who have determined the calcium concentration in the fluids of the guinea-pig ear.

Table I. COMPOSITION OF THE COCHLEAR FLUIDS IN GUINEA-PIG

Substance	Blood Serum	CSF	Perilymph	Endolymph	Reference
Sodium mEq/L.		152	150	16	(a)
	140	150	148	26	(b)
Potassium "		4.2	4.8	144	(a)
	4.5	4.0	5.0	142	(b)
Calcium "		3	3	3	(c)
Magnesium "		2	2	0.9	(c)
Chloride "		122	121.5	107	(a)
		122	120	110	(b)
Protein mg/100 ml		21	50	15	(a)
		20	75	25	(b)
Non-Protein Nitrogen "		21	20	21.5	(b)

(a) Smith, Lowry and Wu (1954)

(b) Citron, Exley and Hallpike (1956)

(c) Citron and Exley (1957)

a) Perilymph

The protein level in perilymph is higher than that of other fluids and may be three times that of endolymph and CSF, possibly due to stagnation (Citron, Exley and Hallpike, 1956). In other respects the composition of perilymph is comparable to that of cerebrospinal fluid (Smith, Lowry and Wu, 1954; Citron and Exley, 1957). The proportions of sodium, potassium, calcium and magnesium are the same, as it also that of the chlorides, and indicates that perilymph is derived mainly from cerebrospinal fluid. Hughes and Chou (1963), by following the passage of radioactive phosphorus (P_{32}) from CSF into the perilymph and endolymph, showed conclusively that the perilymph is mainly composed of CSF which has flowed from the subarachnoid space through the cochlear aqueduct into scala tympani.

b) Endolymph

Endolymph has a high potassium concentration and a low sodium concentration compared to perilymph or CSF. The protein level is the same as that in CSF. The only determinations of calcium (Citron and Exley, 1957) indicate a similar concentration in endolymph, perilymph and cerebrospinal fluid, equal to 3.0 mEq./L. While the high potassium and low sodium content of endolymph is characteristic of intracellular fluid, the magnesium content is not, being in fact lower than that of extracellular fluid (Citron and Exley, 1957).

Engström, Sjöstrand and Spoendlin (1955), Smith (1957), Chou (1961), and Choo and Tabowitz (1964) have indicated considerable evidence to support the belief that the stria vascularis is involved in the formation of cochlear endolymph.

c) Organ of Corti fluid

In view of the high potassium and low sodium concentration of endolymph, Tasaki, Davis and Eldredge (1954), Citron, Exley and Hallpike (1956), and Davis (1961) postulated that the tunnel of Corti could not contain endolymph because the nonmyelinated fibers of the cochlear nerve traversing this space could not function in a high potassium environment. These authors therefore concluded that the tunnel and adjacent space of Nuel and outer tunnel might contain perilymph (Fig. 3).

Engström (1960b) could find no morphological evidence of direct communication between the perilymph of the scala tympani and the tunnel spaces in the embryonic or adult ear. He therefore described the fluids found in the tunnel of Corti, the space of Nuel, the outer tunnel and the space around the hair cells as a separate entity - "Cortilymph".

However, some evidence has accumulated during the past decade which suggests the contrary. The results of Tasaki, Davis and Eldredge (1954) and Tasaki and Fernández (1952) have suggested to these authors that the basilar membrane must be permeable to ions (Davis, 1961), since K^+ ions injected into the scala tympani in higher than normal concentration were able to abolish the CM, presumably by entering the fluids of the organ of Corti.

Rauch (1960) was able by means of several methods to demonstrate that the composition of the fluid in the tunnel and in Nuel's spaces is the same as that of perilymph.

Schuknecht, Churchill and Doran (1959), in their experiments

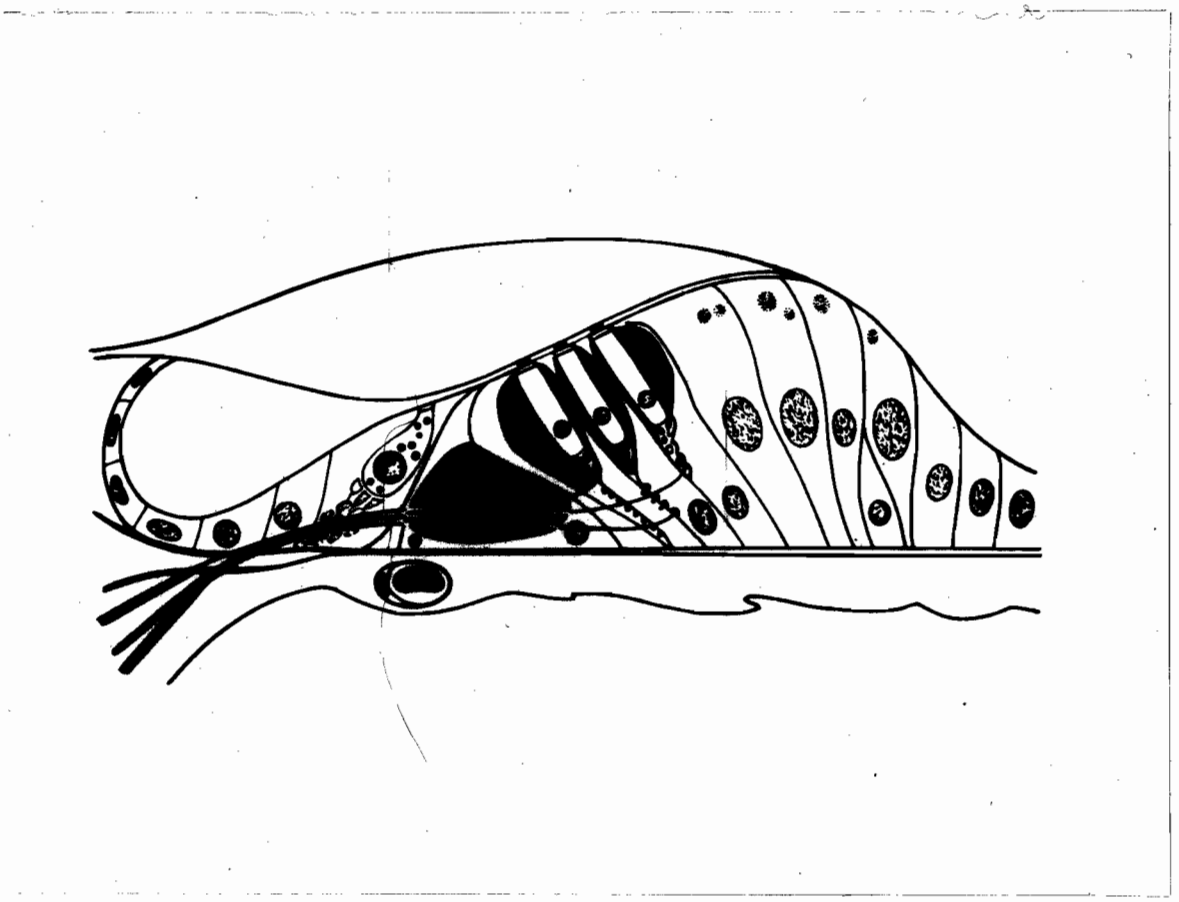

Fig. 3. THE FLUID SPACES OF THE ORGAN OF CORTI

The areas in black are those fluid compartments within the organ of Corti which directly bathe the hair cells, the nerve fiber endings and the peripheral axons of the neurons. These spaces are (from left to right): tunnel of Corti, Nuel's space, spaces around the hair cells, and outer tunnel (Engström, 1960b).

on the distribution of acetylcholinesterase within the cochlea, found evidence of communicating channels through the lower shelf of the osseous spiral lamina into scala tympani, which Schuknecht and Seifi (1963) believe can provide a route whereby perilymph can reach the interior of the organ of Corti (see Fig. 4).

These findings, based on physiological, biochemical and anatomical approaches, support the basic assumption of the present work, that ionic substances injected into scala tympani can rapidly diffuse into the fluids surrounding the hair cells and their associated neural synapses. It remains to be shown, however, what effects will be manifest as a consequence of specific ionic concentration changes.

3. Neural innervation of the cochlea

Anatomical and physiological evidence has revealed that the cochlea is provided with both an afferent and an efferent innervation. The electron microscopy studies of the neurosensory area of the normal cochlea (Engström and Wersäll, 1953; Wersäll, 1956) have disclosed two or more different kinds of nerve endings around the hair cells, which have been described in great detail in the guinea-pig (Smith and Sjöstrand, 1961; Smith, 1961b). The anatomical pattern of fiber distribution within the cochlea has been described by Fernández (1951). No autonomic innervation has yet been demonstrated (Davis, 1962).

a) Afferent innervation

The cell bodies of the primary auditory neurons constitute the spiral ganglion (Fig. 2). Their peripheral processes are nonmyelinated

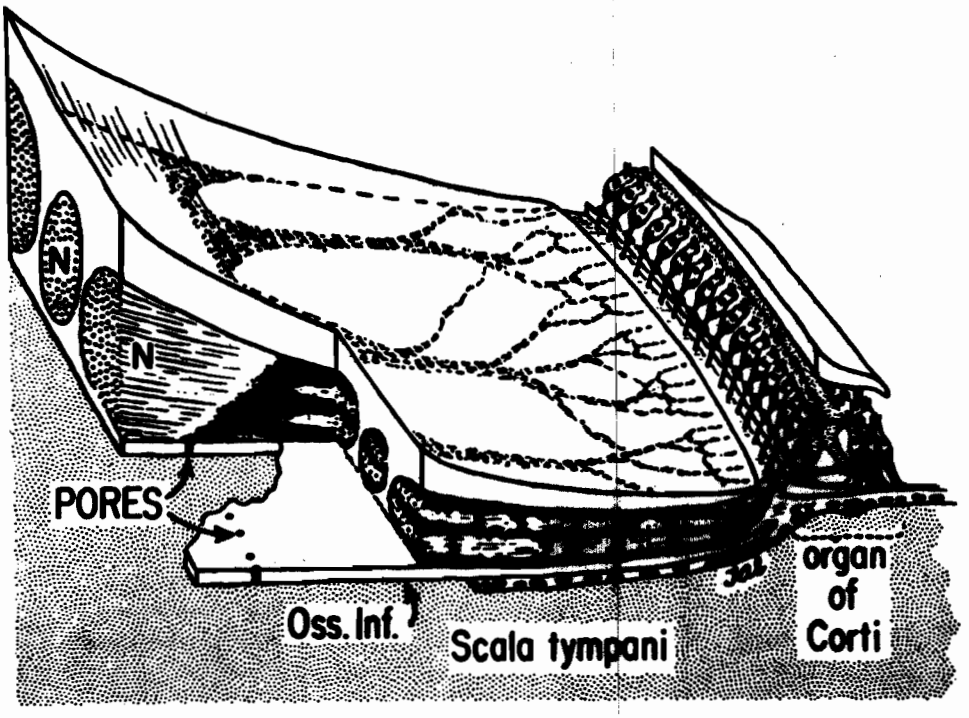

Fig. 4. PERILYMPHATIC CHANNELS OF THE OSSEOUS SPIRAL LAMINA

Artist's impression of the location and distribution of the perilymphatic channels of the osseous spiral lamina, as reconstructed from serial sections of cat cochleas. The system begins at small pores in the inferior shelf of the osseous spiral lamina and passes by a system of interconnected channels among the nerve fibers to reach the habenula perforata (Schuknecht and Seifi, 1963).

beyond the habenula perforata (Smith, 1955) and follow three alternate routes to the hair cells (Fernández, 1951). Afferent fibers which innervate the inner hair cells terminate as elongated endings with few vesicles and ascend the sides of the hair cells (Smith, 1961b). The afferent nerve endings at the external hair cells are now presumed to be the small, non-vesiculated type (Kimura and Wersäll, 1962; Spoendlin and Gacek, 1963) which were described by Engström (1958) and Smith and Sjöstrand (1961).

b) Efferent innervation

A general description of the central and peripheral connections of the efferent innervation of the cochlea can now be formulated, based on the light and electron microscope and histochemical studies summarized by Rossi and Cortesina (1962) and by Smith and Rasmussen (1963).

A contralateral component originating in the accessory superior olivary nucleus (Rasmussen, 1946, 1953), and a homolateral component originating in the main superior lateral olivary nucleus (Rasmussen, 1960) together form the efferent olivo-cochlear bundle which enters the cochlea as the intraganglionic spiral bundle. Like the afferent, they give rise to unmyelinated fibers within the organ of Corti (Smith, 1955).

Fibers destined for the inner hair cells proceed apically or basally as part of the inner spiral bundle (Fernández, 1951), turn toward the hair cells and make extensive synaptic contact with the short, radial cochlear afferents (Smith, 1961b; Smith and Rasmussen, 1963). Other fibers travel with the inner tunnel bundle or traverse the middle of the tunnel of Corti (Spoendlin and Gacek, 1963) to the external spiral bundle with

which they make contact (Smith and Rasmussen, 1963) and terminate as the large, vesiculated endings on the external hair cells (Iurato, 1962; Kimura and Wersäll, 1962; Spoendlin and Gacek, 1963).

c) Transmitter involvement

The olivo-cochlear bundle appears to be part of an efferent system that regulates the auditory responses in the central nervous system (Smith and Rasmussen, 1963). The demonstrations of the cholinergic nature of the olivo-cochlear bundle (Schuknecht, Churchill and Doran, 1959), of the intraganglionic spiral bundle (Rossi, 1960), and of the large vesiculated nerve endings on the hair cells (Hilding and Wersäll, 1962) provide considerable evidence that this system may utilize acetyl choline as its chemical mediator.

B) Physiology of the guinea-pig cochlea

1. Electrical activity of the cochlea

The physiology of the cochlea has been studied extensively by recording electric potentials with electrodes placed in or near the cochlea. Using various combinations of electrodes (Fig. 5), four classes of potentials have been identified and associated with particular sources or generators (Davis, 1957). These potentials are: (a) the direct current resting potentials, intracellular and endocochlear; (b) the cochlear microphonic, or CM, the alternating current response to acoustic stimulation generated in the hair cells; (c) the summating potentials, or SP, either positive or negative, which are direct current responses of the hair cells; and (d) the

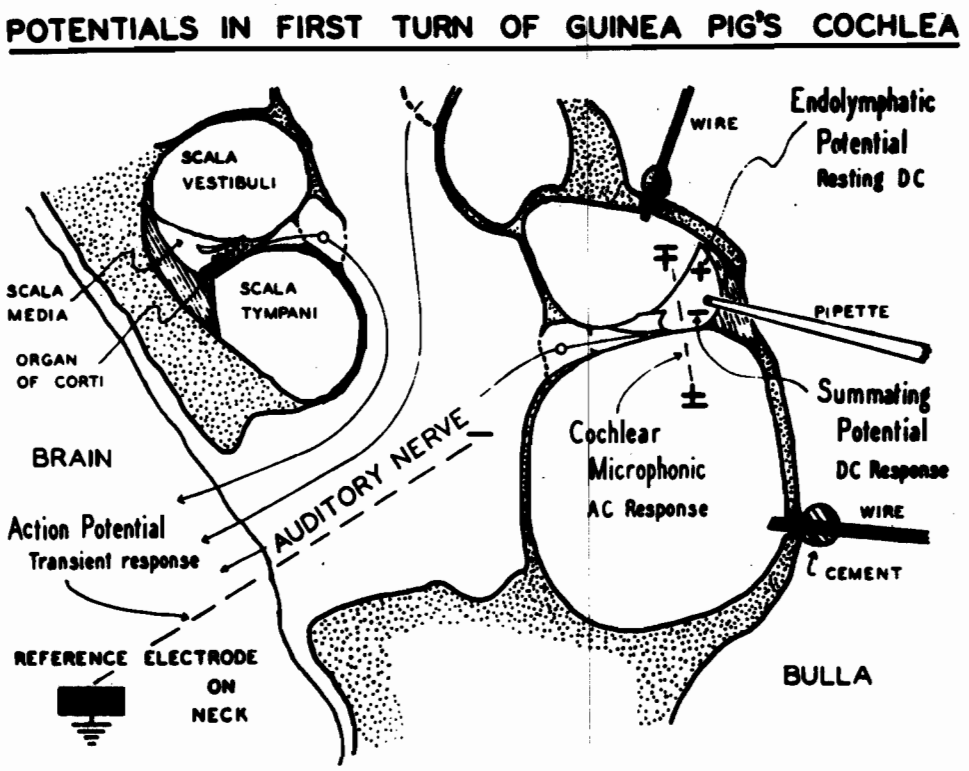

Fig. 5. ELECTRODE PLACEMENT IN COCHLEA OF GUINEA-PIG

Diagram of the electrode placements and orientation of the cochlear potentials in the first turn of the guinea-pig cochlea (Davis, 1956).

action potentials, or AP, of the nerve fibers of the auditory nerve, which carry 'information' about the acoustic stimulus into the central nervous system. The last three are evoked in response to acoustic stimulation and it is these sound-evoked potentials with which the present work is primarily concerned.

2. Characteristics of the cochlear potentials

a) Endocochlear potential

The endocochlear potential (EP), a positive dc resting potential of the endolymph relative to the perilymph, was discovered by Békésy (1951, 1952a). It is approximately +80 mV but varies with position along the cochlear partition and may be as high as 110 to 120 mV (Misrahy et al., 1958b). Three independent studies have verified the source of the EP as the stria vascularis (Davis et al., 1958b; Misrahy et al., 1958a; Tasaki and Spyropoulos, 1959). The dependence of the EP on an adequate oxygen supply has been established (Békésy, 1952a; Davis et al., 1955; Konishi, Butler and Fernández, 1961).

b) Cochlear microphonic

The cochlear microphonic (CM) is an alternating current electrical potential that is proportional, up to a limit, to the displacement of the cochlear partition, and thus indirectly, to the instantaneous acoustic pressure. Vosteen (1961) has summarized the evidence upon which the hair cells are known to be the generators of the CM. Békésy (1952b) and Tasaki (Tasaki, Davis and Eldredge, 1954) have localized the generators of the CM at the level of the reticular lamina where the hairs are located.

Davis (1960) believes that the primary generators of the CM are the external hair cells, while the internal hair cells are the primary generators of the SP (vida infra). The CM is continuously graded, showing no true threshold (Davis, 1957; Wever, Rahm and Strother, 1959), or refractory period (Davis, 1957). It is greatly reduced by oxygen deprivation (Butler et al., 1962) and by alterations in the ionic concentration of cations in the scala media and scala tympani (Tasaki and Fernández, 1952; Tasaki, Davis, and Eldredge, 1954). The amplitude of the CM can also be influenced by direct current applied between scala media and scala tympani (Tasaki and Fernández, 1952).

c) Summating potential

The summating potential (SP), first described by Davis, Fernández and McAuliffe (1950), appears as a shift in the baseline on which the CM is superimposed. Pestalozza and Davis (1956) established the SP as an independent electric response of the cochlea with no detectable latency relative to the CM.

Davis et al. (1958a) investigated the SP in more detail and described most of the characteristics of this response in the guinea-pig. The SP differed markedly from the CM in its resistance to anoxia and was actually increased by anoxia, mild injury, and changes in the chemical composition of the endolymph.

The mechanism of SP generation was linked to specific mechanical movements of the cochlea (Békésy, 1951; Davis et al., 1958a), and probably results from a constant shift of the tectorial membrane relative to the hair cells in the longitudinal direction of the cochlea (Davis, 1958).

Davis et al. (1958b), on the basis of drug toxicity studies, showed that the inner hair cells are the most probable source of the negative SP. Konishi and Yasuno (1963) located the SP generators at the reticular lamina when their micropipette recordings showed that the SP changed in phase at this point.

d) Action potential

The auditory nerve action potential (AP) can be separated from the CM and SP with two intracochlear electrodes and differential amplification (Tasaki, Davis and Legouix, 1952). Recorded in this manner, the AP response to a high frequency tone pip consists of a series of two or three monophasic, negative waves, referred to as N_1, N_2, and N_3 (when present). The total waveform represents an algebraic summation of the 'all-or-none' action potential spikes in the axons of the primary auditory neurons (Davis, 1957; Teas, Eldredge and Davis, 1962). This summation occurs as the impulses pass through the modiolus (Davis, Tasaki and Goldstein, 1952). The amplitude of the N_1 component of the AP is indicative of the number and synchrony of primary auditory neurons stimulated (Tasaki, 1954). The AP is highly sensitive to anoxia (Fernández, 1955; Konishi, Butler and Fernández, 1961) and is also depressed by stimulation of the olivo-cochlear bundle (Galambos, 1956).

3. Effects of cations in the cochlea: sodium and potassium

a) Alteration of perilymph composition

Tasaki and Fernández (1952) showed that if the concentration of KCl in perilymph was increased, the CM and AP were reversibly depressed

to an extent determined by the K^+ concentration and that these potentials could be restored by complete replacement of the perilymph by mammalian Ringer solution which, by itself, produced no effect on the cochlear potentials. KCl acted more readily when applied to scala tympani than when injected into scala vestibuli.

These observations were confirmed and extended by Tasaki, Davis and Eldredge (1954) who noted that the EP was not altered by increasing the KCl concentration in scala tympani. Davis (1959) reported that the SP was also depressed during these same experiments, only more slowly than the CM and AP.

Butler (1964) has reported that a negative dc potential of -80 to -90 mV measured with a micropipette electrode in the organ of Corti, was reduced by addition of KCl into scala tympani.

b) Alteration of endolymph composition

Injections into scala media of isotonic KCl (Tasaki, Davis and Eldredge, 1954) or Tyrode solution having Na^+ and K^+ concentrations comparable to those in endolymph (Davis et al., 1955) did not affect the CM or AP. Injections of Ringer solution (Tasaki, Davis and Eldredge, 1954) or artificial perilymph (high Na^+, low K^+), (Davis, et al., 1955) severely depressed the CM, AP and EP.

c) Implications

i. The electrolyte distribution within perilymph and endolymph plays an important role in the genesis of the cochlear potentials (Tasaki and Fernández, 1952; Tasaki, Davis and Eldredge, 1954; Vosteen, 1961).

ii. The basilar membrane is at least permeable to sodium and potassium ions (Tasaki and Fernández, 1952; Tasaki, Davis and Eldredge, 1954; Tasaki, 1957; Davis, 1957)(1).

C) Theories of cochlear potential generation

A number of theories have been proposed to explain the generation of the sound-evoked cochlear potentials. Although no one theory is without criticism, the mechano-electric theory proposed by Davis (1957), with its modifications, has been most influential in auditory physiology. An outline of several prominent theories found in the current literature follows; the order of the theories is based on the amount of corroborative evidence.

1. Mechano-electric theory: Davis

The essence of Davis' theory (Davis, 1957) is that the original acoustic energy serves to regulate the release of biochemical energy from a pre-existing "pool" or reservoir. The EP and the intracellular polarization of the hair cells make up this pool of immediately available energy, arranged such that these two potentials produce a total dc polarization of about 140 mV across the reticular lamina.

Davis (1961) postulated that, as a result of mechanical deformation, a variable resistance across the cell membrane at the base of the hairs alters its conductance to sodium or potassium ions, thus

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(1) Several years later, Schuknecht, Churchill and Doran (1959) found histochemical evidence of channels linking scala tympani with the fluid spaces of the organ of Corti.

changing the leakage current through the hair cell (Fig. 6). He regards the CM and SP as the receptor potentials[1] of the ear which are generated by the alternating leakage current. While providing for either "ephatic" or "synaptic" excitation of the afferent nerve fibers by the CM and the SP, Davis (1962, 1964) has recently expressed the view that excitation is more likely to be mediated by a chemical transmitter.

2. Critical anodal polarization theory: Tasaki and Spyropoulos

Tasaki and Spyropoulos have recently formulated a theory to explain mechanoreceptor action (Tasaki, 1960). The hair-bearing ends of the hair cells are known to be immersed in a medium containing a high potassium concentration and are exposed to a large anodal polarization by virtue of the endocochlear potential. Under similar conditions of high external potassium and critical polarization, nerve membranes become extremely unstable, shifting suddenly between two stable potential states spontaneously, in response to weak electrical stimuli, hydrostatic changes, and especially mechanical stimuli.

Tasaki (1960) proposed that this mechanism operates in the cochlea. The CM is supposed to result from the large number of cells undergoing large (but not all-or-none) potential variations giving rise to a smooth function. The SP can be interpreted as a shift in the ratio of the number of hair cells at the two potential states.

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(1) "The receptor potential is the graded electric response of a sensory neuron or receptor cell to an external stimulus. It is defined from the point of view of the incident energy of the stimulus. It is the first electric potential to arise in the causal sequence that leads to the nerve impulse" (Davis, 1961).

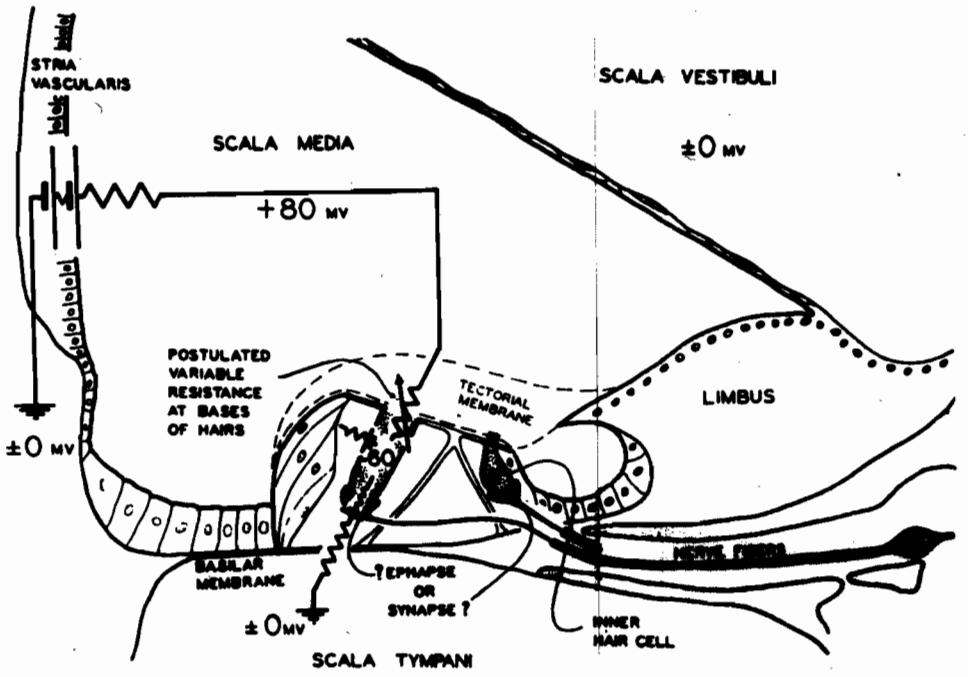

Fig. 6. THE RECEPTOR ACTION OF THE ORGAN OF CORTI: DAVIS

Diagram of the proposed receptor action of the organ of Corti, showing the location of sources of polarization, variable resistance, current path during production of the CM, and two possible modes of nerve ending excitation (Davis, 1961).

3. Cytochemical theory: Vinnikov and Titova

Vinnikov and Titova (1964) proposed the theory that convection currents caused by the vibrations of the organ of Corti are responsible for the initial transport of the acetylcholine in the endolymph to the appropriate hair cells, causing depolarization of the hairs during which the Ach is hydrolized by acetylcholinesterase. However, they deny the mediation of the ionic currents associated with nerve membrane excitation in the generation of the CM and SP.

They claim that the change in ionic equilibrium and the development of electrical potentials are the result of the action of a mediator which, by its energy, sets in motion a series of metabolic changes, initially in the hairs and later in the body of the hair cell. In this latter, during excitation, a complex cycle of metabolic processes takes place. These metabolic processes serve two functions: i) they provide the energy for the release of Ach to excite the afferent fibers, and ii) are accompanied by modifications of the electrolyte composition of the protein substrate of the cell leading to a change in ionic equilibrium and the development of electrical potentials, ie., the CM and SP(1).

4. Potassium displacement-potential theory: Dohlman

By autoradiography, using S^{35}, Dohlman (1959) demonstrated the presence of a protein-bound potassium-muco-polysaccharide complex within the fluids of the tectorial membrane and the cupulae. Because of the highly

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(1) These authors do not specifically differentiate the AP from the CM and SP.

charged negative pole of these molecules, Dohlman suggested that they would be firmly bound by their negative ends to the walls lining the canaliculi of the tectorial membrane, into which the hairs of the hair cells are fitted[(1)], and to the surface of the hairs as well. Movements of the tectorial membrane relative to the hair cells cause the hairs to project more or less into the canaliculi, with a consequent flow or "displacement" of the positive pole of the potassium-muco-polysaccharide complex molecule. The surface membranes of the hairs, being impermeable to ions, would act as a dielectric, causing the cell to act as a condenser. Displacement potentials of the fluid outside the hairs caused by the flow of K^+ ions could thereby produce potentials of opposite phase inside the hair cell by a condenser action.

Dohlman's theory attempts to explain how the fluid oscillations with deformations of the tectorial membrane induced by endolymph movements give rise to the alternating electrical potentials of the cochlea by a mechanism not requiring a concomitant membrane ion flow, and consequently not requiring an added source of energy (Dohlman, 1960).

5. Bent hyaluronate molecule theory: Christiansen

Christiansen (1963) has proposed a model for the generation of the cochlear potentials in which the function of a variable conductance is fulfilled by molecular switches, or hyaluronate molecules. He pictures

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(1) Dohlman's view that the hairs enter the tectorial membrane is at variance with the more recent view emphatically expressed by Engstrom, Ades and Hawkins (1962), that the hairs of the outer hair cells do not enter the tectorial membrane and those of the inner hair cells may not even touch the tectorial membrane when at rest.

the hairs of the hair cells as being covered by a brush-like arrangement of hyaluronate molecules, fixed to the hair membrane and projecting perpendicularly into the fluid or gel-like space around the hairs. Bending of the hyaluronate molecules with vibration of the basilar membrane gives rise to a displacement potential within the cylindrical molecule which can drive ions along its core, either into or out of the cell, via the protoplasmic continuity between the cell and the hairs. Christiansen suggests that the ionic current is carried by hydroxonium ions (H_3O^+) which are driven into the cell through the free end of the hyaluronate molecule by the dc polarization (140 mV) across the hair cell membrane.

D) The effects of calcium on the inner ear

Shimamoto (1954), attempting to find a chemical basis for Ménière's disease, perfused the endolymphatic sac of guinea-pigs with various solutions of drugs and salts including calcium chloride. He reported that potassium chloride solutions evoked spontaneous nystagmus whereas groups of animals given larger injections of calcium chloride, magnesium chloride, atropine, adrenaline, glucose, and streptomycin had no vestibular reaction.

Although Shimamoto reported that magnesium and calcium ions do not exert their effects on labyrinthine activity, Koide and his associates believed that there was no clear-cut evidence available which would not implicate these ions in the chain of events by which cations exert their effects on the labyrinth, since larger injections and complete perfusion of the scala media are complicated by probable mechanical injury.

These authors therefore took up the investigation using different techniques.

Koide (1958) demonstrated that intratympanic injections of isotonic and hypertonic calcium chloride solutions in rabbits produced vestibular reactions indicative of hypofunction of the test side and concluded that the reaction was due to a true toxic effect of the cation.

Using polarographic methods, Koide, Seki and Morimoto (1959) showed that immediately following intratympanic injections of calcium chloride solutions there occurred a drop in oxygen tension in the perilymph in the basal turn of the cochlea, and a maximum fall within 15 minutes. These authors interpreted this "calcium effect" as due primarily to the inhibition of metabolism of the labyrinthine tissues, possibly related to steps in the phosphate transfer system which are sensitive to calcium ion concentration (Brink, 1954), and the drop in oxygen tension being, therefore, of secondary origin. However, neither did they measure cochlear function nor ascribe a possible role for calcium in the bioelectric activity of the cochlea.

E. Role of calcium in neural processes

1. Electrical phenomena of nerve membranes

The role of calcium in neural processes has been extensively discussed by Brink (1954). One of the facts established in his review is that increasing the external calcium concentration raises the threshold, increases membrane resistance and accelerates accommodation.

Frankenhaeuser and Hodgkin (1957) and Frankenhaeuser (1957), working with squid fiber and myelinated frog nerve respectively, have

demonstrated the importance of calcium for the maintenance of membrane potential and for the development of the action potential. They showed that increasing the external calcium concentration is equivalent to increasing the membrane polarization in reducing the membrane permeability.

Increasing the external Ca^{++} concentration prevented the prolongation of the action potential in toad's spinal ganglion cells bathed in TEA or barium rich solutions (Koketsu, Nishi and Soeda, 1963) and in single nodes of toad nerve fiber bathed in hypertonic NaCl plus NiCl solutions (Spyropoulos, 1961). Increasing the internal concentration of Ca^{++} also abolished the conduction of nerve impulse in squid axon (Tasaki, Teorell and Spyropoulos, 1961). Calcium concentration increase probably affects both nonmyelinated (squid) and myelinated (frog) axons in like manner (Ulbricht, 1964).

2. Membrane stability

According to the present view, the nerve membrane is capable of maintaining two different states (Tasaki, 1963; Koketsu, Nishi and Soeda, 1963), either the resting state in which the permeability ratio P_{Na}/P_K is small, or the acting state in which this ratio is large. The principle of the cation-exchange membrane has been applied to explain the reduction of monovalent ion (K^+ and Na^+) flux resulting from addition of divalent ions (Ca^{++}) (Tasaki, Teorell and Spyropoulos, 1961).

A considerable portion of the negative sites of the membrane are occupied by Ca^{++} in the resting state (Spyropoulos, 1961). Removal of this calcium through external influence results in stimulation by allowing

a rapid flow of Na^+ or K^+ to the sites thus exposed. However, this dissociation of membrane calcium will be impeded when the concentration of Ca^{++} in the external solution is high (Wright and Ooyama, 1962; Koketsu, Nishi and Soeda, 1963).

3. Neurohumor release and synaptic transmission

Synaptic transmission can be altered by such factors as Ca^{++} concentration influencing transmitter release. A dependence of neurohumoral release upon the presence of calcium ion in the bathing medium has been demonstrated in the superior cervical ganglion (Harvey and MacIntosh, 1940; Hutter and Kostial, 1954), in the adrenal medulla (Douglas and Rubin, 1961), and at the neuromuscular junction (Katz and Miledi, 1964).

Hutter and Kostial (1954) demonstrated that perfusion of sympathetic ganglia with media containing elevated concentrations of calcium ions resulted in an increase in the amount of Ach liberated by preganglionic stimulation. Acetylcholine release from the small intestine in guinea-pig was increased fourfold when incubated in a solution containing four times the usual concentration of calcium (Gerhards, Röttcher and Straub, 1964).

Takeshige and Volle (1964) suggested, on the basis of their own and other evidence, that calcium ions delivered rapidly and in large amount, can penetrate nerve terminals to effect the release of acetylcholine.

III. METHODS AND MATERIALS

A) Techniques

1. Selection of experimental animals

Experimental animals were healthy adult guinea-pigs weighing between 300 and 400 g. with normal Preyer reflexes and free of any signs of middle ear infection.

2. Operative preparation

Animals were anaesthetised with Dial in Urethane (Ciba) 0.5 cc/kg. body weight intraperitoneally. With supplementary doses of 0.02 cc. animals could be maintained adequately for up to eight hours without artificial respiration. Tracheotomy was done only when necessary.

Using a Zeiss binocular operating microscope the right cochlea was exposed by a ventral approach to the bulla through a skin incision along the mandible. With a needle sharpened to a three-sided point, 25μ holes were drilled by hand into scala vestibuli and scala tympani respectively in the first turn of the cochlea. Next, a hole was drilled into scala tympani, at a point between the first hole and the round window, for insertion of a glass pipette. The pipette, hand drawn from 1 mm. Pyrex glass tubing, was approximately 8 mm. long and had a tip diameter of 90μ. The pipette was inserted snugly and cemented to the edge of the bulla with zinc silicate dental cement. As soon as the pipette was inserted perilymph flowed into it, the level rising noticeably with each contraction of the middle ear muscles. This indicated a patent cannulation. A hole was made later at the apex of the cochlea to provide an outlet during perfusion if the preparation proved satisfactory for use.

The recording electrodes were 1 mil (25μ diam.) insulated nichrome-steel wire, from which the enamel had been scraped for approximately 100μ at the tip, supported by a cotton thread. The bare tips of the wires were inserted into the holes and the electrodes cemented to the edge of the bulla. A tiny, spindle-shaped bead of cement limited the depth of the electrodes (see Fig. 5). Figure 7 illustrates the location of the pipette and the electrodes in the cochlea.

An otological speculum was sewn into the guinea-pig's ear before cementing the pipette and electrodes in place. The animal was transferred to the sound proof room and the quality of the preparation checked. The animal's body temperature was maintained at 37°C with a hot water bottle.

3. Technique of perfusing the cochlea

The technique developed for perfusing the cochlea while recording the cochlear potentials permitted repeated changes of test solutions and added negligible dead space to the perfusion route. Solutions were introduced into the cochlea with a 1 cc. tuberculin syringe connected by a 23 gauge hypodermic needle to a 25 cm. length of PE 50 polyethylene tubing. Several sets of syringes and needles were used to avoid contamination of solutions. The glass pipettes were drawn to an internal diameter which received the PE 50 tubing with a snug, sliding fit. The tips of the polyethylene tubes were also tapered so that they entered the pipette as deeply as possible.

A syringe was filled with the test solution (carefully excluding air bubbles) and, with the needle and tubing attached, warmed

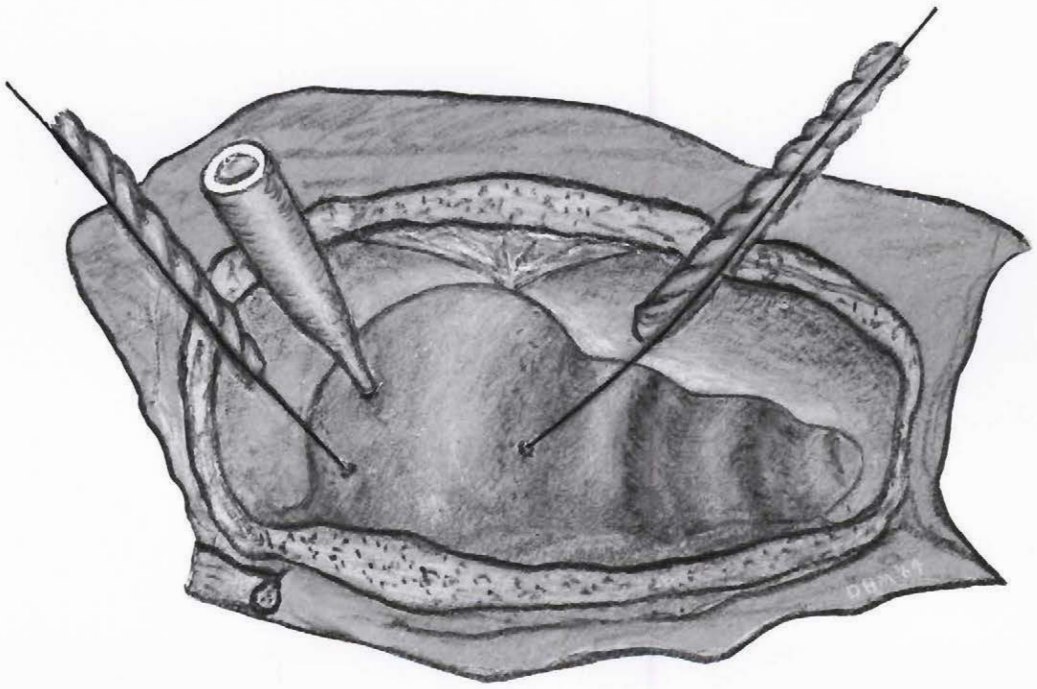

Fig. 7. POSITION OF DIFFERENTIAL ELECTRODES AND PIPETTE IN THE COCHLEA

Drawing of the cochlea as seen through the operating microscope. The cut edge of the bulla is stippled. The 1 mil wire electrodes supported by thread are inserted into holes drilled in the scala tympani (left) and scala vestibuli (right) of the first turn and are shown being supported against the edge of the bulla. A Pirex glass pipette is inserted into a 100μ hole in scala tympani closer to the round window. Dental cement, which is used to fasten the electrodes and pipette to the edge of the bulla, is not shown in this drawing for the sake of clarity.

to body temperature. One half cc. of the solution was ejected through the tubing and the plunger adjusted to a convenient mark. The tip of the tubing, filled with warm solution, was carefully inserted into the glass pipette, thereby displacing the perilymph in the pipette without introducing air bubbles. Continuity between the tubing and the cochlea was thus established with a minimum of dead space. With the tubing held securely in the pipette, the plunger of the syringe was slowly advanced so that exactly 0.05 cc. of the test solution was injected in approximately 30 seconds to prevent large or sudden pressure changes within the cochlea which could adversely affect its function. The fluid thus passed through the whole scala tympani from the round window to the helicotrema, where the apex had been opened for the effluent.

The tubing was then carefully withdrawn from the pipette while an amount of the solution, just sufficient to fill the space left vacant by the retreating tip of the tubing, was injected in order to prevent a sucking action from being transmitted to the cochlea and drawing air into the latter. This step also insured that the pipette remained filled with fluid for the next perfusion.

The quantity of fluid injected was determined in the following way. Using the above technique, a solution stained with India ink was injected under microscopic observation. Ink particles appeared in the effluent at the apex when 0.02 cc. had been injected. Two and one-half times this volume (0.05 cc.) was considered adequate to insure complete change of fluid in the scala tympani. Greater volumes would have required longer perfusion times.

4. Acoustic stimulation

The acoustic stimuli were 7,000 cps tone bursts, at 75 per min., generated by gating the output of an audio oscillator (Hewlett-Packard). After amplification (MacIntosh, 75 watt), the resulting signal was fed to a University 50 watt loudspeaker driver coupled by a rubber hose to the speculum sewn into the animal's ear. The same intensity of stimulus (80 db SPL) was used in all experiments.

5. Electrical recording

The scala vestibuli and scala tympani electrodes and a reference electrode, clipped to the neck of the animal, were connected through cathode followers to a special balancing network (Tasaki, Davis and Legouix, 1952), the outputs of which were amplified differentially (Grass, model P6), displayed on two oscilloscopes (Hewlett-Packard, model 122) and photographed at 10 frames per minute with an oscilloscope camera (Grass, model C4). This arrangement produced two traces, one containing the AP response, the other containing the CM and SP responses (Davis et al, 1958a). Typical normal responses are shown in Figure 8. Precision attenuators (Daven), inserted ahead of the oscilloscope, were used to adjust the amplitude of the control responses to a convenient size. The same setting was used throughout a given experiment.

B) Composition of perfusion media

1. Preliminary experiments

A series of preliminary trials was carried out using various

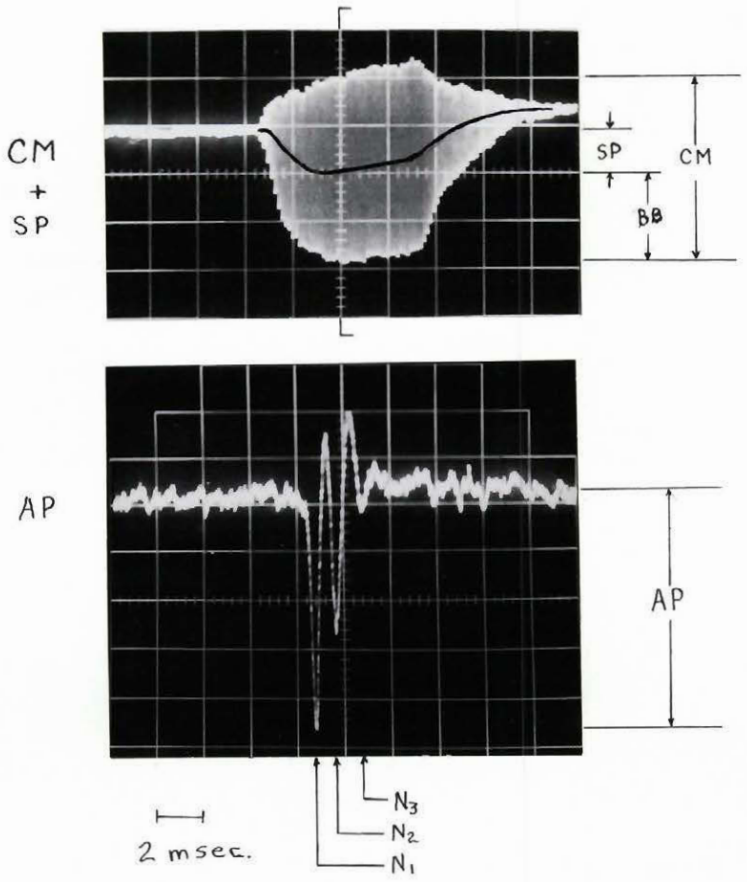

Fig. 8. COCHLEAR POTENTIAL RESPONSES TO TONE BURSTS

Cochlear microphonic and summating potential (above) and action potential (below) from the first turn of the guinea-pig in response to a 7000 cps tone burst. The CM is superimposed on a negative (downward) SP, drawn as a black line through the middle of the CM response. The AP trace shows three successive volleys of synchronized nerve impulses (N_1, N_2 and N_3) following the onset of the microphonic. The amplitudes of the CM, SP, BB and AP are indicated.

physiological solutions with calcium concentrations of 3 and 20 mEq/L. Fourty-four perfusions were done in 24 experimental animals to evaluate these solutions as suitable perfusion media. The solutions used and the number of trials each is given in Table II.

2. Elliott's solution

Elliott's solution was used in a series of perfusions in which the effects of graded concentrations of calcium were studied. Normal Elliott's solution had the following composition[1]: NaCl, 150 mM; KCl, 4 mM; $CaCl_2$, 1.36 mM; $MgSO_4$, 1.2 mM. Calcium concentrations of 2.7, 10, 20, 40, 60, and 80 mEq/L were prepared by adding the appropriate volume of $CaCl_2$ solution. The pH was adjusted to 7.4 with NaOH. $CaCl_2$ stock solution was made on the day of the experiment. Twenty ml. of test solution with the required calcium concentration was prepared immediately before each perfusion.

C) Analysis of data

Each perfusion yielded 60 to 200 frames of film which were numbered consecutively from the start of the perfusion. The amplitudes of the CM, AP and the distance from the baseline to the bottom of the CM (referred to as BB) were recorded in mm. together with the corresponding frame number[2] with the aid of a film reader (Recordak). In the preliminary

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(1) The use of Elliott's solution was suggested by Dr. S. Lowden. The composition given here differs from that originally described (Elliott and Henderson, 1948).

(2) Each frame represented 1/10 minute. The frame number therefore indicated the time in tenths of a minute since the beginning of the perfusion. Negative numbers were used for control frames.

Table II. SOLUTIONS TESTED IN PRELIMINARY EXPERIMENTS

Solution	No. of Trials
Mammalian Ringer Solution * (Bicarbonate buffer)	7
Mammalian Ringer Solution (TRIS buffer) **	14
Krebs Ringer Solution *** (Bicarbonate or Phosphate buffer)	9
Krebs Ringer Solution (Bicarbonate buffer plus pooled CSF)	5
Elliott's Solution, pH 7.4 **** (not buffered)	9
Total no. of trials	44

Animals perfused: 24

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* (Tasaki and Fernández, 1952)

** (Gomori, 1946)

*** (Krebs, 1950)

**** Modified from Elliott and Henderson (1948)

experiments (Table II), the SP was computed from the relation SP = $\frac{1}{2}$ CM - BB and the amplitudes of the potentials (CM, SP and AP) were plotted versus time for each perfusion.

The results of 69 perfusions with Elliott's solution were analyzed using a high speed electronic computer. The frame number, CM, BB and AP amplitudes were punched onto IBM data cards together with the guinea-pig number, perfusion number and the concentration of calcium injected. A programme written in Fortran for the IBM 7040 computer performed the following operations: i) computed the SP; ii) computed the mean of the control period; iii) plotted a graph of the CM, SP and AP amplitudes versus time for each perfusion; iv) computed a set of average curves (CM, SP and AP) for each of the six concentrations of calcium used. The control means were automatically assigned a value of 100 and all amplitudes were expressed relative to the control; v) plotted the data as curves showing the average recovery time course of the cochlear potentials following injection of each of the six concentrations of calcium.

IV. RESULTS AND DISCUSSION

The present work is divided into two sections. The first section includes the preliminary experiments in which the techniques were developed and several types of solutions were tested. The second section includes the major series of experiments on the effects of calcium on the cochlear potentials. The results of these two sections will be presented and discussed separately.

A) Results of preliminary experiments

1. Effects of normal mammalian Ringer solution

Six cochlear perfusion were completed using bicarbonate buffered Ringer solution (Tasaki and Fernández, 1952) containing 3 mEq/L. Ca^{++}. The transient effects on the cochlear potentials were a result of the small pressure fluctuations during the perfusion (Davis et al., 1958a) and manipulations while drying the bulla. These effects were: i) reduction of the CM amplitude lasting 30 to 40 sec. with complete recovery to control value within 60 sec.; ii) reduction of the negative SP amplitude, sometimes associated with a reversal of the SP polarity, lasting 30 to 60 sec.; iii) immediate abolition of the AP response, with gradual recovery within 1.5 min. and complete recovery in less than 2 min. These effects on the CM and AP were similar to those reported by Tasaki and Fernández (1952).

2. Effects of elevated calcium concentrations

a) Bicarbonate and phosphate buffered solutions

Solutions containing calcium concentrations of 3 and 20 mEq/L. were used. In some cases, following perfusion with the high Ca^{++} concentrations, depression of the AP persisted for several minutes although this effect was not obtained consistently. Precipitation of calcium in the solution and in the pipette was noted in several instances.

b) TRIS-Ringer solution

Ringer solution buffered with 0.05 M and 0.03 M TRIS(1) buffer (Gomori, 1946) containing 3 and 20 mEq/L. Ca^{++} was used in 14 perfusions. Prolonged depression of the AP was noted following normal and high Ca^{++} concentrations. Consistent differentiation between low and high Ca^{++} levels could not be demonstrated.

c) Elliott's solution

Initial experiments with Elliott's solution containing 2.6 mEq/L. Ca^{++} produced results similar to those obtained using Tasaki's mammalian Ringer solution. Only transitory depression of the CM and AP were noted. These potentials recovered fully in less than 2 min. The SP was variably affected.

Figure 9 illustrates the results of an experiment on one guinea-pig in which Elliott's solution containing Ca^{++} concentrations of 2.6, 20, and 20 mEq/L. plus pooled human CSF were perfused through the cochlea.

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(1) Tris-(hydroxymethyl) aminomethane-HCl (Sigma)

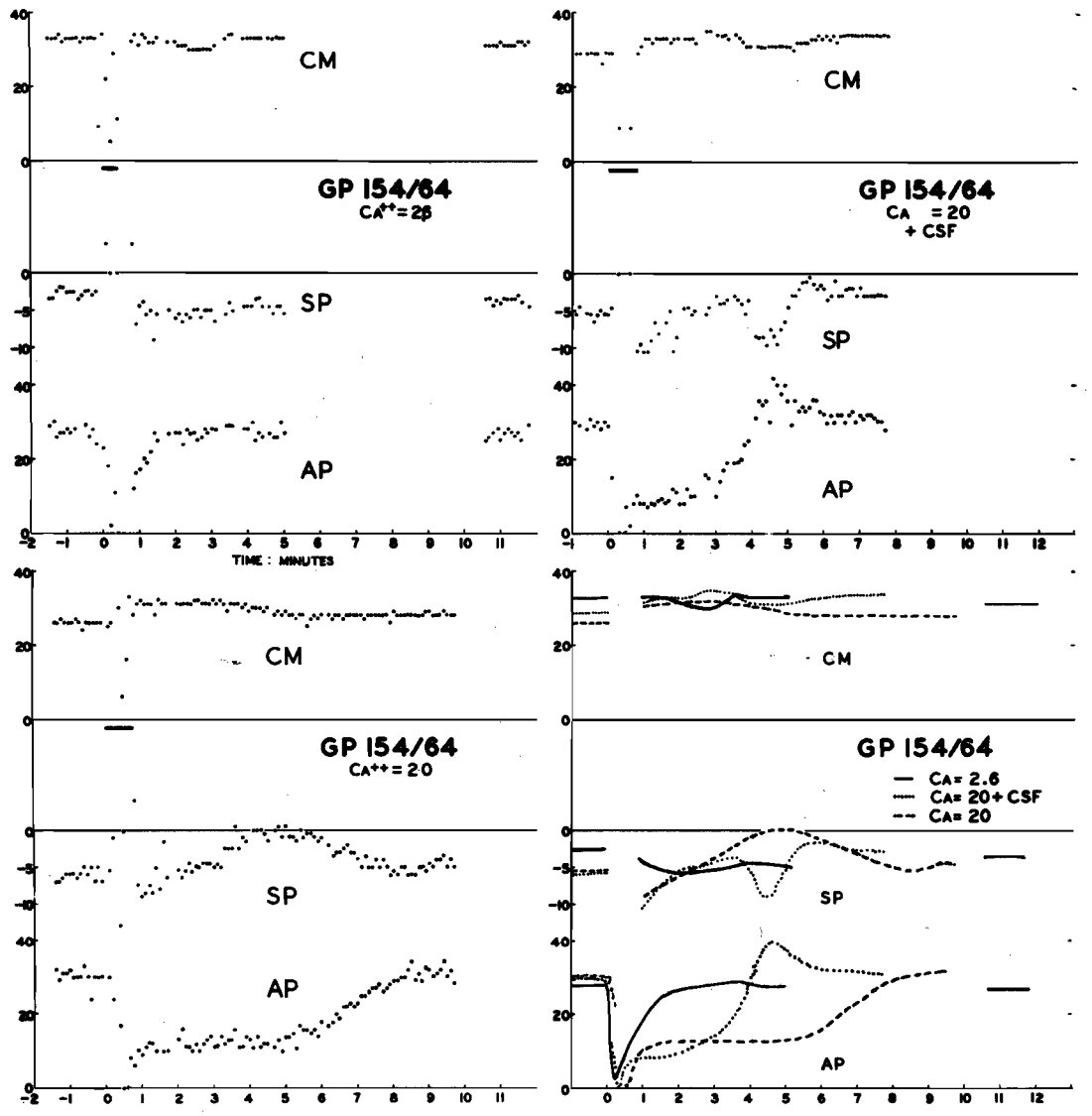

Fig. 9. COCHLEAR PERFUSIONS WITH ELLIOTT'S SOLUTION: PRELIMINARY RESULTS

Results of perfusing scala tympani of the cochlea in the guinea-pig with Elliott's solution. Abscissa: time in minutes relative to beginning of perfusion; ordinate: amplitude in mm. of the cochlear potential response on oscilloscope (see Fig. 8). Horizontal lines are zero reference for the CM, SP and AP. Solid horizontal bar beginning at time zero indicates the duration of perfusion. Individual measurements are plotted in a, b and c.

a) Ca= 2.6 mEq/L; note rapid recovery of CM and AP.
b) Ca= 20 mEq/L; AP is depressed for 8.5 min., CM augmented for 5 min. SP and AP recover simultaneously.
c) Ca= 20 mEq/L plus CSF; pattern of effects is very similar to c) except that overall time course appears shortened.
e) Comparison of a, b and c (results from one animal).

Following perfusion with 2.6 mEq/L. Ca^{++} (as in Fig. 9A), the CM and SP are depressed for 1 min. and the AP for 1.5 min. Comparison with Figure 9B shows the striking difference of increasing the Ca^{++} concentration to 20 mEq/L. The CM recovers rapidly and is augmented for 4 min. while the AP is reversibly depressed for 8.5 min. The SP is affected in a more complex way.

The same solution perfused a second time produced identical results in the same animal (not shown in Fig. 9).

As is seen in Figure 9C, CSF has the effect of shortening the duration of action of the high Ca^{++} solution by a factor of almost 2. A more direct comparison of the results is seen in Figure 9D.

B) Discussion of preliminary results

1. Feasibility of perfusing the cochlea

The preliminary experiments indicated that scala tympani of the guinea-pig cochlea could be perfused with an artificial perilymph solution simultaneously with recording the sound-evoked cochlear potentials. Tasaki and Fernández (1952) and Tasaki, Davis and Eldredge (1954) have shown that, following the perfusion of scala tympani with mammalian Ringer solutions the AP and CM are not adversely affected following the termination of pressure. These observations have been repeated and confirmed in the present investigation. In addition, the actual recovery of the AP has been plotted.

The effects of increasing the pressure in scala tympani during perfusion resulted in depression of the CM, SP and AP, and reversal of SP polarity. The same effects have been illustrated by Davis et al. (1958a).

2. Effects of elevated calcium concentrations

The differential effects of high calcium concentrations on the cochlear potentials, depression of the AP with augmentation of the CM, were not a consistent finding when bicarbonate or phosphate buffers were used in the perfusing fluids. However, Elliott's solution with 20 mEq/L. Ca^{++} did produce consistent effects on the AP and furthermore, these effects were attenuated by incorporating CSF into the perfusing medium.

Elliott's solution differed from ordinary Ringer solutions in that it did not contain bicarbonate or phosphate buffers which tend to limit the concentration of calcium in the ionic form. Also, CSF, because of its protein content, undoubtedly bound the available calcium when added to the high Ca^{++} concentration solution, thereby lowering the ionic calcium level. The observations of a reduced or abolished effect of high calcium concentrations under circumstances which lower ionic calcium levels, indicated that the effects of Ca on the AP were related to the level of ionic calcium (Ca^{++}) in the solutions.

3. Artificial perilymph substitutes

A suitable artificial substitute for perilymph in the present investigation had to be one which was compatible with high ionic calcium concentrations. Two approaches to this problem were investigated: TRIS-buffered Ringer solution and unbuffered Elliott's solution.

a) TRIS-Ringer solution

TRIS-Ringer solution was unsuitable for perfusing the cochlea,

although the Ca^{++} remained stable in solution (Gomori, 1946). The AP was generally depressed for longer periods following perfusion with TRIS-Ringer as compared to bicarbonate buffered solutions and occasionally both normal and high Ca^{++} concentrations resulted in similarly prolonged AP depression.

b) Elliott's solution

Elliott's solution, an unbuffered salt solution with pH adjusted to 7.4 by the addition of NaOH, was found to be the most suitable medium for perfusing the cochlea with both normal and high concentrations of Ca^{++}. Complete recovery of the cochlear potentials following perfusion with 2.6 mEq/L. Ca^{++} was always within 1.5 to 2 min. This solution was therefore adequate in view of the fact that Tasaki and Fernández (1952) reported that the AP required 2 min. to recover following cochlear injections in their experiments using bicarbonate buffered Ringer solution. Furthermore, preliminary experiments indicated that the perfusion of 0.05 cc. volumes of the solution could be repeated several times without causing obvious deterioration of the cochlear potential responses. Elliott's solution therefore seemed to be a suitable medium for studying the effects of graded increases in the Ca^{++} concentration of scala tympani.

C) Effects of graded concentrations of Ca^{++} on the sound-evoked cochlear potentials: results

Sixty-eight successful perfusions were completed using Ca^{++} concentrations of 2.7, 10, 20, 40, 60 and 80 mEq/L. in Elliott's solution. The results of these perfusions are presented in Figure 10. The corresponding Ca^{++} concentrations have been identified at points along the curves where

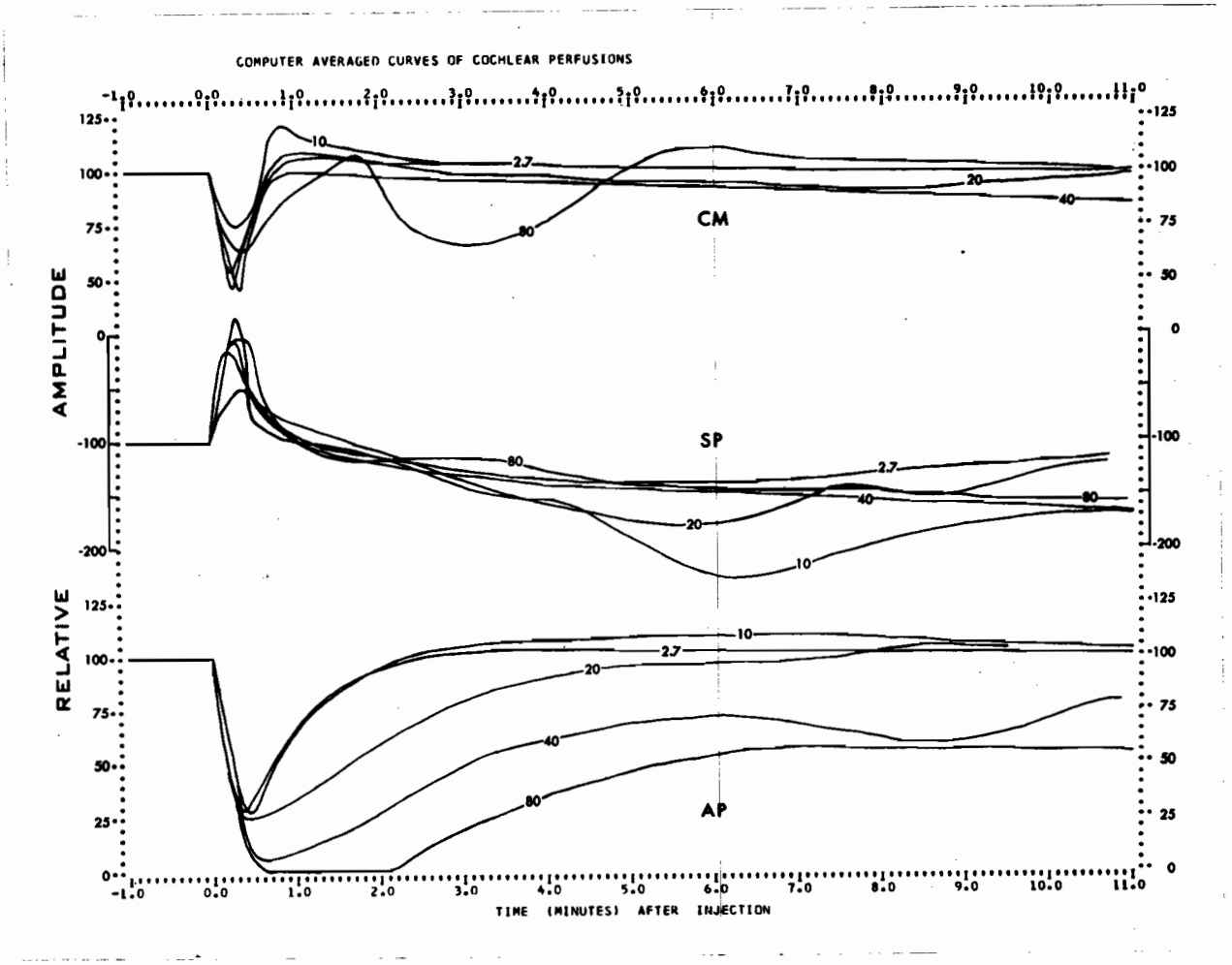

Fig. 10. EFFECTS OF CALCIUM ON THE COCHLEAR POTENTIALS

Computer-averaged curves of 67 perfusions in the guinea-pig cochlea. Results are shown for Ca concentrations of 2.7, 10, 20, 40 and 80 mEq/L. One perfusion with 60 mEq/L. is not included. The averaged results plotted by the computer as individual points were converted manually into line curves and superimposed. The original graphs contained 35 minutes of data but only the first 11 minutes following perfusion are of special interest. See text for discussion.

Number of perfusions in each group: 2.7 (25); 10 (8); 20 (18); 40 (12); 80 (4).

individual variations are evident. One perfusion using 60 mEq/L. Ca^{++} has been omitted from the composite graph. The curves for that experiment were similar to the ones drawn for 80 mEq/L. Ca^{++}.

The most striking result seen in this graph is a graded effect of Ca^{++} concentration on the depression of the AP. The AP is depressed for 2 min. following perfusion of 2.7 and 10 mEq/L. Ca^{++} but is normal for several minutes thereafter. The AP responses following 20, 40 and 80 mEq/L. are depressed for periods qualitatively related to the Ca^{++} concentration of the perfusate.

By contrast, the CM and SP responses are augmented by Ca^{++} concentrations of 20 mEq/L. or lower. All SP and CM curves (except 80) are at normal levels within 45 seconds after the beginning of the perfusion. The CM curve for 80 mEq/L. Ca^{++} is based on only four perfusions and may therefore be influenced by artifacts. Augmentation of the CM responses is noted following perfusion of 2.7, 10 and 20 mEq/L. Ca^{++}.

The SP effects all follow the same pattern. The amplitude of the negative SP is actually increased, this effect being most prominent following 10 and 20 mEq/L. Ca^{++}.

D) Discussion

The effects of perfusing scala tympani of the cochlea in the guinea-pig with Elliott's solution containing graded concentrations of Ca^{++} between 2.7 and 80 mEq/L. were noted as a differential action of Ca^{++} on the sound-evoked cochlear potentials. The CM and SP recovered quickly and were augmented in amplitude by moderate increases in Ca^{++} concentration

while the AP was depressed to an extent qualitatively related to the Ca^{++} concentration of the perfusate. Preliminary experiments related these effects to the concentration of ionic calcium in the perfusing fluid while further experiments have more clearly demonstrated a complex action of Ca^{++} on the cochlear potentials, involving both the neural and receptor potential responses. These effects can be explained by a direct action of Ca^{++} on the neural component and an indirect action on the receptor potential components.

1. Direct effects of calcium on the AP

Tasaki and Fernández (1952) and Tasaki, Davis and Eldredge (1954) presented evidence that Na^{+} and K^{+} ions can enter the organ of Corti from the scala tympani. Schuknecht, Churchill and Doran (1959) later showed evidence of communicating channels connecting scala tympani with the organ of Corti (see Fig. 4). On the basis of this evidence, the present author suggested that Ca^{++} ions can also diffuse from scala tympani to the organ of Corti.

In the preliminary experiments it was established that the method developed for perfusing the guinea-pig cochlea with normal mammalian Ringer solutions did not adversely affect the cochlear potentials and using Elliott's solution, high concentrations of Ca^{++} (in ionic state) could be produced throughout scala tympani. The AP depression seen in the experiments can be explained, on the basis of the known effects of Ca^{++} in blocking nerve excitation and conduction (Brink, 1954; Tasaki, Teorell and Spyropoulos, 1961), by the entry of Ca^{++} into the organ of Corti or into the habenula perforata. If the Ca^{++} entered via the route illustrated by Schuknecht

and Seifi (1963), then nerve conduction was blocked in the myelinated section of the nerve fibers between the spiral ganglion and the organ of Corti (see Figs. 2 and 4). Immediate depression of the AP responses could result as soon as the calcium entered the osseous spiral lamina.

2. Imitation of olivo-cochlear bundle activation

Besides the direct effect of Ca^{++} on the myelinated nerve fibers within the osseous spiral lamina, Ca^{++} appears to have affected the hair cells, as evidenced by the augmentation of the CM and SP. Electrical stimulation of the olivo-cochlear bundle has been shown to reduce the action potential (Galambos, 1956; Fex, 1959; Desmedt and Monaco, 1961) and to augment the cochlear microphonic (Fex, 1959; Desmedt, 1962). These same effects were also seen following perfusion with 10 mEq/L. Ca^{++}. In other words, a moderately elevated Ca^{++} concentration in the cochlea appears to have mimiced the effects of olivo-cochlear bundle stimulation. An explanation of this effect is warranted, in view of the current concepts of the efferent mechanism of the cochlea.

Considerable evidence exists to support the present view that acetylcholine is the chemical mediator of the olivo-cochlear bundle (Schuknecht, Churchill and Doran, 1959; Rossi, 1960; Hilding and Wersäll, 1962; Smith and Rasmussen, 1963). Desmedt and Monaco (1961) proposed that the efferent neurons (of the olivo-cochlear bundle) produce a post-synaptic inhibition at the endings of the afferent neurons by liberating an inhibitory transmitter. They also proposed that a certain amount of this substance would diffuse onto the membrane of the hair cell whose resting potential would thereby be increased, consequently resulting in an increase in the "receptor potential" (CM) amplitude.

Gisselsson has demonstrated that iontoelectrophoretic injection of acetylcholine into scala media close to the hair cells brings about an increase in the amplitude of the CM. His observation seems to indicate that the CM augmentation noted on stimulation of the olivo-cochlear bundle is a consequence of the release of acetylcholine by the efferent neurons.

3. Indirect action of calcium on the hair cells

It has been shown that the liberation of acetylcholine may be potentiated or even effected by increases in Ca^{++} concentration (Hutter and Kostial, 1954; Gerhards, Röttcher and Staub, 1964; Takeshige and Volle, 1964).

While it is not known for certain whether the olivo-cochlear fibers carry tonic activity or are specifically activated during acoustical stimulation, the release of acetylcholine from the terminals of these neurons may be produced by Ca^{++} even in the absence of impulse activity (Takeshige and Volle, 1964). On the basis of the action of calcium ions enumerated above, it is possible to explain the Ca^{++} induced augmentation of the CM and SP by postulating an indirect action of the ion through the liberation of acetylcholine from the olivo-cochlear terminals at the base of the hair cells.

The SP is believed to be generated by the same energy-transducing mechanism as the CM (Davis, 1961). The potentiating effect of the Ca^{++} on both the CM and the SP, if brought about indirectly through the liberation of acetylcholine, suggests that the SP should increase following olivo-cochlear bundle stimulation. This effect on the SP has

not been described, in the investigations in which tone bursts were used as stimuli (Desmedt and Monaco, 1961).

4. Factors affecting recovery

In the guinea-pig, cerebrospinal fluid enters the scala tympani via the cochlear aqueduct (Hughes and Chou, 1963) and by the perineural and perivascular spaces in the internal auditory meatus, especially when an artificial opening is made into the cochlea (Davis, 1957). This was born out in the present experiments by the consistent observation of perilymph flowing into the pipette as soon as it was inserted into the cochlea, and that the pipette refilled with fluid in a few minutes each time it was dried out for any reason. The fluid perfused into scala tympani was therefore replaced and diluted by CSF whose high protein content would ultimately bind almost all available ionic calcium.

The time taken for the gradual recovery of the AP responses was quantitatively related to the original Ca^{++} concentration. In a preliminary experiment CSF, when added to the perfusing medium, reduced the depressing effect of 20 mEq/L. Ca^{++} on the AP. Recovery of the AP was therefore governed by the rate at which Ca^{++} was bound and/or diluted, which in turn, was limited by the rate at which natural perilymph replaced the perfusate in scala tympani.

In view of the very high protein concentration of perilymph (Citron, Exley and Hallpike, 1957) the amount of ionic calcium is very small compared to the total calcium concentration of 3 mEq/L. measured by Citron and Exley (1957). It therefore follows that perfusion of scala tympani with

Elliott's solution containing 2.7 mEq/L. Ca^{++} can also produce a temporary elevation of the ionic calcium concentration in the scala.

5. Relation to other receptors

The normal effects of calcium on mechanoreceptors is to reduce their sensitivity. Paintal (1957) found that $CaCl_2$ reduced the peak frequency of discharge in normal pulmonary and atrial stretch receptors. The results of the present investigation on the action potential of the auditory nerve suggest a similar effect of calcium on the mammalian cochlea.

E) Implications on theories of cochlear action

1. Role of calcium in the cochlea

The present investigation of the effects of increasing the concentration of Ca^{++} in scala tympani has demonstrated a depression of the AP response which is adequately accounted for by a diffusion of Ca^{++} into the osseous spiral lamina via the route suggested by Schuknecht and Seifi (1963). However, the concomitent augmentation of the CM can best be explained by comparison with the similar effect associated with olivo-cochlear bundle stimulation (Fex, 1959; Desmedt, 1962).

In view of Gisselsson's (1960) observation that acetylcholine injected into the hair cell region augmented the CM, and the ability of Ca^{++} to effect acetylcholine release (Takeshige and Volle, 1964), the effects of Ca^{++} noted on the CM support the assumption that injected Ca^{++} reached the organ of Corti and ultimately gave rise to an increased concentration of Ca^{++} in the neighbourhood of the hair cells.

2. Mechanism of generation of the cochlear receptor potentials

Although no direct proof of the above assumption can be shown in the present investigation, it does, however, suggest that increasing the Ca^{++} concentration at the hair cell membrane does not directly interfere with the receptor potential generating mechanism.

Fourtes (1959) has suggested that the generator potentials of the limulus eye (and possibly of the crustacian stretch receptor) arise as a consequence of a change in ion permeability of the sensory cell's membrane. Davis (1961) tentatively suggested that, by analogy, the receptor potentials of the cochlear hair cells may also depend on permeability changes to Na^{+} or K^{+}. However, an important difference between these receptors must be taken into account. The generator potential of the visual cell in the limulus eye and of the crustacian stretch receptor are dendritic responses (Fourtes, 1959) whereas the cochlear receptor potentials arise in cells other than the primary neuron or any of its parts, ie., in the hair cell (Davis, 1957).

Loewenstein, Terzuolo and Washizu (1963) have shown that the spike and generator potential process are independent events in the crustacian stretch receptor and the mammalian Paccinian corpuscle. The spike was selectively blocked by tetrodotoxin without affecting the generator potential. These authors suggested that the spike potential (action potential) and generator potential (dendritic response) of sensory receptors depend on different mechanisms. The receptor potentials of the cochlear hair cells (CM and SP) may be generated by yet a third mechanism different from the other two. This point of view is reflected in the

. theories proposed by Dohlman (1959, 1960), Christiansen (1963) and to some extent, by Vinnikov and Titova (1964).

The results of the present investigation can be interpreted as suggesting that the mechanism underlying the generation of the CM and SP does not depend on increased permeability of the hair cell membrane.

V. SUMMARY

The present investigation was carried out for the purpose of studying the effects of changing the concentration of calcium in the cochlea on the sound-evoked cochlear potentials. As a preliminary step a method was developed for perfusing scala tympani in the guinea-pig cochlea with solutions of graded concentrations of calcium while recording the electrical responses generated within the cochlea.

The most suitable perfusing medium was found to be Elliott's solution, an unbuffered salt solution which was adjusted to pH 7.4 by the addition of NaOH.

When the cochlea was perfused with normal levels of calcium in this solution, disruption of the electrical activity lasted no more than 2 min., and was due mainly to mechanical factors.

Preliminary experiments indicated that elevated concentrations of calcium prolonged the depression of the auditory nerve action potential but augmented the cochlear microphonic and summating potential responses. It was also shown that the effect was related to the concentration of calcium ions in the perfusing solution.

A series of perfusions were carried out with graded concentrations of Ca^{++} ranging from 2.7 to 80 mEq/L. The results of 68 perfusions were averaged in 6 groups corresponding to Ca^{++} concentrations of 2.7, 10, 20, 40, 60 and 80 mEq/L.

The average effect on the AP was a prolonged depression of this response in accord with increasing the level of Ca^{++} bathing nerve tissues.

However, the CM and SP responses were augmented by Ca^{++} concentrations up to 20 mEq/L.

This effect of Ca^{++}, described as mimicing olivo-cochlear bundle stimulation, was explained on the basis of a potentiated or stimulated release of acetylcholine from the efferent neuron endings by the elevated Ca^{++} concentrations.

The above interpretation of the results implied that the Ca^{++} concentration increased in the vicinity of the hair cell membrane but did not adversely affect the receptor generator mechanism. This finding is opposite to what would be expected on the basis of Davis' theory that the generation of the receptor potentials of the cochlea derive from an increase in Na^{+} or K^{+} permeability of a part of the hair cell membrane.

In view of this disparity, it was therefore suggested by the author that the receptor potential mechanism may not be ion flux dependent.

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