The effect of
tetracycllin on the processes of calcification of the
otoliths in the developing chick embryo

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It is known that tetracycllin is fixed in calcified tissues and that it can have an inhibitory action on calcification processes under certain conditions (Saxén, 1966). The mechanism by which tetracycllin is bound to the calcified structures is not clearly understood. Some workers believe that there is simple chelation between the antibiotic and calcium (Albert, 1953); others believe that the fixation of tetracycllin is mediated by more complex mechanisms (Sternberg, 1966; Saxén, 1966). The latter hypothesis is based on the observation of the presence of both a stable complex of tetracycllin-Ca and an unstable form from which either the calcium or the tetracycllin can undergo rapid changes.

Histochemical and autoradiographic studies have been made on the localization of mucopolysaccharides and on the exchange of calcium in the membranous labyrinth of the developing chick embryo during the processes of calcification of the otoliths. These studies have demonstrated an intimate, precocious correlation between $^{45}$Ca and the mucoprotein which constitutes the matrix of the otoliths (de Vincentiis, Marmo & Materazzi, 1964; de Vincentiis & Marmo, 1966a, b; Balsamo, de Vincentiis, Marmo & Materazzi, 1969). Inhibition of the morphogenesis of the otoliths has been observed in the presence of specific inhibitors of carbonic anhydrase (de Vincentiis & Marmo, 1965, 1966a, 1968; Marmo, 1965, 1966a, b; Balsamo, de Vincentiis, Marmo & Parisi, 1969). In order to elucidate more clearly the mechanisms of morphogenesis and calcification of the otoliths it seemed of interest to conduct experiments utilizing tetracycllin. This was administered to chick embryos at different stages of development and its localization and effect on the structures under investigation were studied.

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MATERIALS AND METHODS

From 2 to 4 mg of HCl tetracyclin (kindly furnished by Pfizer Co.) dissolved in dimethyl-sulphoxide (Fluka) were inoculated into chick eggs on the 4th, 10th and 12th days of incubation (stages 23, 36 and 38 according to Lillie, 1952) and 0.1 ml of the antibiotic solution was injected into the egg white according to the method of Conti & Milio (1964). At the same time the necessary controls were made, injecting 0.1 ml of dimethylsulphoxide into eggs at the same stage of development. The dose of 2-4 mg of HCl tetracyclin was chosen because with this dose maldevelopment can still be observed in the chick embryos inoculated on the 4th day (Genazzani, Pellagalli, Pagnini & Di Carlo, 1965), even though there is a high mortality rate of about 70%. The embryos treated on the 4th day of incubation were fixed on the 5th or 8th day (stage 26 or 34 according to Lillie); those treated on the 10th and 12th days were fixed 24 h after the injection, on the 11th and 13th days of incubation respectively (stages 37 and 39 according to Lillie). The heads of the embryos were fixed in 4% neutral formalin, embedded in paraffin wax and sectioned at 7 μ. The observations were made with a fluorescent microscope (Ortolux Leitz). On some sections both the Alcian-PAS reaction (Mowry, 1956) and the Kossa reaction for calcium were carried out. Sections of control embryos were deparaffinized and treated with 0.8% tetracyclin dissolved in dimethylsulphoxide (Steendjik, 1964).

RESULTS

Observations were made on a total of twenty-two embryos and the results can be summarized as follows:

(1) Under the experimental conditions used tetracyclin does not seem to exert any apparent influence on the morphogenesis of the otoliths since they remain morphologically and cytochemically similar to the otoliths of controls.

(2) In embryos inoculated with 4 mg/egg of tetracyclin on the 4th day of incubation and sacrificed on the 8th day, there was an intense fixation of the antibiotic by the otoliths of the sacculus and little or no fixation of the antibiotic in the otoliths of the utriculus (Fig. 1). The Kossa reaction at this stage of development was more intense at the level of the otoliths of the sacculus than in those of the utriculus. A weak fluorescence was also observed in the epithelium of endolymphatic sac and duct. In the embryos treated on the 10th day of incubation and sacrificed on the 11th and 13th days of incubation respectively, there was no visible difference in the intensity of the fluorescence in the otoliths of the sacculus and the utriculus: both were intensely fluorescent (Fig. 2). The intensity of the Kossa reaction was also the same at both sites. The otoliths of the lagena are also present at this stage and appear intensely fluorescent. The epithelium of the sacculus, the utriculus, the tegumentum vasculosum and the endolymphatic sac and duct is also weakly fluorescent.
It is interesting to note that the fluorescence is mainly located at the periphery of the body of the otolith (Fig. 3) and appears similar to the distribution of the Alcian–PAS-positive material (Fig. 4). Otoliths of 13-day embryos, which were slowly decalcified in physiological solution at pH 6.4 at 37 °C according to the method of Palladini, Alfei & Conforti (1965), lost their fluorescence between the

Fig. 1. Membranous labyrinth of chick embryo of 8th day of development inoculated with 4 mg/egg of tetracyclin on 4th day of incubation. There is a more intense fluorescence in the otoliths of the sacculus than in those of the utriculus. × 80 approx. (s = sacculus; u = utriculus.)

Fig. 2. Membranous labyrinth of chick embryo of 13th day of development inoculated with 4 mg/egg of tetracyclin on the 4th day of incubation. There is the same intensity of fluorescence in the otoliths of the sacculus and those of the utriculus. × 250 approx.

Fig. 3. The utriculus otoliths of chick embryo of 13th day of development inoculated with 4 mg/egg of tetracyclin on the 4th day of incubation. Notice the peripheral localization of the fluorescence. × 600 approx.

Fig. 4. The utriculus otoliths of chick embryo of 13th day of development treated with Alcian–PAS. Notice the peripheral localization of the positive reaction. × 600 approx.
10th and 13th h of incubation. The Kossa reaction of material incubated in this way is negative.

(3) Treatment of deparaffinized sections with tetracyclin has shown incorporation of the antibiotic only in the otoliths of the sacculus in the 8-day embryo. This is analogous to experiments in vivo. Embryos after 13 days of development show, on the contrary, fluorescence in the otoliths of both the sacculus and the utriculus; the antibiotic is localized at the periphery of the otoliths.

**DISCUSSION**

An examination of the literature regarding the teratogenic effect of the antibiotics (Saxén, 1966) shows contrasting results. Some workers describe maldevelopment in some bones (femor, tibia) after treatment with tetracyclin; others, however, have not observed maldevelopment after similar treatment. This discrepancy could be due to the various types of tetracyclin used (tetracyclin [T], 5-oxytetracylin [OT], 6-methylene-5-oxytetracyclin [MOT]) or to the doses used; in general, the higher and therefore more toxic doses manifest teratogenic effects (Genazzani et al. 1965).

An important fact which the work reported here has uncovered is that the otoliths of the sacculus are in a more advanced stage of calcification than those of the utriculus in the 8-day embryo. This is demonstrated by the fixation of tetracyclin in deparaffinized sections and in vivo, and by the intensity of the Kossa reaction in these structures. These observations should be considered in relation to the general problem of the genesis and mode of formation of the otoliths. In the chick the development of the otoliths takes place in two stages (de Vincentiis & Marmo, 1966a): (1) an initial phase characterized by the formation of the organic matrix which consists of mucopolysaccharides and proteins (this matrix is secreted in certain zones of the membranous labyrinth—endolymphatic sac and macular zone); (2) a phase of mineralization characterized by the deposition of calcium carbonate in the organic matrix.

The mode and site of mineralization of the otoliths is suggested in the above results. The intervention of the endolymphatic sac is indicated during some stages of the morphogenesis of the otoliths. This conclusion is in agreement with the data presented by Vasquez (1955), who observed calcareous formations in the endolymphatic sac and duct in the 5-day chick embryo; these formations tend to decrease in number and volume as development progresses. This hypothesis is supported by the following experimental data:

(a) the biochemical demonstration that carbonic anhydrase is located in the epithelium of the primordial endolymphatic sac in the 5-day chick embryo and disappears before hatching (Marmo, 1966a).

(b) The exchange of $^{46}\text{Ca}$ is particularly high in this area in embryos after 5 days of incubation (de Vincentiis & Marmo, 1966c).

(c) Morphogenesis of the otoliths is inhibited by treating the embryos with
specific inhibitors of carbonic anhydrase on the 4th day of development (de Vincentiis & Marmo, 1965, 1966a, 1968; Marmo, 1965, 1966a, b; Balsamo, de Vincentiis, Marmo & Parisi, 1969).

The hypothesis of an active participation of the endolymphatic sac in the morphogenesis of the otoliths is reinforced by the observation that the mineralization of the otoliths of the sacculus takes place before that of the utriculus. The sacculus in the chick embryo is in direct communication with the endolymphatic sac through the endolymphatic duct.

The experimental data can be interpreted as follows. Carbonic anhydrase located in the endolymphatic sac accelerates the reaction \( H_2O + CO_2 \rightleftharpoons H_2CO_3 \). The carbonic acid produced in this way combines with calcium to form crystals of \( CaCO_3 \) which then migrate through the endolymphatic duct and invade first the sacculus and then the utriculus and the lagena.

The \( CaCO_3 \) is trapped in the matrix present in these zones and in this way leads to the growth of the otoliths. The peripheral location of the tetracyclin in the otoliths of the chick is in agreement with the situation described in the rat by Bélanger (1960). In the chick, as in the rat, the otoliths do not have a homogeneous structure, but seem to form by process of fusion and accretion.

**SUMMARY**

A study of the localization of tetracyclin hydrochloride in the membranous labyrinth of developing chick embryos is reported. The results have shown:

1. In 8-day embryos there is more intense fixation of tetracyclin in the otoliths of the sacculus than in the otoliths of the utriculus. This difference disappears in 13-day embryos.
2. Tetracyclin is deposited at the periphery of the otoliths.

These results are extended by cytochemical observations on the calcium content and the organic matrix of the otoliths and are discussed in the light of previous observations. A hypothesis regarding the mechanism of morphogenesis of the otoliths is discussed.

**RIASSUNTO**

Viene effettuato uno studio sulla localizzazione del cloridrato di tetraciclini nel labirinto membranoso dell'embrione di pollo nel corso della sviluppo

I risultati hanno mostrato che:

1. In embrioni di 8 giorni di incubazione vi è una maggiore fissazione di tetraciclini negli otoliti del sacculo rispetto agli otoliti dell'utricolo. Questa differenza scompare negli embrioni di 13 giorni.
2. La tetraciclini si deposita alla periferia degli otoliti. Questi risultati, estesi da osservazioni citochimiche sul contenuto in Ca e sulla matrice organica degli otoliti, sono discusse alla luce di precedenti osservazioni.
REFERENCES


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