The Effect of 4,5-Dichlor-1,3-Benzendisulfonamide and of 4,5-Dichlor-1,3-Benzendisulfonylaniline on the Morphogenesis of the Otoliths in the Chick Embryo

4, 5-dichlor-1, 3-benzendisulfonamide (dichlorphenamide), which along with other sulfonamide derivates has a specific inhibitory action on carbonic anhydrase, has been shown to be particularly effective in the inhibition of the morphogenesis of the otoliths in the chick embryo[1-6]. The various inhibitors which have been used belong to different classes of chemical compounds; all have in common, however, a free sulfonamidic group, SO_2NH_2, which is believed to be the active group in the inhibition of carbonic anhydrase[7-12].

The results of experiments conducted with 4, 5-dichlor-1, 3-benzendisulfonamide and with 4, 5-dichlor-1, 3-benzendisulfonylaniline (derived from dichlorphenamide to which a benzenic radical has been substituted for an H atom in each of the $-SO_2NH_2$ groups) are reported in this paper.

Material and methods. 4, 5-dichlor-1, 3-benzendisulfonamide and 4, 5-dichlor-1, 3-benzendisulfonylaniline were synthesized in our laboratory. The reaction between *o*-dichlorobenzene and chlorosulforic acid gave 4, 5-dichlor-1, 3-benzendisulfonylchloride, m.p. 115–116 °C, which after reacting with ammonium hydroxide and crystallization from ethanol gave 4, 5-dichlor-1, 3-benzendisulfonamide, m.p. 240 °C (UV-spectrum in ethanol conc. = 100 γ/ml, λ_{max} = 285 nm, log. ε = 3.17, λ_{max} = 294 nm, log. ε = 3.15). Chromatography on a thin layer of Kieselgel G in an ethanol:chloroform:petroleum ether (1:1:1) system gives a spot visible in UV-light λ = 254 nm, Rf = 0.8. A reaction between 4, 5-dichlor-1, 3-benzendisulfonylchloride and aniline gave, after crystallization from ethanol, 4, 5-dichlor-1, 3-benzendisulfonylaniline, m.p. 195–198 °C (chromatography on a thin layer of Kiesielgel G in an ethanol:chloroform:petroleum ether (1:1:1) system gives a spot visible in UV-light λ = 254 nm, Rf = 0.55.

The action on carbonic anhydrase (Sigma) was tested using the method of NATELSON[13]. The variations in pH were followed on a Beckman expandomatic titrater with a system of registration on paper (velocity of the paper 2.54 cm/min). The blank, made up of the reagents only, was subjected to a continuous flow of carbonic anhydride at a pressure of 60 mm of mercury; it required 73 sec to disrupt the buffering capacity of the solution. A quantity of enzyme corresponding to 1 enzymatic unit dissolved in 0.5 ml of physiological solution requires 41 sec.

Both 4, 5-dichlor-1, 3-benzendisulfonamide and 4, 5-dichlor-1, 3-benzendisulfonylaniline were tested. Both substances were dissolved in dimethylsulfoxide and physiological solution. The 2 substances gave times of 73 and 40 sec respectively; this clearly demonstrates the inhibitory effect of the first and the lack of inhibition of the second. To obtain the maximum inhibitory effect, it was necessary to use 20 γ of 4, 5-dichlor-1, 3-benzendisulfonamide for each enzymatic unit. Whereas 100 γ per enzymatic unit of 4, 5-dichlor-1, 3-benzendisulfonylaniline did not show inhibitory action.

The substances were dissolved in dimethylsulfoxide and 0.1 ml[14] were inoculated into the egg white of an embryo on the fourth day of incubation (stage 23 according to LILLIE[15]); at this stage the morphogenesis of the otoliths has not been initiated. It precedes, by a short time, the beginning of active turnover of ^{45}Ca at the level of the membranous labyrinth[16]. The 2 substances were inoculated in equimolecular concentration: 4 mg/egg for 4, 5-dichlor-1, 3-benzendisulfonamide and 6 mg/egg for 4, 5-dichlor-1, 3-benzendisulfonylaniline. A concentration was chosen which showed a low toxicity (the mortality rate was very little higher than that of the controls) and in the case of 4, 5-dichlor-1, 3-benzensulfonamide the maximum inhibitory action on the morphogenesis of the otoliths[1-3]. At the same time, the corresponding controls were made by inoculating the embryos with 0.1 ml of dimethylsulfoxide.

The embryos were fixed on the eighth day of incubation in 4% neutral formalin. The heads were embedded in paraffin and section were stained with Alcian and treated with PAS[17].

Results. In Figure 1 the results of the determinations purified made with carbonic anhydrase are shown. It is clearly evident that only 4, 5-dichlor-1, 3-benzendisulfonamide inhibits enzymatic activity. The experiments carried out by inoculating the 2 substances into the egg white of the embryos on the fourth day of incubation

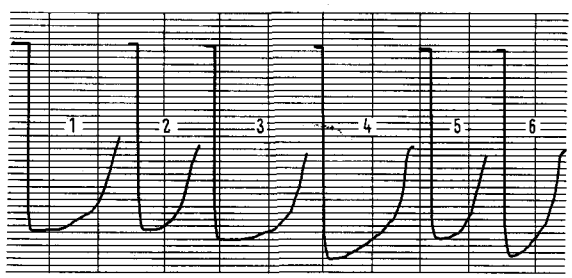
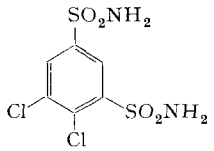


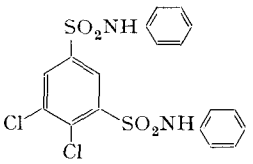
Fig. 1. (1) Blank; (2) effect of carbonic anhydrase (1 unit) on the blank; (3) total inhibition of carbonic anhydrase (1 unit) by 20 γ of 4, 5-dichlor-1, 3-benzendisulfonamide (10 mg dissolved in 5 ml of dimethylsulfoxide and 45 ml of sodium chloride 0.9%; 0,1 ml used); (4) partial inhibition of carbonic anhydrase (1 unit) by 10 γ of 4, 5-dichlor-1, 3-benzendisulfonamide (1:1 dilution of the preceding solution); (5) lack of inhibition of carbonic anhydrase (1 unit) by 20 γ of 4, 5-dichlor-1, 3-benzendisulfonylaniline (10 mg dissolved in 5 ml of dimethylsulfoxide and 25 ml of 0.9% sodium chloride; 0.1 ml were used); (6) lack of inhibition of carbonic anhydrase (1 unit) by 100 γ of 4, 5-dichlor-1, 3-benzendisulfonylaniline (10 mg dissolved in 5 ml of dimethylsulfoxide and 5 ml of 0.9% sodium chloride; 0.1 ml were used).

[1] M. DE VINCENTIIS and F. MARMO, Atti Accad. naz. Lincei Rc. *39*, 601 (1965).
[2] M. DE VINCENTIIS and F. MARMO, Acta med. romana *4*, 43 (1966).
[3] M. DE VINCENTIIS and F. MARMO, Experientia *24*, 818 (1968).
[4] F. MARMO, Boll. Zool. *322*, 231 (1965).
[5] F. MARMO, Atti Soc. pelorit. Sci. fis. mat. nat. *12*, 671 (1966).
[6] F. MARMO, Rass. Med. sper. *13*, 251 (1966).
[7] R. O. ROBLIN and J. W. CLAPP, J. Am. chem. Soc. *72*, 4890 (1950).
[8] R. W. BERLINER and J. ORLOFF, Pharmac. Rev. *8*, 137 (1965).
[9] T. MANN and D. KEILIN, Nature *146*, 164 (1940).
[10] H. A. KREBS, Biochem. J. *43*, 525 (1948).
[11] A. DOBRY-DUCLAUX, C. r. hebd. Séanc. Acad. Sci., Paris *261*, 2131 (1965).
[12] N. W. H. ADDINK and L. BASTINGS, Nature *216*, 72 (1967).
[13] S. NATELSON, *Microtechniques of Clinical Chemistry* (C. C. Thomas, Springfield 1961).
[14] G. CONTI and G. MILIO, Experientia *20*, 110 (1964).
[15] F. R. LILLIE, *Development of the chick* (H. Holt and Co., New York 1952).
[16] M. DE VINCENTIIS and F. MARMO, J. Embryol. exp. Morph. *15*, 349 (1966).
[17] R. W. MOWRY, J. Histochem. Cytochem. *4*, 407 (1956).



p.m. 305.08 ⟶

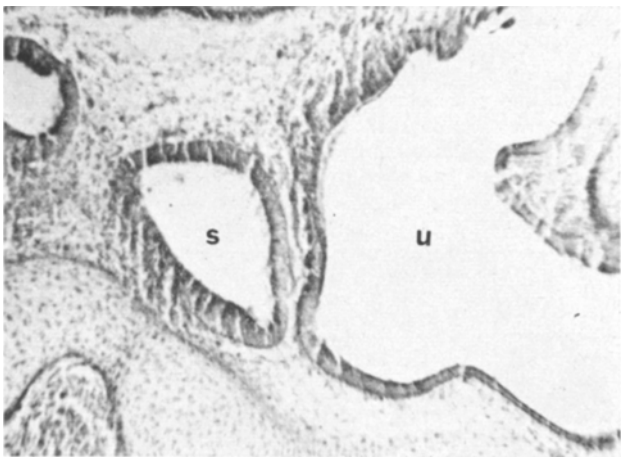
4, 5-dichlor-1, 3-benzendisulfonamide inoculated in chick embryos on the 4th day of incubation (13 embryos).



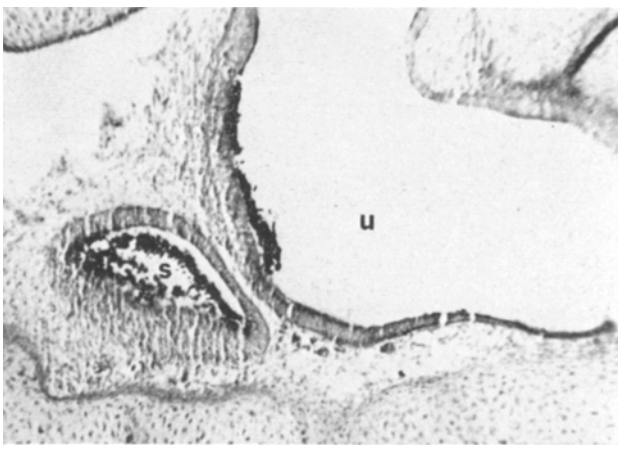
p.m. 457.2 ⟶

4, 5-dichlor-1, 3-benzendisulfonylaniline inoculated in chick embryos on the 4th day of incubation (22 embryos).

Fig. 2.



Membraneous labyrinth in the chick embryo on the 8th day of development. Selective inhibition of the otoliths in the sacculus and utriculus in presence of 4, 5-dichlor-1, 3-benzendisulfonamide. (S = sacculus; U = utriculus). × 80.



Membraneous labyrinth in the chick embryo on the 8th day of development. Normal morphogenesis of the otoliths in the sacculus and utriculus in presence of 4, 5-dichlor-1, 3-benzendisulfonylaniline. (S = sacculus; U = utriculus). × 80.

all show that only in the presence of 4, 5-dichlor-1, 3-benzendisulfonamide is a selective inhibition of the morphogenesis of the otoliths observed. The other structures of membranous labyrinth are normal (Figure 2).

Interesting are the relationships of the 2 substances in vitro on the carbonic anhydrase and in vivo on the morphogenesis of the otoliths.

Discussion and conclusions. The results obtained show the intervention of carbonic anhydrase at the molecular level during the morphogenesis of the otoliths in the chick embryo.

In fact with 4, 5-dichlor-1, 3-benzendisulfonamide, in which 2 free sulfonamidic group ($-SO_2NH_2$) are present, (4, 5-dichlor-1, 3-benzendisulfonamide is active in the inhibition of carbonic anhydrase) there is an arrest in the morphogenesis of the otoliths. Using the derivate in which a hydrogen atom of each of the SO_2NH_2 group has been substituted by phenyl (a derivative which shows no inhibitory activity on carbonic anhydrase) there is no arrest in the morphogenesis of the otoliths. The intervention of carbonic anhydrase in the morphogenesis of the otoliths of the chick embryo is necessary because the reaction $H_2O + CO_2 \rightleftarrows H_2CO_3$ must be accelerated to give the maximum formation of $CaCO_3$ which has a high turnover rate demonstrated with ^{45}Ca [16] at the level of the membranous labyrinth where the otoliths form [18].

Riassunto. Inoculando in embrioni di pollo di 4 giorni di sviluppo un inibitore dell'anidrasi carbonica (4, 5-Dicloro-1, 3-Benzendisolfonamide) o un suo derivato inattivo nel quale un atomo di idrogeno dei 2 gruppi SO_2NH_2 è sostituito da radicali fenilici (4, 5-Dicloro-1, 3-Benzendisolfonilanilina), si nota una inibizione selettiva della morfogenesi degli otoliti in presenza di 4, 5-Dicloro-1, 3-Benzendisolfonamide.

G. BALSAMO, M. DE VINCENTIIS, F. MARMO and G. PARISI

Institute of Histology and Embryology, University of Camerino (Group for research in Embryology of the C.N.R.), and Institute of General Biology and Genetics of the University, 80134 Napoli (Italy), 29 July 1968.

[18] We wish to thank L. BOTTE for skilled technical assistance.