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METABOLISM OF PREGNENOLONE BY RAT NORMAL FIBROBLASTS IN CULTURE *IN VITRO*

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SUMMARY

Rat embryo fibroblasts were cultured in monocellular layers in the presence of [4^{14}C]-pregnenolone. The labelled pregnenolone was added to the culture medium and the cells cultured for 72 h in its presence. The transformation products were extracted from the nutritive medium and from the fibroblast pellet. The results obtained demonstrate a transformation of the pregnenolone. Ninety-eight per cent of the total radioactivity was found in the medium and 2% in the cellular pellet. By means of radiochromatography and G.L.C. coupled with mass spectrometry, we identified products different from pregnenolone. From the culture medium we extracted product A (3.7% of the total radioactivity) whose structure is currently being studied and product B (1.9% of the total radioactivity) of mass 360 and with R_F value and mass spectrum identical to those of cortisone. This hypothesis was confirmed by isotope dilution in the presence of radioinert cortisone. A product C was extracted from the cellular sediment: it could be an ester of cortisone as a product with an R_F identical to this steroid was obtained by alkaline hydrolysis.

These results confirm the hypothesis that fibroblasts are capable of synthetizing cortisone from pregnenolone. This steroidogenesis may participate in the modulation of certain inflammatory processes in the tissues.

INTRODUCTION

Numerous experiments have shown that connective tissue fibroblasts have the enzyme apparatus permitting them to transform and utilize corticosteroids. These cells possess a microsomal enzyme, 5-ene-3β-hydroxysteroid-dehydrogenase, which permits the oxidation of the 5-ene-3β-hydroxy OH group of steroid to form 4-ene 3 keto and thus the transformation of pregnenolone into progesterone [1, 2, 4]. It has also been demonstrated that fibroblasts can transform 17 hydroxy progesterone into 11-deoxycortisol and into cortisol [5, 6]. In the present study, we have investigated the metabolism of an earlier precursor in the biosynthesis of cortisol and cortisone, namely pregnenolone.

MATERIALS AND METHODS

Cell cultures

A strain of rat embryo fibroblasts was used which have been cultivated in the laboratory for several years and which have always maintained the same characteristics. The fibroblasts were cultured in monocellular layers in Earle flasks, the culture medium consisting of Eagle's Minimum Essential Medium (M.E.M. Gibco) and decomplementized foetal calf serum (Gibco). The cells were regularly detached by trypsinization and the nutritive medium was changed every four days.

The cells were used in the tests immediately after subculture. 16×10^6 cells were introduced into 80 ml of medium, pregnenolone was added to the medium and the cells cultivated for 72 h in its presence. The transformation products were extracted while the cells were in the growth phase at which point approximately 30×10^6 fibroblasts were counted in 80 ml of medium. A pool of six culture flasks was used for each experiment.

Study of the pregnenolone transformation products

Introduction and incubation of the precursor. [4^{14}C]-labelled pregnenolone was used with a specific activity of 50 mCi/mol (CEA SACLAY, Division of Labelled molecules). The chemical and radiochemical purity was tested by t.l.c. 5.6×10^5 d.p.m. were added per Earle flask (or 2 μg for 16×10^6 cells) and incubation lasted 72 h. The study included ten experiments, i.e. 60 flasks. In each case a control was included consisting of the labelled precursor added to cultures previously killed by autoclaving.

After the incubation period, the cells were detached from the bottom of the flask by trypsinization and the fibroblasts were separated from the extracted culture medium by centrifugation. The cells were washed in Hanks' medium and collected by centrifugation.

Isolation of metabolites of the culture medium. Four successive chloroform extractions were carried out, the emulsions were centrifuged at 10,000 *g* for 10 min, the chloroform phases were mixed, dried with Na_2SO_4 and evaporated in a vacuum at 40°C. The sediment was deposited on a silica plate (Merck F 254) in the presence of controls of known unlabelled metabolites of pregnenolone in order to compare the R_F values of the isolated products. The plates were

eluted with successively 4 solvents: chloroform, 90:10 acetone–methylene chloride, 60:40 benzene–acetone, 50:50 benzene–acetone.

Isolation of metabolites of the cellular fraction. The cellular pellet (180×10^6 cells) was homogenized in phosphate buffer pH 7.2; 4 successive chloroform extractions were carried out following the same procedure as that described for the medium. The sediment was deposited on the same silica plates (Merck F 254) and spelt out with chloroform. The same operations were carried out on the medium and the cellular pellet of the killed control cultures.

Identification of the transformation products. The radioactive spots were located by applying a Panax counter to the chromatographic plates and by radioautography (Kodak Ready Pack Film) after an exposure period of 7 days. The radioactive spots were recovered by scraping the silica gel and elution in a 2:5:3 mixture of methanol–chloroform ethyl ether. Their radiochemical purity was tested by t.l.c. and their R_F values compared to that of the radioinert products deposited on the plate.

The nature of the products obtained was confirmed by isotope dilution and successive recrystallization of the radioactive metabolites was carried out in hexane in the presence of radioinert controls and repeated until a constant specific activity per mg of crystals was obtained.

The structure of the isolated metabolites was studied by gas liquid chromatography (G.L.C.) coupled with mass spectrometry.

RESULTS

After 72 h of culture in the presence of [4^{14}C]-pregnenolone, 98% of the radioactivity was recovered in the culture medium and 2% in the fibroblast pellet.

The chromatograms of the product extracted from the medium showed the presence of two products, A and B, which were more polar than pregnenolone (Fig. 1). In the cellular sediment, only one product, C, was found which was less polar than the precursor (Fig. 2). The transformation rates as compared to the pregnenolone initially introduced into the culture were 3.7% for product A, 1.9% for product B and 1% for product C.

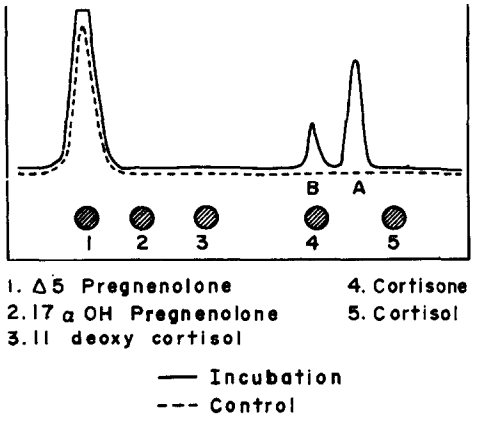

Fig. 1. Chromatograms of the product extracted for the medium.

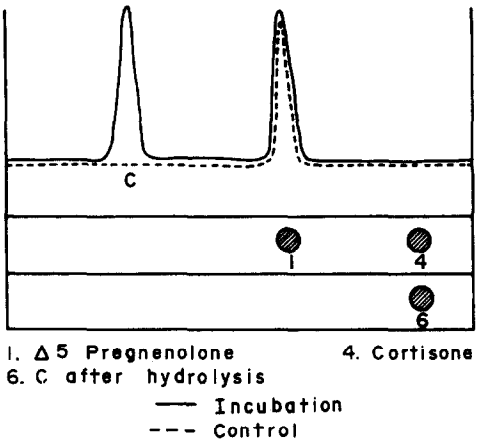

Fig. 2. Chromatograms of the product extracted for the cellular sediment.

Product A had a mass of 530 and an R_F value intermediary between that of cortisol and cortisone. It resisted hydrolysis by 20% potassium methanol for 5 h. Its structure is in the process of being determined.

Product B has a mass of 360 and an R_F value identical to that of cortisone in the system previously described. It resisted hydrolysis carried out under the same conditions as for product A.

Product C had a mass of 546. It could be an ester of product B as a product with an R_F value identical to that of B was obtained by basic hydrolysis.

Structure of product B

Successive recrystallization in the presence of cold cortisone showed that from the second recrystallization there was no loss of specific activity per mg of crystals, the latter having been 95 d.p.m./mg for the first and 93 d.p.m./mg for the second.

The mass spectrum (Fig. 3) showed the molecular peak M^+ at 360. The principal fragmentation peaks, *m/e* 300, 257, 244, 163 and 122, were found in the mass spectrum of the standard cortisone. The *m/e* ion (M 60) resulted from cleavage of the C 17-C20 bond with a concomitant loss of one hydrogen. The base peak, *m/e* 122, resulted from cleavage of the bonds shown in Fig. 3 which are characteristic of an 11 substituted Δ4-3-oxo steroid.

These data conform to the literature [3, 7, 8] and allowed us to identify product B as cortisone.

CONCLUSION

These chromatographic and spectrometric data confirm the fact that fibroblasts cultivated *in vitro* can, in proliferative phase, synthetise cortisone from pregnenolone introduced into the cell culture. This

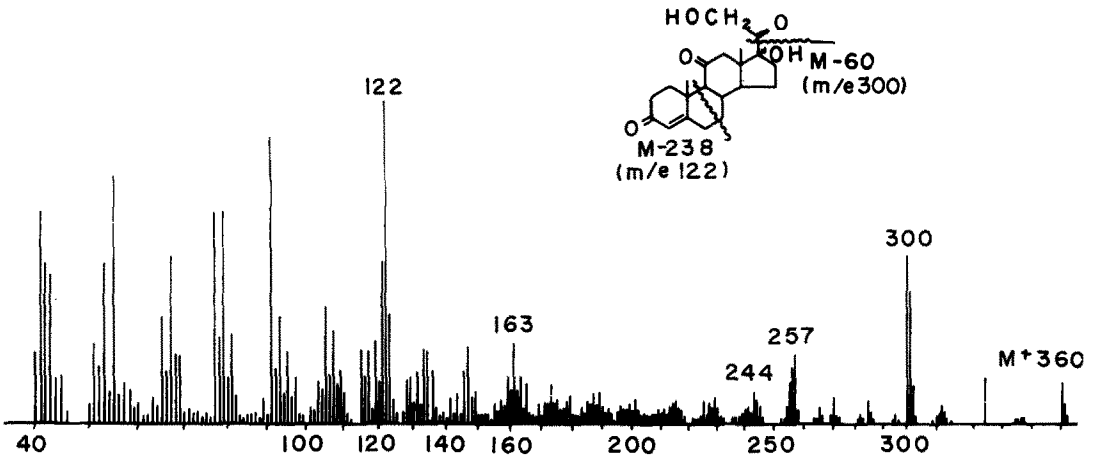

Fig. 3. Mass spectrum of cortisone extracted for the medium.

cortisone certainly participates in the metabolism of fibroblasts. However, the extent of fibroblastic proliferation during the inflammatory process in the tissues and the selective effect of the antiinflammatory steroids on these events indicate that this endogenous steroidogenesis could have a more specific rôle. The steroids of the fibroblasts therefore could actively participate in modulating the tissue defence mechanisms.

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