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7-HYDROXYLATION OF DEHYDROEPIANDROSTERONE BY A HUMAN LYMPHOBLASTOID CELL LINE

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SUMMARY

The enzymic liberation of tritium from [7-^{3}H]-dehydroepiandrosterone was used as an *in vitro* assay for the possible presence of 7-hydroxylase in human lymphocytes. Indications of low activity were found in mitogen transformed lymphocytes from healthy donors and in lymphocytes from some patients with acute and chronic lymphocytic leukaemia. However, a lymphoblastoid cell line (McB) derived from a subject with Hodgkin's Disease proved exceptionally active when assessed by this procedure.

An incubation of 10^8 McB cells with [7-^{3}H]-dehydroepiandrosterone produced [^{3}H]-7α-hydroxydehydroepiandrosterone in 12.2% yield. Identification of this product was established by crystallizations to constant specific activity.

INTRODUCTION

Cultured human lymphocytes after mitogen transformation have been shown to possess an oxygenase which hydroxylates aryl hydrocarbons [1, 2]. The possibility that such lymphocyte preparations may also hydroxylate steroids has been studied but only the 16α-hydroxylase acting on estradiol [3] has been reported to date.

As dehydroepiandrosterone (DHA) has been hydroxylated in the 7 position by a number of human tissues [4–11], evidence for the presence of a DHA 7-hydroxylase was sought in lymphocytes derived from healthy donors and subjects with lymphoproliferative malignancies.

MATERIALS AND METHODS

Steroids. DHA acetate was obtained from Sigma, St. Louis, U.S.A. 7α-Hydroxy DHA diacetate was synthesised from DHA acetate [12, 13] and its identity confirmed by comparison with authentic material (kindly supplied by Dr D. N. Kirk, M.R.C. Steroid Reference Collection), separate and mixed m.p. 169–170°C (uncorr.).

7α-hydroxy DHA was prepared from the diacetate by two procedures, chemical [13] and enzymic (α-amylase [14]). Each produced products which were indistinguishable, m.p. 178–179°C (uncorr.).

[7-^{3}H(N)]-DHA, specific activity 20 Ci/mmole, was obtained from New England Nuclear, Boston, U.S.A.

Reagents. Chemicals and solvents were of analytical grade. Thin-layer chromatography was carried out on precoated 0.25 mm silica gel 60 F_{254} 5 × 20 cm glass plates obtained from E. Merck., Darmstadt, Germany. NADPH and NADH were from Sigma, St. Louis, U.S.A.

Radioactivity measurements. Radioactivity was detected on thin-layer chromatography plates by means of a Varian Berthold radio scanner Model LB2722 with a windowless gas-flow counter.

Quantitation of tritium in water was carried out by mixing precisely 1 ml with 10 ml of Insta-gel (Packard Instrument Pty. Ltd, Melbourne, Australia) and counting on a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375. Counting efficiency for ^{3}H was 38.3%.

Radioactivity in steroids was assessed by dissolving a weighed amount of dry steroid in 10 ml toluene phosphor solution (0.5% w/v Packard Permablend 11) and counting by liquid scintillation. Tritium counting efficiency in toluene was 56.4%.

Lymphocyte preparations. Fresh heparinized blood was treated to obtain a lymphocyte-rich suspension by means of a single stepped gradient centrifugation using ficoll hypaque [15]. Culturing was carried out in RPMI 1640 (GIBCO) with 10% foetal calf serum (Commonwealth Serum Labs) and containing 25 mg penicillin G and 20 mg streptomycin/l. Cells were suspended at 2 × 10^6/ml in plastic flasks (Falcon-3012) and cultured at 37°C in 10% CO_2, 90% air mixture in a Kevatron forced-air humidified incubator. Mitogen stimulation was performed with 5 μl/ml Wellcome phytohaemagglutinin HA15, a dose used routinely by this laboratory in microculture assays for lymphocyte function. After 72 h the transformed lymphocytes were harvested, washed in Dulbecco's phosphate buffered saline and suspended in phosphate buffer (see Incubation Procedure) at a concentration of 2.5 × 10^6 cells/ml and stored at −20°C.

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The lymphoblastoid cell lines were maintained in the same tissue culture medium and flasks at concentrations of 0.5–2 × 10^6 cells/ml. "G.K." cells are a line produced by Ian Jack (Royal Childrens' Hospital, Melbourne) from human peripheral blood lymphocytes infected with Epstein–Barr virus. "McB" cells arose spontaneously in culture from spleen cells obtained from a patient with Hodgkin's disease. These cells had surface IgM, did not rosette with sheep erythrocytes (a T cell marker) or with erythrocytes coated with IgG or complement; they did not phagocytose latex particles or produce lysozyme. These cells were similarly washed and resuspended in phosphate buffer either at 2.5 × 10^6 or 50 × 10^6 cells/ml. They were also preserved at −20°C prior to use in incubation.

Lymphocytes from subjects with lymphoproliferative malignancies were supplied by Miss Ying Mei Huang through the courtesy of Dr I. A. Cooper, Head of the Haematology Research Unit. They were obtained by the single stepped gradient centrifugation of peripheral blood samples or lymphocyte-rich preparation from the centrifuge. They were washed, suspended in buffer, and preserved as described above.

Incubation procedure. A freshly thawed suspension of cells in 2 ml of Na_2HPO_4/NaH_2PO_4 buffer (50 mM, pH 7.4) was mixed with 25 μl ethanol solution of [7-^{3}H]-DHA (0.9 μCi). To this was added 0.2 ml of buffer containing 1.55 mg NADH and 1.55 mg NADPH and the mixture was incubated at 37°C with gentle shaking for 3 h.

DHA 7-hydroxylase screening assays. Based on the liberation of tritium from the substrate, [7-^{3}H]-DHA, when hydroxylation occurs, quantitation of [^{3}H]-H_2O in the incubations was carried out using the following procedures.

For the initial experiments incubations containing 3–5 × 10^6 cells were transferred to round bottom flasks and cooled with swirling in an ethanol–solid CO_2 mixture. These were lyophilized in a closed system and the water distillates assessed for tritium content (see radioactivity measurements). A disposable charcoal column method [16] was subsequently found to give the same results and was used because of its greater convenience.

An incubation containing no cells was always carried through the procedure with each batch of assays and used to correct for the tritium liberated by autooxidation of the substrate. Thus, the assay measured the net liberation of tritium from 0.9 μCi [7-^{3}H]-DHA (45 pmol) by a known number of cells in 3 h at 37°C. It has been expressed as a percentage per 5 × 10^6 cells.

7α-hydroxy DHA identification procedure. An incubation of 100 × 10^6 cells was stopped by extraction with 10 ml CH_2Cl_2 containing 25 μg 7α-hydroxy DHA. Two more extractions were carried out with 10 ml volumes of CH_2Cl_2 and the combined extract cooled to −20°C. After filtration, solvent was removed at room temperature with dry N_2. The residue was dissolved in 2 ml ethanol and 0.2 ml taken for radioactivity measurement. The remainder, after removal of some solvent, was applied to the origin of a 0.25 mm silica gel thin-layer chromatography plate and developed twice with chloroform:ethanol (9:1 v/v). That region of the plate corresponding to the mobility of 7α-hydroxy DHA was removed and eluted with 3 × 5 ml methanol.

After evaporation, the extract was made up to 2 ml with ethanol and 0.2 ml taken for tritium measurements. The remainder was divided into two parts and used for crystallizations to constant specific activity: one with 7α-hydroxy DHA and the other (after acetylation overnight with 0.1 ml pyridine and 0.1 ml acetic anhydride) with the diacetate of 7α-hydroxy DHA.

RESULTS

Table 1 records the results obtained when the screening procedure was applied to normal and abnormal lymphocytes of human origin. The lymphoblastoid cell line (McB) was shown to be exceptionally active by liberating 1.67% of the tritium from the [7-^{3}H]-DHA substrate.

An incubation of 100 × 10^6 McB cells with [7-^{3}H]-DHA was performed. The CH_2Cl_2 extract, containing 84.2% of the original substrate radioactivity, was chromatographed and 14% of the substrate tritium found in a region of the thin layer plate with the same mobility as 7α-hydroxy DHA. Identification of this product was established by crystallizations to constant specific activity of (a) product plus unlabelled 7α-hydroxy DHA, and (b) acetylated product plus unlabelled 7α-hydroxy DHA diacetate (Table 2). Calculations based on the mean specific activity of the final three crystallizations show that 12.2% of the tritium contained in the substrate was isolated as [^{3}H]-7α-hydroxy-DHA.

DISCUSSION

It has been demonstrated that the human lymphoblastoid cell line, McB, exhibits a high level of DHA 7-hydroxylase activity. The observed conversion of 12.2% of the original substrate to 7α-hydroxy DHA must be a gross underestimate as it takes no account of tritium, presumably at the C_7 position of DHA, which was converted into [^{3}H]-H_2O. Our data (Table 1) indicate that one-twentieth of the cells used to obtain this conversion liberated 1.67% of the tritium from the substrate, [7-^{3}H]-DHA.

It is not known what proportion of the ^{3}H in the substrate is in the 7 position; the suppliers stated that this varied from lot to lot with approximately 10% of the ^{3}H residing at C_4. However, it is possible that even if the specificity of the labelling was known, a paradoxical result may have been obtained similar to that described by Couch *et al.*[9] with human mammary tissue.

To explain their data, they proposed that an epi-

Table 1. DHA 7-hydroxylase screening assay

Cells	% ^{3}H liberated from [7-^{3}H]-DHA per 5 × 10^6 cells[1]
Mitogen transformed lymphocytes from 4 healthy subjects	
(a)	0.011
(b)	0.015
(c)	0.011
(d)	0.030
Chronic lymphocytic leukaemia (CLL) Lymphocytes from 6 subjects	
(a)	<0.002
(b)	<0.002
(c)	0.010
(d)	0.073
(e)	0.043
(f)	0.010
CLL (T cell) lymphocytes	0[2]
Acute lymphoblastic leukaemia cells from 2 subjects	
(a)	0[2]
(b)	0.148
Sezary lymphocytes	0.108
Lymphocytic lymphosarcoma cells	<0.002
Lymphoma (leukaemic phase) cells	0[2]
Lymphoblastoid cell line "G.K."	0.024
Lymphoblastoid cell line "McB"	1.67

[1] Cells were incubated for 3 h at 37° C with [7-^{3}H]-DHA (0.9 μCi, 45 pmol) in phosphate buffer (2.2 ml, pH 7.4, 50 mM) containing NADH (0.89 mM) and NADPH (0.79 mM). Correction was made for ^{3}H liberated by a control incubation containing no cells.

[2] Control incubation liberated more ^{3}H than incubation with cells.

Table 2. Specific activities of 7α-hydroxy DHA and its diacetate derivative

Crystallization	Solvent pair	Specific activity (d.p.m./mg) Crystals	Mother liquors
7α-hydroxy DHA			
1	acetone/hexane	4816	4984
2	methanol/water	4493	6803
3	benzene/hexane	4335	4186
4	acetone/hexane	4534	4509
7α-hydroxy DHA diacetate			
1	methanol/water	4083	10149
2	acetone/pet. ether	3883	10445
3	acetone/water	3688	4780
4	methanol/water	3580	—

Presumptive [^{3}H]7α-hydroxy DHA was isolated from an incubation with 100 × 10^6 McB lymphoblastoid cells and [7-^{3}H]-DHA (2.02 × 10^6 d.p.m.). A portion of the isolated material (124 × 10^3 d.p.m.) was successively crystallized with 24.5 mg unlabelled 7α-hydroxy DHA. The remainder (117 × 10^3 d.p.m.) was acetylated and recrystallized with 27.2 mg of the diacetate of 7α-hydroxy DHA.

merase may be present which could convert 7β-hydroxy DHA to the 7α epimer with complete retention of the label at the 7 position. Until this matter is resolved, it is not certain that an observed conversion of DHA to 7α-hydroxy DHA necessarily proves the presence of a DHA 7α-hydroxylase.

The McB lymphoblastoid cell line was isolated from the spleen of a patient with Hodgkin's Disease. Further studies would be required to determine whether there could be an association between DHA 7-hydroxylase and Hodgkin's Disease. It has been reported that splenic microsomal cytochrome P450

is significantly higher than normal in patients with Hodgkin's Disease [17].

The screening assay has indicated the possibility of a low level of DHA 7-hydroxylase activity in peripheral lymphocytes grown in culture with phytohaemagglutinin. It is possible that indications of a higher level of enzyme activity may have been observed if an inducing agent had been added to the culture, as was the case with the induction of the 16α-hydroxylase [3] and the aryl hydrocarbon hydroxylase [18, 19].

An intriguing observation recorded in Table 1 are those neoplastic cells which assayed "below zero". The presence of these cells in an incubation apparently protected [7-^{3}H]-DHA from chemical oxidation which, over a period of 3 h at 37°C, liberates about 0.012% of the tritium in the substrate. It could mean that while the cells have completely lost the capacity for 7-hydroxylation, they possess, or have retained, the ability to bind DHA at a concentration of 20.5 nM.

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