20 α -Hydroxysteroid Dehydrogenase of Cultured Mammalian (HeLa S₃) Cells

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Synopsis

The activity of pyridine nucleotide-linked 17 β - and 20 α -hydroxysteroid dehydrogenase(s) was detected in HeLa S₃ cells. Addition of progesterone to cultures failed to increase the 20 α -hydroxysteroid dehydrogenase activity of the cells. The enzyme was partially purified by ammonium sulfate fractionation and chromatography on Bio-gel P-100, and the molecular weight of the enzyme was estimated at approximately 30,000. The enzyme(s) could operate progesterone, 17 α -hydroxyprogesterone (20 α -hydroxysteroid dehydrogenase) and androst-4-ene-3, 17-dione (17 β -hydroxysteroid dehydrogenase) in the presence of either NADH or NADPH. However, there was a small difference of elution patterns from the Bio-gel column between the NADH- and NADPH-linked enzymes, while the NADPH-linked 17 β -hydroxysteroid dehydrogenase activity was eluted at the same void volume as the NADPH-linked 20 α -hydroxysteroid dehydrogenase activity.

Various mammalian cells which have been cultured *in vitro* for many years possess enzymic activities to metabolize hormonal steroids (Sweat *et al.*: 1958, Perlman *et al.*: 1960, Pierson, 1967, Shin, 1967, Shikita and Sato, 1969). Among several enzymes concerned with the steroid metabolism, 20 α -hydroxysteroid dehydrogenase seems to be preponderant in all cell-cultures hitherto studied, but the physiological meaning of this enzyme is not known at present. Similar 20 α -hydroxysteroid dehydrogenase is found in various endocrine and non-endocrine organs of the Mammalia (Recknagel, 1957, Wiest, 1959, Little *et al.*: 1959, Thomas *et al.*: 1960, Matthijssen *et al.*: 1964, Shikita and Tamaoki, 1965, Rembiesa, 1969). This paper

describes some properties of the 20 α -hydroxysteroid dehydrogenase of HeLa S₃ cells (tissue-culture cells originated from human cervix carcinoma).

Materials and Methods

Chemicals

(4-¹⁴C) Progesterone, (4-¹⁴C) androst-4-ene-3, 20-dione and (4-¹⁴C) 17 α -hydroxyprogesterone were purchased from New England Nuclear Corp. These radioactive steroids were mixed with respective non-radioactive steroid and dissolved in methanol in a concentration of 500,000 cpm and 8 μ moles/ml. NADH and NADPH were obtained from Sigma Chemical Co. Standard proteins for the molecular-weight determination were obtained commercially. β -Mercaptoethylguanidine (MEG) was prepared by spontaneous transformation of β -aminoethylisothiuronium in the aqueous solution.

Purification of 20 α -hydroxysteroid dehydrogenase of HeLa S₃ cells

Methods used for culture of the cells were described in a previous paper (Shikita and Sato, 1969). The cells

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* In a paper published previously (Shikita and Sato, 1969), it was reported by error that progesterone was added to the HCT cell culture in a concentration of 12 mM. This should be corrected to 12 μ M.

grown to full-sheet in 250-ml rectangular glass bottles were rinsed three times with 0.9% NaCl solution (pH 7.0) and then removed from the glass surface with a hooked plastic rod. The cells from 10 culture-bottles were suspended in 0.02 M phosphate buffer (10 ml, pH 7.0) and treated with 20 kc sonic oscillation for one min at 0°. Insoluble substances were removed by centrifugation ($25,000 \times g$, 10 min). Microscopic examination of this precipitate showed that all of the cells were disrupted by this sonic treatment.

To the $25,000 \times g$ supernatant, solid ammonium sulfate was added to 80% saturation at pH 7.0. The solution was stirred for 2 hr and centrifuged as above. The resultant precipitate was taken in 1.5 ml of 0.1 M

phosphate buffer (pH 7.0) containing 10 mM MEG. The mixture was centrifuged once more at $25,000 \times g$ for 10 min and the sediment was discarded. The supernatant (1.4 ml) was applied onto a Bio-gel P-100 column (9×600 mm) which had been equilibrated with the phosphate-MEG buffer. The enzyme was eluted from the column with the same buffer at a flow rate of 6 ml/hr, and each 1.2 ml fraction was collected. All these procedures were performed at 5°.

Assay of enzymes

The enzyme solution was incubated with the radioactive steroid (10,000 cpm, 0.16 μ mole) in a final volume of 1.0 ml of phosphate buffer (0.1 M, pH 7.0).

Fig. 1. Silica-gel thin layer chromatography of the metabolites of ($4\text{-}^{14}\text{C}$) progesterone. Radioactive progesterone ($50 \mu\text{g}$, 10,000 cpm) was incubated in a flask containing monolayer culture of HeLa S_3 cells for 24 hr. The steroids were extracted and chromatographed in a solvent system of benzeneacetone (4:1). The figure shows the autoradiograph of the chromatogram. Following steroids were used as the marker; P=progesterone, A=androst-4-ene-3, 17-dione, 17=17 α -hydroxy-progesterone, T=testosterone. The radioactive steroids were identified by the methods reported previously (Shikita and Sato, 1969).

Unless otherwise specified, NADPH (0.36 μ mole/flask) was added as the cofactor of the enzyme. The reaction was started by the addition of the steroid, continued for 30 min at 37°, and then stopped with methylene dichloride. The methylene dichloride extracts were concentrated to dryness under vacuum and chromatographed on silica-gel thin layer with benzene-acetone (4:1). Identification and determination of the amount of the steroids were performed by the methods reported previously (Shikita and Tamaoki, 1965, Shikita and Sato, 1969). Protein concentration was determined by the method of Lowry *et al.* (Lowry *et al.*, 1951).

Results and Discussion

Presence of a 20 α -hydroxysteroid dehydrogenase activity in the HeLa S₃ cell cultures (S₃-1-164) was reported previously (Shikita and Sato, 1969). The same HeLa S₃ line has been maintained in the culture system for 4 years since then, and the metabolism of (4-¹⁴C)progesterone was examined again in one of the cultures (S₃-9-IV-288). It was shown by the thin-layer chromatography that the pattern of the steroid metabolism was very much similar to that observed before, and 20 α -hydroxypregn-4-en-3-one was the major metabolite (Fig. 1). By a separate experiment, it was shown that the 20 α -hydroxysteroid dehydrogenase was present in the soluble fraction of the cell-homogenate as it was observed previously in the case of HTC cells (Shikita and Sato, 1969).

Non-radioactive progesterone was added to the culture medium in concentrations of 12 μ M*, and 60 μ M. It was found that there was no increase of the enzyme activity in the sonic extract of the cells after such incubations of the cells with progesterone (Fig. 2). This result is not reconcilable with the previous observation that the enzyme activity increased 3 fold when HTC cells were incubated with progesterone in a same way as above (Shikita and Sato, 1969).

When the supernatant of the high-speed centrifugation of the sonic extract of HeLa S₃ cells (3 mg protein per ml) was left standing

Fig. 2. 20 α -Hydroxysteroid dehydrogenase activity of HeLa S₃ cells: Effect of progesterone. The cells (10 flasks) were cultured in the medium containing progesterone, 12 μ M (open circles) and 60 μ M (enclosed circles), for various periods of time as indicated on the abscissa. The cells in each flask were treated with sonic waves and the soluble fraction of the sonicate was examined for the NADPH-linked 20 α -hydroxysteroid dehydrogenase activity as described in MATERIALS AND METHODS.

for 4 hr in an ice-water bath, the 20 α -hydroxysteroid dehydrogenase activity was decreased by 45%. Thus the enzyme was precipitated by ammonium sulfate as soon as the supernatant was obtained. It was then dissolved in the phosphate-MEG buffer in a concentration of 1 mg protein per ml. In this way, 73% of the initial enzyme activity was recovered and rendered stable.

Similar ammonium-sulfate fractions prepared from the extract of the cells which had been cultured with or without progesterone were chromatographed separately on the Bio-gel P-100 column (Figs. 3 and 4). The 20 α -hydroxysteroid dehydrogenase activity was eluted from the column at the void volume of 1.5, and the pre-treatment of the cells with progesterone did not change this profile of the enzyme. Based on this exclusion volume, the molecular weight of the 20 α -hydroxysteroid dehydrogenase was estimated at approximately 30,000 (See the insert of Fig. 3). The magnitude of overall purification was about 4.

Fig. 3. Bio-gel P-100 chromatography of 20α -hydroxysteroid dehydrogenase of progesterone-treated HeLa S_3 cells. The cells (10 flasks) were cultured with progesterone ($12 \mu\text{M}$) for 20 hr. The enzyme activity of the sonic extract of the cells was precipitated by ammonium sulfate and then subjected to the chromatography. Details are described in the text. Open circles represent 280 nm absorption; enclosed circles represent the NADPH-linked 20α -hydroxysteroid dehydrogenase (See the legend of Figure 4). The relation between the molecular weight and the exclusion volume is shown in the insert: 1, cytochrome C (Sigma); 2, chymotrypsinogen (Sigma); 3, lactoglobulin (N.B. Co.); 4, peroxidase (Sigma); 5, ovalbumin (N.B. Co.); 6, α -amylase (Seikagaku Fine Chem.); 7, bovine serum albumin (N.B. Co.); 20α -HSD, 20α -hydroxysteroid dehydrogenase.

The fractions obtained by the Bio-gel chromatography were assayed also for the 17β -hydroxysteroid dehydrogenase activity with use of androst-4-ene-3, 17-dione as the substrate. It may be seen in Figure 4 that the peak of the 17β -hydroxysteroid dehydrogenase activity coincided with that of the 20α -hydroxysteroid dehydrogenase activity. However, it is difficult to make a conclusion at the present moment whether there are two distinct dehydrogenases of similar molecular weight or a single species of enzyme exhibits broad

substrate specificity.

Figure 4 also shows the distribution of the NADH-linked 20α -hydroxysteroid dehydrogenase activity among the fractions of the Bio-gel chromatography. The NADH-linked enzyme was eluted faster than the NADPH-linked enzyme. The result suggests that there are at least two enzymes with different specificity towards the pyridine nucleotides. In another experiment, the Bio-gel fractions were pooled between 1.2 and 1.6 void volumes and assayed both for 17β - and 20α -hydroxysteroid

Fig. 4. Bio-gel P-100 chromatography of 17 β - and 20 α -hydroxysteroid dehydrogenase(s) of non-induced HeLa S₃ cells. Chromatography was performed in a same way as in the case of progesterone-treated cells (Fig. 3). Aliquots of each fraction were incubated with (4-¹⁴C) progesterone or with (4-¹⁴C) androst-4-ene-3, 17-dione in the presence of NADPH or NADH. The enzyme activity is expressed by μ moles of 20 α -hydroxypregn-4-en-3-one or that of testosterone computed for the total amount of the fraction. Details of incubation are described in the MATERIALS AND METHODS. Open circles represent 280 nm absorption.

dehydrogenases with either NADH or NADPH. The result given in Figure 5 shows that there is larger NADPH-linked enzyme activity than the NADH-linked one both for progesterone and androstenedione. This result agrees with that given in Figure 4 where the

peak for the NADH-linked 20 α -hydroxysteroid dehydrogenase is smaller than that of the NADPH-linked 20 α -hydroxysteroid dehydrogenase.

Some other properties of the NADPH-linked 20 α -hydroxysteroid dehydrogenase

Table 1. Effect of inhibitors on the NADPH-linked 20 α -hydroxysteroid dehydrogenase of HeLa S₃ cells. Bio-gel P-100 peak fractions were pooled and aliquots (0.5 mg protein in each) were incubated for the 20 α -hydroxysteroid dehydrogenase assay. Expt 1 to 3 represent three batches of enzyme prepared separately from different cultures (non-induced). In Expt 1, the enzyme solution was dialyzed against 0.1 M potassium phosphate (pH 7.0) prior to this inhibitor study.

Substrate	Additions	Reaction product (nmoles)		
		Expt 1	Expt 2	Expt 3
(4- ¹⁴ C)Progesterone	—	1.7	2.9	5.3
	MEG, 10 mM	4.4	3.3	—
	PCMB, 10 μ M	0.4	1.2	—
	EDTA, 50 mM	2.0	—	—
(4- ¹⁴ C)17 α -Hydroxyprogesterone	—	—	2.0	2.3

Fig. 5. NADPH-linked and NADH-linked 17 β - and 20 α -hydroxysteroid dehydrogenase(s) of HeLa S₃ cells. Pooled peak fractions of Bio-gel P-100 chromatography were incubated with either (4-¹⁴C) progesterone or (4-¹⁴C) androst-4-ene-3, 17-dione with NADPH or NADH of various concentrations as indicated in the Figure.

were studied with use of combined Bio-gel peak fractions. It was observed that the enzyme activity was inhibited by a sulfhydryl blocker, p-chloromercuribenzoate, and that the decreased enzyme activity after dialysis could be restored by MEG. It was also observed that 17 α -hydroxyprogesterone was less reactive than progesterone for the 20-ketone reduction by the enzyme (Table 1).

References

- Little, B., J. Dimartinis, J. and B. Nyholm (1959). *Acta Endocrinol.* **30**, 530.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). *J. Biol. Chem.* **193**, 265.
- Matthijssen, C., J. E. Mandel and P. T. Seiden (1964). *Biochim. Biophys. Acta* **89**, 363.
- Perlman, D., P. W. Jackson, N. Giuffre and J. Fried (1960). *Can. J. Biochem. Physiol.* **38**, 393.
- Pierson, R. W., Jr. (1967). *Endocrinology* **81**, 693.
- Recknagel, R. O. (1957). *J. Biol. Chem.* **227**, 273.
- Rembiesa, R. (1969). *Endocrinology* **84**, 973.
- Shikita, M. and F. Sato (1969). *Arch. Biochem. Biophys.* **133**, 336.
- Shikita, M. and B. Tamaoki (1965). *Biochemistry* **4**, 1189.
- Shin, Seung-Il (1967). *Endocrinology* **81**, 440.
- Sweat, M. L., B. I. Grosser, D. L. Berliner, H. E. Swim, C. J. Nabors, Jr. and T. F. Dougherty (1958). *Biochim. Biophys. Acta* **28**, 591.
- Thomas, P. Z., E. Forchielli and R. I. Dorfman (1960). *J. Biol. Chem.* **235**, 2797.
- Wiest, W. G. (1959). *J. Biol. Chem.* **234**, 3115.