Hydrolysis of Dehydroepiandrosterone Sulphate by Human Placental Steroid 3 β -Sulphatase in [^{18}O]Water

By GEOFFREY G. LOGAN AND JAMES C. WARREN

*Departments of Obstetrics, Gynaecology and Biochemistry,
University of Kansas School of Medicine, Kansas City, Kansas 66103, U.S.A.*

(Received 22 April 1969)

1. Dehydroepiandrosterone sulphate was hydrolysed by human placental steroid 3 β -sulphatase in H_2^{18}O . Equimolar amounts of dehydroepiandrosterone and sodium sulphate were similarly incubated as controls. After incubation, unconjugated steroid was extracted with ether and sulphate precipitated as barium sulphate. Both were analysed for ^{18}O -content by mass spectroscopy, the sulphate as carbon dioxide after initial pyrolysis with graphite. 2. In duplicate experiments, amounts of ^{18}O equivalent to 67% and 72% respectively of the theoretical content calculated for rupture of the O-S bond were present in the sulphate. As the enzyme preparation used was a microsomal preparation containing unenriched endogenous sulphate and phosphate, and as no incorporation of isotope was found in the steroid, it is concluded that the placental enzyme effects hydrolysis by rupture of the O-S bond.

The presence of a sulphatase in human placenta capable of hydrolysing steroid 3 β -sulphates was demonstrated by Pulkkinen (1961) and Warren & Timberlake (1962). Further investigations in this laboratory have shown that placenta is the richest human source of this activity (Warren & French, 1965), which is localized in the microsomal fraction (French & Warren, 1966) and is dependent upon the structural integrity of the microsomal particles to which the enzyme is bound (French & Warren, 1967).

The present investigation was undertaken to examine the mechanism of placental hydrolysis of steroid 3 β -sulphates by determination of the cleavage site.

EXPERIMENTAL

The following trivial names are used in this paper: dehydroepiandrosterone for 3 β -hydroxy-5-androsten-17-one and dehydroepiandrosterone sulphate for 17-oxo-5-androsten-3 β -yl sulphate.

Materials. Steroids were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and used without further purification. Anhydrous sodium sulphate, reagent grade, was obtained from Mallinckrodt Chemical Co., St. Louis, Mo., U.S.A. The H_2^{18}O (1.5 atoms %) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Preparation of placental microsomes. Freshly delivered placentas were obtained from the delivery room, briefly rinsed with tap water and placed on ice. All subsequent procedures were carried out at 0–4°. The preparation was

carried out by a modification of the method of Schneider & Hogeboom (1950). Trophoblastic tissue (3 g./ml. of 0.05 M-tris-HCl buffer, pH 7.8, with 0.25 M-sucrose) was homogenized for 2 min. in a Waring Blendor. The homogenate was centrifuged initially at 10000g for 45 min. and the supernatant then centrifuged again for a further hour at 105000g in a Spinco model L ultracentrifuge. The final microsomal pellet was resuspended in water, so that 1.0 ml. of suspension was equivalent to 5 g. of fresh placenta, and stored at –20° until needed.

One day before the experiment the frozen microsomes were thawed, centrifuged at 105000g for 45 min. and resuspended up to the original volume in ^{18}O -enriched water and stored overnight at –20°. Immediately before use the microsome suspension was thawed, again centrifuged and resuspended in fresh ^{18}O -enriched water with 1.0 ml. of suspension equivalent to 5 g. of trophoblastic tissue.

Incubations. Tris-HCl buffer (0.1 M) was prepared by dissolving the calculated amount of Trizma base in 35 ml. of ^{18}O -enriched water and adjusting to pH 7.5 with conc. HCl.

Dehydroepiandrosterone sulphate solution was prepared in ^{18}O -enriched water at a concentration of 2.0 $\mu\text{moles/ml}$. A volume of the microsomal suspension described above equivalent to 6 g. of fresh placenta was mixed with 6.0 ml. of tris-HCl buffer solution in an incubation flask and placed in a constant-temperature water bath at 37°. The incubation was commenced by addition of 6.0 ml. of substrate solution (12.0 μmoles of dehydroepiandrosterone sulphate) and the mixture subjected to continuous agitation during the incubation period of 5 hr.

Duplicate incubations were carried out, and duplicate controls were prepared by mixing 12.0 μmoles of Na_2SO_4 dissolved in 6.0 ml. of ^{18}O -enriched tris-HCl buffer with amounts of microsomal suspension identical with those

described above. A solution of dehydroepiandrosterone was prepared by dissolving the steroid initially in a minimum amount of 50% (v/v) ethanol and ^{18}O -enriched water and subsequently diluting to a concentration of $2.0\ \mu\text{moles/ml.}$ with ^{18}O -enriched water. Of this solution 6 ml. was added to the mixture of Na_2SO_4 solution and microsomes, and the incubation carried on for 5 hr. The control samples are referred to as such in the subsequent tables, and the samples obtained after incubation of dehydroepiandrosterone sulphate with microsomes are called test samples. The total incubation volume of each sample was 13.2 ml. In providing appropriate controls it was hoped to allow for non-specific exchange of isotope with either the steroid or the inorganic sulphate.

After 5 hr., the incubation was terminated by the addition of 25 ml. of ice-cold ether to each incubation flask, which was then shaken vigorously for approx. 1 min. When the aqueous and ether layers had separated, the aqueous layer was frozen and the ether decanted. The extraction was repeated with two further 25 ml. volumes of ether.

The pooled ether extracts were back-washed twice with 10 ml. of distilled water in a separatory funnel and then evaporated to dryness under vacuum. Subsequently the steroid was dissolved in 5 ml. of methanol and transferred to a small vial, dried under N_2 and stored at room temperature until mass spectroscopy could be performed.

The aqueous layer was allowed to thaw and was then centrifuged at 105000g to deposit the microsomes. The supernatant was aspirated and to it was added sufficient 60% perchloric acid to make the final concentration 7.5% (v/v). The protein precipitate was centrifuged down at 3000g for 30 min., the supernatant again aspirated and its pH adjusted to 7.5 with 5M-NaOH solution. Saturated BaCl_2 solution (2 ml.) was added to each tube and the resultant BaSO_4 precipitate deposited by centrifugation at 3000g for 15 min.

The precipitate was digested in 2 ml. of 0.5M-NaOH solution for 10 min. and then was washed thrice in 2 ml. of water, thrice in 2 ml. of 0.5M-HCl to remove any Ba(OH)_2 and lastly in 2 ml. each of absolute ethanol and ether.

Mass spectroscopy. Analysis of dehydroepiandrosterone was carried out in a Varian-Mat CH 4-B single focusing mass spectrometer set for a resolution of 1000. The ion-source temperature was 250° and the electron energy 70 eV. The samples were introduced from a low-mass gold crucible that was located on a line-of-sight 10 mm. from the ionizing electron beam. The crucible temperature was set and maintained at 30° independent of the ion-source temperature throughout the experiment. Under these conditions the molecular ion of dehydroepiandrosterone (mass number 288) was the base peak of the spectrum.

Pyrolysis and analysis of the samples of BaSO_4 were performed according to the method of Rafter (1967). After degassing the samples at 300° for 10 min. under high vacuum the BaSO_4 was pyrolysed at 1100° with pure graphite. The resultant mixture of CO and CO_2 was collected at the temperature of liquid N_2 and then sparked at 20000 v to convert CO into CO_2 , the latter being collected in a solid CO_2 -acetone trap. The gas was redistilled from this trap, the yield measured and the gas subsequently analysed in a model 21-401 mass spectrometer made by Consolidated Engineering Corp., Pasadena, Calif., U.S.A., and specially modified for ^{18}O assays in the Department of Chemistry, University of California at Los Angeles.

RESULTS

Analysis of hydrolysed dehydroepiandrosterone.

Under the conditions of the experiment the molecular ion ($m/e=288$) formed the base peak of the spectrum and the sensitivity of the recorder was adjusted so that this signal occupied the full width of the paper. Ions of mass 289 (i.e. molecules of steroid containing one atom of the isotopes ^{13}C , ^2H or ^{17}O) produced a signal approximately one-quarter of the intensity of that from the molecular ion; ions of mass 290 (i.e. molecules of steroid containing two atoms of the isotopes ^{13}C , ^2H or ^{17}O , or alternatively incorporating one ^{18}O atom) were present in much smaller quantity and yielded a signal whose intensity was only about one-fiftieth of that of the molecular ion.

The height of each signal peak was measured in millimetres and the mean of four separate measurements calculated for each peak. Ratios of the mean peak heights were then determined with reference to that of the molecular ion as 100 (see Table 1). In neither of the test samples was the 290 peak greater than that of the controls.

Analysis of barium sulphate. One of the control sulphate samples was lost in preparation and thus data are given for content of ^{18}O in one control and two test samples in Table 2.

The ^{18}O content of the control sample, however, is very close to the reported natural abundance of the isotope of 0.204 atom % (Weast, 1966). Furthermore, Spencer (1958) carried out incubations for up to 24 hr. at pH values with greater variation from neutrality than pH 7.5 and found a maximum exchange of ^{18}O between H_2^{18}O and sulphate of

Table 1. Ratios of signal peak heights in mass-spectrographic analysis of dehydroepiandrosterone after hydrolysis in ^{18}O -enriched water

Dehydroepiandrosterone sulphate was hydrolysed in ^{18}O -enriched water, 1.5 atoms %, by the action of placental steroid 3β -sulphatase. After incubation free dehydroepiandrosterone was extracted with ether, dried and analysed by mass spectroscopy under conditions where the molecular ion (mass number 288) formed the base peak of the mass spectrum. The mean heights of the signals from ions of mass number 288, 289 and 290 were determined and the ratios below calculated with reference to the 288 peak as 100.

Sample	Ratio of peak heights at mass		
	288	289	290
Control 1	100	24	2.2
Control 2	100	20	2.8
Test 1	100	23	1.9
Test 2	100	24	2.1

Table 2. *Percentage of ^{18}O in oxygen obtained as carbon dioxide after pyrolysis of barium sulphate*

After enzymic hydrolysis of dehydroepiandrosterone sulphate (DOSO_3H in the table below) by placental steroid 3β -sulphatase in ^{18}O -enriched water the liberated inorganic sulphate was precipitated as BaSO_4 and subjected to multiple washings with dilute NaOH , water, HCl , ethanol and ether. The barium sulphate was then de-gassed, dried and pyrolysed with pure graphite. The resulting CO_2 was analysed by mass spectroscopy for ^{18}O content.

Sample	Content	^{18}O (atom %)		
		Expected enrichment for $\text{D}:\text{OSO}_3\text{H}$	Expected enrichment for $\text{DO}:\text{SO}_3\text{H}$	Actual enrichment
Control	0.202	0	—	0
Test 1	0.436	0	0.325	0.234
Test 2	0.420	0	0.325	0.218

only 0.018 atom %. Therefore although only one control value is presented there is good reason to believe that it is reliable.

The actual enrichment in the sulphate liberated as a result of hydrolysis of dehydroepiandrosterone sulphate by placental steroid 3β -sulphatase clearly rules out hydrolysis by rupture of the C—O bond and in the two experiments is 72% and 67% respectively of that theoretically calculated for rupture of the O—S bond. The discrepancies between theoretical and experimental values can be attributed to: (a) adsorption of oxygen-containing organic compounds (possibly even substrate) to the barium sulphate (cf. Spencer, 1958); (b) possible exchange of ^{18}O out of the sulphate during the washing procedures; (c) most importantly, contribution of non-enriched, endogenous sulphate and phosphate ions from the microsomes themselves. This is particularly pertinent as microsomes from 6g. of placental tissue were utilized for each incubation and the average amount of intracellular sulphate in tissues is 20m-equiv./l. And so, even though these values fall short of the 85% of theoretical values obtained with solubilized enzymes (Spencer, 1958), the considerations above and the clear absence of ^{18}O -enrichment in the steroid moiety indicate that enzymic hydrolysis occurs by breaking of the O—S bond.

DISCUSSION

Certain speculations are possible about the site of bond cleavage during hydrolysis of dehydroepiandrosterone sulphate even without the isotope data presented in this manuscript. It is reasonable to assume that the cleavage results from a displacement reaction. If, after hydrolysis, the steroid has undergone Walden inversion it is certain that the C—O bond must have been broken by a one-step displacement reaction ($\text{S}_\text{N}2$). However, it has been shown by Warren & Timberlake (1962)

that the product of this reaction is dehydroepiandrosterone and not the 3α -epimer. Therefore, if the C—O bond is broken, hydrolysis must proceed by a double displacement reaction. Alternatively cleavage of the O—S bond may occur. The results of the present investigation indicate that the O—S bond is split.

The placental steroid 3β -sulphatase thus may be thought of as a sulpho-transfer sulphuryl enzyme. By analogy with phosphate-transfer mechanisms (Axelrod, 1956) the enzyme may be regarded as an attacking nucleophilic reagent and the reaction most suitably explained as:

where D is dehydroepiandrosterone. In these reactions water is shown as the acceptor. Should some other molecule be capable of acting as acceptor the enzyme could, in fact, function as a sulpho-transferase.

Spencer (1958), studying the hydrolysis of aryl sulphates by aryl sulphatases from human and non-human sources, demonstrated that aryl sulphatases also effected cleavage of the O—S bond and considered them as potential sulphotransferases. However, attempts to demonstrate the transfer of sulphate from *p*-nitrophenyl sulphate to a number of simple alcohols, sugars, choline and tyrosine in the presence of purified human aryl sulphatases A and B were unsuccessful [K. S. Dodgson & F. A. Rose, unpublished work cited by Spencer (1958) and Dodgson & Tudball, (1960)].

This work was supported by U.S. Public Health Service grant no. AM-05532. Geoffrey Logan was a Ford Foundation Fellow and James C. Warren a career development awardee of the National Institute of Child Health and Human Development. The authors thank Dr William

Budde of the Midwest Research Institute and Dr T. Peter Stein of the Department of Chemistry, University of California at Los Angeles for carrying out mass spectroscopy.

REFERENCES

- Axelrod, B. (1956). In *Advances in Enzymology*, vol. 17, p. 159. Ed. by Nord, F. F. New York: Interscience Publishers Inc.
- Dodgson, K. S. & Tudball, N. (1960). *Biochem. J.* **74**, 154.
- French, A. P. & Warren, J. C. (1966). *Steroids*, **8**, 79.
- French, A. P. & Warren, J. C. (1967). *Biochem. J.* **105**, 233.
- Pulkkinen, M. O. (1961). *Acta physiol. scand.* **52** (Suppl.), 180.
- Rafter, T. A. (1967). *N.Z. J. Sci.* **10**, 493.
- Schneider, W. C. & Hogeboom, G. M. (1950). *J. biol. Chem.* **183**, 123.
- Spencer, B. (1958). *Biochem. J.* **69**, 155.
- Warren, J. C. & French, A. P. (1965). *J. clin. Endocrin. Metab.* **25**, 278.
- Warren, J. C. & Timberlake, C. E. (1962). *J. clin. Endocrin. Metab.* **22**, 1148.
- Weast, R. C. (Ed.-in-Chief). (1966). *Handbook of Chemistry and Physics*, 47th ed., p. B7. Cleveland, Ohio: The Chemical Rubber Co.