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ON STEROID CONJUGATES IN PLASMA

XXIII—BIOSYNTHESIS OF STEROID SULFATIDE FROM A CYTIDINE PHOSPHOSULFATE STEROID COMPLEX

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SUMMARY

The assumed intermediate in the biosynthesis of DHEA sulfatide, a cytidine phosphosulfate DHEA complex, was synthesized from 7 α-^{3}H-DHEA sulfate and cytidine monophosphate by the carbodiimide method and properly characterized. By incubation of C-P-S-DHEA with mitochondria from guinea pig liver 7 α-^{3}H-DHEA sulfatide was formed. The K_M-value of the diglyceride transferase, its pH- and temperature optimum as well as its dependancy on co-enzymes are reported.

ON THE basis of preceding experiments [1] it had been assumed that the biosynthesis of steriod sulfatides from steroid sulfates proceeds by a two-step reaction. In analogy to the biogenesis of phosphatides [2, 3] the first step seems to consist of the formation of a cytidine phosphosulfate steroid complex (C-P-S-Steroid) from steroid sulfate and cytidine triphosphate. In the second step the C-P-S-Steroid complex probably reacts with diglyceride, yielding the lipophile steroid sulfatide.

For further support of this hypothesis the present communication deals with the preparation of the C-P-S-Steroid complex and the conversion of C-P-S-DHEA (DHEA, dehydroepiandrosterone, 3 β-hydroxy-5-androsten-17-one) to the corresponding steroid sulfatide by incubation with mitochondria.

EXPERIMENTAL

Synthesis of cytidine phosphosulfate 7 α-^{3}H-DHEA

10 μMoles (3·90 mg) 7 α-^{3}H-DHEA sulfate, sodium salt with 30–100 μCi ^{3}H are converted into the acid form or the pyridinium salt by passing an acetone-dimethyl formamide (4 : 1 v/v) or pyridine solution through a column (0·5 × 5 cm) of acidic cation exchange resin (ion exchange resin I; Merck AG, Darmstadt), washed with methanol and the above solvents. In a similar manner 10 μMoles (4·75 mg) cytidine-5′-monophosphate, disodium salt in water are converted into the acid form on an acidic cation exchange resin.

The reaction of both components is carried out in 90% pyridine (or 90% acetone-dimethyl formaide (4 : 1 v/v), using 50 μMoles (10·30 mg) dicyclohexyl carbodiimide as condensing agent. The reaction mixture is stirred for 18 h at 4°C and 1 h at room temperature. Following 1 h cooling at −5°C and filtration the filtrate is reduced to 2–3 ml *in vacuo* and extracted two times with 5 ml hexane for removal of excess dicyclohexylcarbodiimide. The lower phase is evaporated in vacuo at maximal 35°C and the residue submitted to preparative thin layer

chromatography on silica gel G in chloroform-methanol-ammonia (15 : 5 : 0·2 v/v). The radioactive zone with a R_f-value between 0·6 and 0·8 is eluted with methanol and the desired complex precipitated with ether-cyclohexame (1 : 1 v/v). The yields of C-P-S-DHEA varied between 8 and 14%.

ANALYSIS : calculated for $C_{28}H_{43}O_{12}N_4Ps$ (690·6) P = 4·49% S = 4·63%
found P = 4·40% S = 4·65%.

The ratio DHEA: cytidine, as measured by the Oertel-Eik-Nes-reaction[4] and UV-absorption at 270 nm was found to be 1·00 : 1·03. The infrared spectrum of the complex in potassium bromide pellets indicated the presence of the 17-oxo group (1743 cm^{-1}), of sulfate (1208 and 1230 cm^{-1}) and phosphate (1041, 1066 and 1096 cm^{-1}). The UV-absorption spectrum of the complex, dissolved in methanol, exhibited an absorption maximum at 272 nm and a minimum near 253 nm, reflecting the presence of the cytidine moiety. In the sulfuric acid absorption spectrum maxima near 403, 483 and 512 nm could be observed, indicative of the DHEA moiety.

Conversion of cytidine phosphosulfate 7 α-^{3}H-DHEA into 7 α-^{3}H-DHEA sulfatide.

Mitochondria from guinea pig liver were obtained as described in a previous publication (5). Unless otherwise stated, incubations were carried out for 30 minutes at 37°C, using 0·2 *M* Tris buffer of pH 7·4, 1 μMole of magnesium chloride and 1 μMole cysteine. The final volume of the incubates amounted to 5 ml.

Effect of substrate concentration

0·1 ml of the enzyme preparation was incubated with 1, 2, 5, 10, 20, 30 and 50 nMoles C-P-S-DHEA as substrate (Table 1, Fig. 1).

Table 1. Effect of substrate concentration upon the formation of 7 α-^{3}H-DHEA sulfatide

Substrate: C-P-S-DHEA		Product: 7 α-^{3}H-DHEA sulfatide	
nMoles	cpm ^{3}H	nMoles	cpm ^{3}H
1	643	1·00	649
2	1290	1·91	1230
5	3220	3·96	2540
10	6430	7·51	4830
20	12900	10·8	6940
30	19300	11·2	7230
50	32200	15·6	9040

Effect of enzyme concentration

0·1–0·6 ml of the enzyme preparation, equivalent to 3·7 to 22·2 mg mitochondrial protein were incubated with 50 nMoles C-P-S-DHEA (Fig. 2).

Effect of pH

Substituting a 0·2 *M* phosphate-acetate buffer of varying pH for the regular Tris buffer the influence of pH changes upon the formation of 7 α-^{3}H-DHEA sulfatide was studied (Fig. 3).

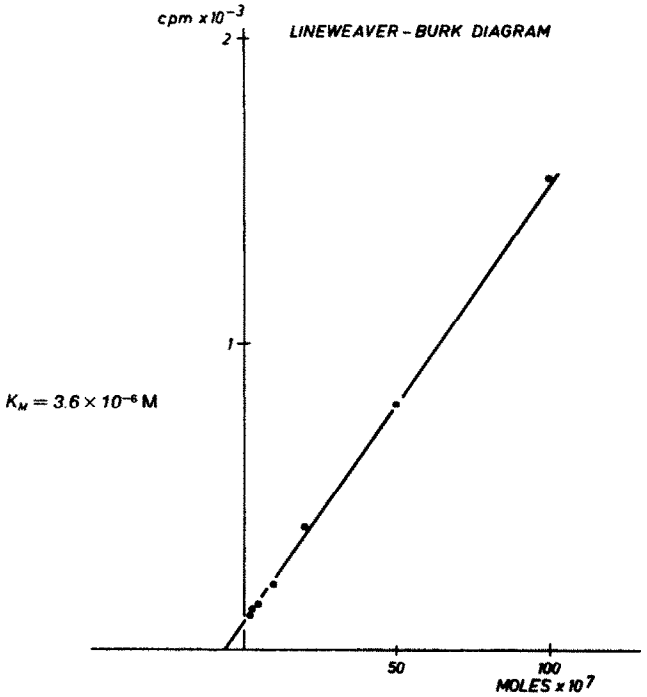

Fig. 1. Lineweaver–Burk diagram derived from results in Table 1.

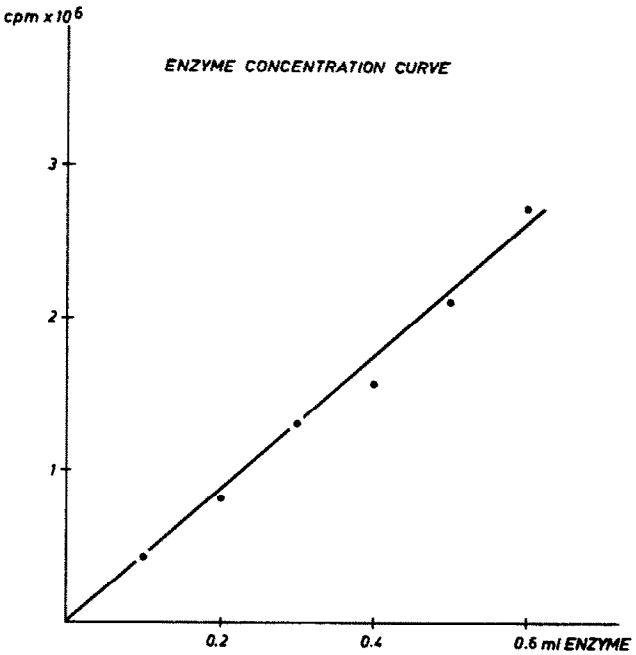

Fig. 2. Effect of enzyme concentration on the formation of 7 α-^{3}H-DHEA sulfatide.

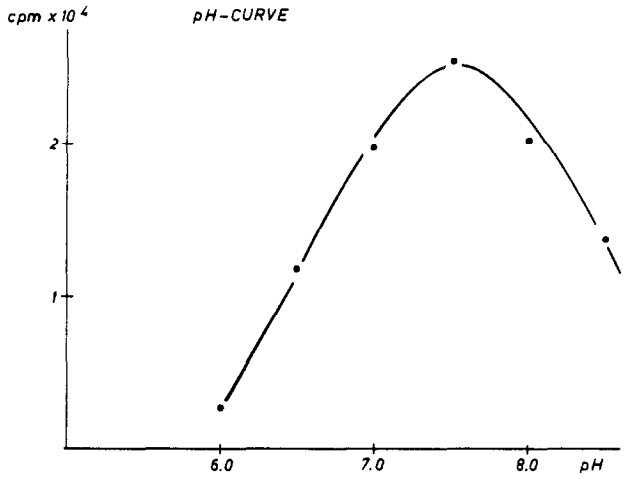

Fig. 3. Effect of pH on the formation of 7 α-^{3}H-DHEA sulfatide.

Effect of incubation time

For determination of the conversion rate the duration of incubations, carried out with 0·5 ml of the enzyme preparation and 100 nMoles C-P-S-DHEA was varied from 5 to 30 min (Fig. 4).

Effect of temperature

In order to gain information on the influence of the incubation temperature upon the yields of 7 α-^{2}H-DHEA sulfatide 0·5 ml of the enzyme preparation and 100 nMoles C-P-S-DHEA were incubated at 20, 30, 37 and 40°C (Fig. 5).

Isolation of 7 α-^{3}H-DHEA *sulfatide*

The procedures used for the isolation of steroid sulfatides from such incubates

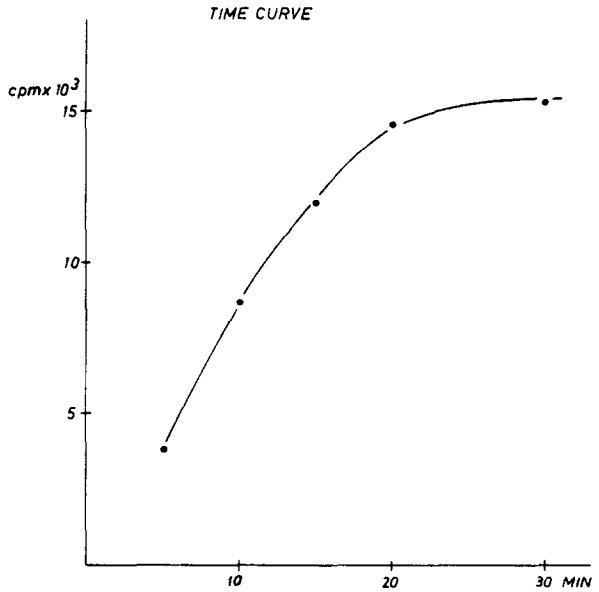

Fig. 4. Effect of incubation time on the formation of 7 α-^{3}H-DHEA sulfatide.

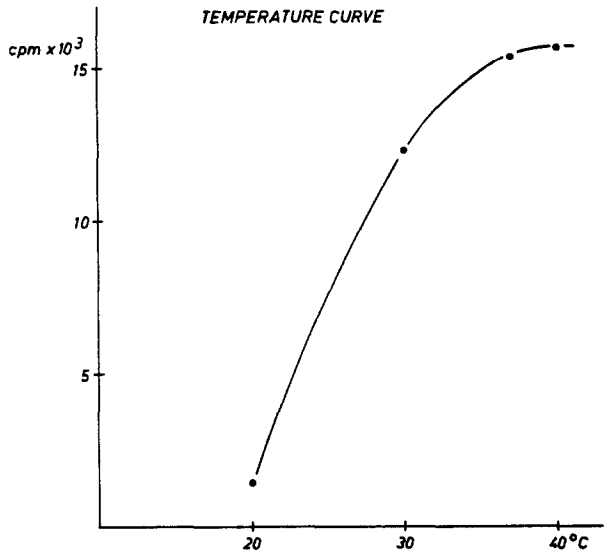

Fig. 5. Effect of temperature on the formation of 7 α-^{3}H-DHEA sulfatide.

are outlined in an earlier communication [6]. Separation of steroid sulfatides from C-P-S-DHEA was achieved by column chromatography of extracts on polyamide. Following the solvolysis of steroid sulfatides DHEA (and metabolites) was isolated by thin layer chromatography, converted into its 2, 4-dinitrophenylhydrazone and eventually purified to constant specific ^{3}H-activity.

RESULTS

The identity of the C-P-S-DHEA complex could be established by its elemental analysis, its IR, UV and sulfuric acid absorption spectrum with their characteristic absorption maxima and a composition corresponding to the assumed structure.

The incubation of constant amounts of enzyme with increasing concentrations of substrate led to the results compiled in Table 1. By evaluation according to the method of Lineweaver and Burk (Fig. 1) a K_M-value of $3{\cdot}6 \times 10^{-6}$ M was obtained. When excess substrate was incubated with increasing amounts of the mitochondrial enzyme a linear increase in the formation of steroid sulfatide could be demonstrated (Fig. 2). The effects of pH changes of the incubation medium are shown in Fig. 3. Figure 4 reveals the influence of the temperature upon the conversion of C-P-S-DHEA to DHEA sulfatide. Prolonging the time of incubation stepwise from 5 to 30 min the yields of DHEA sulfatide reflected an initial conversion rate of 0·32 nMoles/min/18·5 mg protein. (Fig. 5). The addition of 1 μMole ATP or CTP to the incubation medium did not affect the conversion of C-P-S-DHEA, whereas 5 μMoles of p-chloromercuribenzoate caused an 83% decrease in the formation of 7 α-^{3}H-DHEA sulfatide.

DISCUSSION

As previously reported the conversion of 7 α-^{3}H-DHEA sulfate to 7 α-^{3}H-DHEA sulfatide by mitochondria was greatly stimulated by the addition of CTP, suggesting the formation of a C-P-S-DHEA complex as intermediate in analogy

to the biosynthesis of phosphatides [1–3]. In fact, when these incubates were examined for the presence of such an intermediate by thin layer chromatography of appropriate extracts a ^{3}H-labeled, UV absorbing compound with a R_f-value of 0·68 could be detected. In the same solvent system the synthetic C-P-S-DHEA complex and DHEA sulfate exhibited a R_f-value of 0·69 and 0·54 resp.. The analysis of the biosynthetic complex revealed the presence of sulfate as well as phosphate, indicating its identity with C-P-S-DHEA.

By incubation of 0·1 ml of the mitochondrial enzyme preparation with 50 nMoles C-P-S-DHEA in the presence of 1 μMole CTP 34 nMoles of the substrate were converted into steroid sulfatide as compared to only 7% when the same amount of 7 α-^{3}H-DHEA sulfate was employed. Such data seems to be quite compatible with the aforementioned concept of a two-step reaction sequence, leading from steroid sulfate over a C-P-S-DHEA complex to the steroid sulfatide. The K_M-value of the so-called diglyceride transferase, eventually identical with the choline phosphotransferase (EC 2 . 7 . 8 . 2), turned out to be lower than that of the overall system ($3{\cdot}6 \times 10^{-6}$ *M* v $6{\cdot}6 \times 10^{-6}$ *M*) pointing at the high affinity of the diglyceride transferase for the C-P-S-DHEA. Whereas the cytidine transferase, responsible for the activation of the steriod sulfate by its transformation into the C-P-S-Steroid complex, has not yet been localized in mitochondrial components the diglyceride transferase obviously resides in the outer membrane of mitochondria just like the choline phosphotransferase [7]. As to be expected in enzyme reactions the increase in the amount of enzyme, e.g. mitochondria resulted in a corresponding rise of sulfatide yields. In contrast to the complete enzyme system [1], possessing optimal activity near pH 8·0 the pH curve of the diglyceride transferase showed a maximum near pH 7·6. The rapid transformation of the C-P-S-DHEA complex may be derived from Fig. 4. At the beginning of the reaction 0·32 nMoles of substrate were converted into sulfatide within 1 min by 18·5 mg of mitochondrial protein. The fact, that p-chloromercuribenzoate significantly suppressed the conversion of C-P-S-DHEA into DHEA sulfatide hints at SH-groups in the enzyme molecule.

Whether the enzyme system involved in the biosynthesis of steriod sulfatide from steroid sulfate is present in mitochondria of every tissue remains to be seen. At least, following the *in vivo* perfusion of human gonads with 7 α-^{3}H-DHEA ^{35}S-sulfate a sizeable percentage of double-labelled sulfoconjugates occured in the fraction of steroid sulfatides, isolated from the venous effluent [8, 9]. Furthermore, the isolation of steroid sulfatides from human adrenal tissue [10] and the demonstration of higher concentrations of steroid sulfatides in human adrenal vein blood as compared to peripheral or hepatic vein blood [11] may actually reflect an adrenal biosynthesis of lipophile steroid sulfatide and its release into the general circulation. Here, the most prominent steroid sulfatide, namely DHEA sulfatide appears to participate in the regulation of at least the red blood cell glucose-6-phosphate dehydrogenase [12, 13].

Finally, it should be pointed out that during the incubation of mitochondria with the C-P-S-DHEA complex also a minor metabolism of the steroid could be verified, producing preferably androstenediol (5-androstene -3 β, 17 β-diol).

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