an argon atmosphere. A slight excess of aluminium was used in order to counterbalance the loss by vaporization of this metal. The X-ray powder pattern showed the product to be a single phase.

Single crystals of $IrAl_3$ were obtained from the crushed melt. The Weissenberg data showed hexagonal symmetry and the unit-cell dimensions derived from a Guinier photograph (*cf.* Table 1) were:

$$a = 4.246 \text{ Å}, \quad c = 7.756 \text{ Å}$$

The single-crystal data were collected along an a-axis using CuK radiation. The multiple film technique was used and the intensities were estimated visually. The data obtained showed $IrAl_3$ to have a $D0_{18}$-type of structure. In this type of structure there is only one atomic parameter to be refined and this was done from successive ϱ_o and ϱ_c syntheses based on the $h0l$ reflexions. The following structure was thus derived:

Unit cell content: $2IrAl_3$
Space group: $P6_3/mmc$ (No. 194)

Ir in $2c$ $\frac{1}{3},\frac{2}{3},\frac{1}{4}$; $\frac{2}{3},\frac{1}{3},\frac{3}{4}$
Al_1 in $2b$ $0,0,\frac{1}{4}$, ; $0,0,\frac{3}{4}$
Al_2 in $4f$ $\frac{1}{3},\frac{2}{3},z = 0.575$; $\frac{2}{3},\frac{1}{3},\bar{z}$; $\frac{2}{3},\frac{1}{3},\frac{1}{2}+z$; $\frac{1}{3},\frac{2}{3},\frac{1}{2}-z$

Table 1 gives a comparison between calculated and observed powder intensity data.

The interatomic distances are given in Table 2. The environment of iridium consists of eleven aluminium atoms at distances between 2.45 and 2.80 Å and the average distance is 2.65 Å. These distances may be compared to the Os—Al distances in Os_4Al_{13}.[1] In this structure the Os—Al distances are in the range 2.46—2.86 Å and there are two kinds of osmium atoms. One has eleven aluminium neighbours at an average distance of 2.65 Å and the other ten at an average distance of 2.64 Å. Those average distances are thus practically the same in $IrAl_3$ and Os_4Al_{13} and this is in concordance with the very similar metallic radii found in the pure elements (r_{Os} = 1.353 Å and r_{Ir} = 1.357 Å).

Table 2. Interatomic distances in $IrAl_3$.

Atom	Neighbour	C.N.	Distance(Å)
Ir	Al_1	3	2.45
	Al_2	2	2.52
	Al_2	6	2.80
Al_1	Ir	3	2.45
	Al_2	6	2.80
Al_2	Ir	1	2.52
	Ir	3	2.80
	Al_1	3	2.80
	Al_2	1	2.71
	Al_2	3	2.71

The arc-melted $IrAl_3$ sample was also studied at lower temperatures by heat treatments in evacuated silica tubes followed by quenching in water. The $D0_{18}$ phase was thus found to be retained in specimens quenched from 950°C but not in such that were quenched from 850°C. The stability region of $IrAl_3$ ($D0_{18}$-type) obviously extends from approximately 900°C upwards.

Further studies on phases of this system are in progress.

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1. Edshammar, L.-E. *Acta Chem. Scand.* **18** (1964) 2294.

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On the Conversion of Cholest-5-ene-3β,7α-diol to 7α-Hydroxycholest-4-en-3-one in Rat Liver Homogenates

Bile Acids and Steroids 186

OLLE BERSÉUS and KURT EINARSSON

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

The first step in the degradation of cholesterol to cholic acid is a hydroxylation at position C-7 to yield cholest-5-ene-3β,7α-diol.[1,2] Cholest-5-ene-3β-,7α-diol in turn is converted into 7α,12α-dihydroxycholest-4-en-3-one either by means of the intermediary formation of 7α-hydroxycholest-4-en-3-one or of cholest-5-ene-3β,7α,12α-triol.[2-4] Recently, Hutton and Boyd [5] reported studies on the conversion of cholest-5-ene-3β,7α-diol into 7α-hydrox-

ycholest-4-en-3-one by rat liver homogenates. The reaction was found to be catalyzed by the mitochondrial as well as the microsomal fraction, the microsomal fraction being the more efficient. Pyridine nucleotides were required and with the concentrations used NADP was found to be as efficient as NAD. In connection with studies on the formation and metabolism of cholest-5-ene-3β,7α,12α-triol,[4] it was found that the conversion of cholest-5-ene-3β,7α,12α-triol into 7α,12α-dihydroxycholest-4-en-3-one was catalyzed mainly by the microsomal fraction and required the addition of pyridine nucleotides. In this case, NAD was found to be several times more efficient than NADP. In view of these results, it was considered of interest to examine whether or not the same difference in extent of stimulation between NAD and NADP could be observed in the conversion of cholest-5-ene-3β,7α-diol into 7α-hydroxycholest-4-en-3-one.

Experimental. Materials. Cholest-5-ene-3β,7α-diol-7β-^{3}H was prepared as described previously [2] and had a specific activity of 10 μC per mg. NAD and NADP were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of homogenates. White male rats of the Sprague-Dawley strain weighing about 200 g were used. Homogenates, 20 % (liver wet weight/volume), were prepared in a modified Bucher medium [6] with a Potter-Elvehjem homogenizer using a loosely fitting teflon pestle. The homogenate was centrifuged at 800 *g* for 10 min and the supernatant fluid obtained was centrifuged at 20 000 *g* for 10 min. The 20 000 *g* supernatant fluid was centrifuged at 100 000 *g* for 2 h. The microsomal fraction was resuspended in the homogenizing medium by homogenization with a loosely fitting pestle. The suspension was centrifuged at 800 *g* for 5 min and the supernatant fluid was used as microsomal fraction. In experiments with the mitochondrial fraction, the 800 *g* supernatant fluid was centrifuged at 8500 *g* for 10 min and the mitochondrial fraction was obtained as described by Wilgram and Kennedy.[7] One ml of enzyme solution was diluted with 2 ml of homogenizing medium and 100 mμmoles of substrate were added dissolved in 50 μl of acetone. Incubation was run for 20 min at 37°.

Table 1. Metabolism of cholest-5-ene-3β,7α-diol-7β-^{3}H in fractions of rat liver homogenate. The amounts of enzyme fraction used corresponded to 1 ml of 800 *g* supernatant fluid. 100 mμmoles of substrate and 0.7 μmoles of NAD were added and incubations were run for 20 min. The percentages were calculated from the amounts of radioactivity in the different zones of the thin layer chromatograms.

	Products %				
Enzyme fraction	Cholest-5-ene-3β, 7α-diol	7α-Hydroxy-cholest-4-en-3-one	7α, 12α-Dihydroxy-cholest-4-en-3-one	Compounds more polar than 7α, 12α-dihydroxy-cholest-4-en-3-one	Other unidentified compounds
800 *g* supernatant	54.0	22.7	5.7	10.8	6.8
Mitochondria	88.1	9.5	0.4	1.0	1.0
20 000 *g* sediment	80.5	14.4	0.3	1.3	3.5
20 000 *g* supernatant	74.2	15.4	4.5	3.1	2.8
Microsomes	67.8	26.7	0.6	2.3	2.6
100 00 *g* supernatant	95.6	1.4	0.2	0.4	2.4

Analysis of homogenates. Incubation was terminated by addition of 20 volumes of chloroform-methanol (2:1). The precipitate was filtered off and 0.2 volumes of 0.9 % sodium chloride solution were added. The chloroform phase was taken to dryness and the residue together with internal standards was subjected to thin layer chromatography with benzene-ethyl acetate (1:1) as solvent. The compounds were located by iodine vapor.[2] After evaporation of the iodine at room temperature, the appropriate zones of the chromatoplate were eluted with methanol according to the technique described by Matthews, Pereda and Aguilera.[8] Radiactivity was measured on aliquots of the fractions with a methane gas flow counter. Under the conditions employed 1 μC of ^{3}H corresponds to 6×10^5 cpm.

Results and discussion. Table 1 summarizes the results of incubations of cholest-5-ene-3β,7α-diol-7β-^{3}H with different fractions of a rat liver homogenate. Each fraction was fortified by addition of NAD. Two main metabolites were formed with the chromatographic properties of 7α,12α-dihydroxycholest-4-en-3-one and 7α-hydroxycholest-4-en-3-one. 7α,12α-Dihydroxycholest-4-en-3-one, the identity of which has been previously established,[2] was formed mainly in the 800 *g* and 20 000 *g* supernatant fluid. The formation of 7α-hydroxycholest-4-en-3-one was catalyzed most efficiently by the microsomal fraction and the compound was identified by crystallization to constant specific activity after addition of authentic material (Table 2). 7α-Hydroxycholest-4-en-3-one was also

Table 2. Identification of 7α-hydroxycholest-4-en-3-one. 7α-Hydroxycholest-4-en-3-one was isolated by means of thin layer chromatography after incubation of cholest-5-ene-3β, 7α-diol-7β-^{3}H with the microsomal fraction fortified with NAD. All specific activities are given in thousands of counts per min per mg.

Solvent	No. of crystallizations	Weight mg	Specific activity
None	0	16.9	1.21
Methanol-water	1	10.8	1.16
Methanol-water	2	6.0	1.17
Methanol-water	3	5.0	1.20

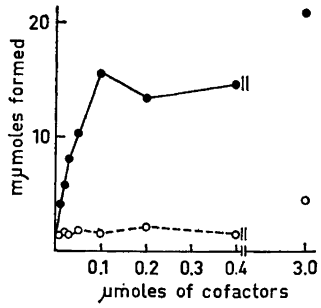

Fig. 1. Effect of increasing amounts of cofactors on the rate of formation of 7α-hydroxycholest-4-en-3-one. Cholest-5-ene-3β,7α-diol-7β-^{3}H (0.24×10^6 cpm, 100 mμmoles) was incubated with 1 ml of microsomal fraction (corresponding to 1 ml of 20 000 *g* supernatant fluid). Incubation was run for 20 min. ○, NADP, ●, NAD.

formed in the presence of mitochondrial fraction and 20 000 *g* sediment but to a less extent than in the presence of microsomal fraction. The rate of formation of 7α-hydroxycholest-4-en-3-one with time in the microsomal fraction fortified with NAD was nearly linear during the first 30 min and an incubation time of 20 min was chosen for the experiments. Under these assay conditions proportionality between reaction rate and protein concentration was observed. Fig. 1 shows the stimulatory effect of varying concentrations of NAD and NADP on the formation of 7α-hydroxycholest-4-en-3-one from cholest-5-ene-3β,7α-diol in the presence of microsomal fraction. The addition of NADP in amounts less than 0.4 μmoles did not result in any significant stimulation, whereas stimulation was observed with NAD already by addition of 0.01 μmoles. When 3 μmoles of NADP were added, the reaction rate was about 20 % of that observed with the corresponding amount of NAD.

The present results concerning the conversion of cholest-5-ene-3β,7α-diol to 7α-hydroxycholest-4-en-3-one in the presence of microsomal fraction of rat liver are very similar to those obtained in a previous investigation on the conversion of cholest-5-ene-3β,7α,12α-triol to 7α,12α-dihydroxycholest-4-en-3-one.[4] In both cases the reaction probably involves the intermediary formation of a Δ^5-3-keto steroid and requires two enzymes, a Δ^5-3β-hydroxy-

steroid dehydrogenase and a Δ^5-3-ketosteroid isomerase. The Δ^5-3β-hydroxysteroid dehydrogenase(s) involved requires a pyridine nucleotide as cofactor and the results make it probable that the enzyme(s) utilizes NAD preferentially. Thus, if the preference of pyridine nucleotide observed were due to an activation of the Δ^5-3-ketosteroid isomerase(s) similar to that described by Oleinick and Koritz [9] for the Δ^5-3-ketosteroid isomerase(s) in rat adrenal small particles, one would expect an accumulation of 7α-hydroxycholest-5-en-3-one in incubations with NADP. No significant amounts of labeled material with chromatographic properties of this compound could be detected. The present results do not provide any information concerning the existence of an activation of the Δ^5-3-ketosteroid isomerase(s) involved by diphosphopyridine nucleotides as described by Oleinick and Koritz.[9] To detect such an activation one would have to have access to the substrates for this enzyme(s), *i.e.* 7α-hydroxycholest-5-en-3-one and 7α,12α-dihydroxycholest-5-en-3-one.

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1. Mendelsohn, D., Mendelsohn, L. and Staple, E. *Biochim. Biophys. Acta* **97** (1965) 379.
2. Danielsson, H. and Einarsson, K. *J. Biol. Chem.* **241** (1966) 1449.
3. Mendelsohn, D., Mendelsohn, L. and Staple, E. *Biochemistry* **5** (1966) 1286.
4. Berséus, O., Danielsson, H. and Einarsson, K. *J. Biol. Chem.* **242** (1967) 1211.
5. Hutton, H. R. B. and Boyd, G. S. *Biochim. Biophys. Acta* **116** (1966) 336.
6. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **9** (1955) 34.
7. Wilgram, G. F. and Kennedy, E. P. *J. Biol. Chem.* **238** (1963) 2615.
8. Matthews, J. S., Pereda, A. L. and Aguilera, A. *J. Chromatog.* **9** (1962) 331.
9. Oleinick, N. L. and Koritz, S. B. *Biochemistry* **5** (1966) 715.

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Analysis of Individual Molecular Species of Phospholipids

V. Separation of Dinitrophenylated and Methylated Ethanolamine Phosphatides of Hens' Eggs*

OSSI RENKONEN

Department of Serology and Bacteriology, University of Helsinki, Helsinki, Finland

Different molecular species of nonpolar phospholipid derivatives are easier to separate from each other by liquid chromatography than the original phosphatide molecules. For instance diglyceride acetates,[1,11] ceramide diacetates,[2] free,[3] and tritylated diglycerides,[4] and dimethyl phosphatidates [5,6] derived from native phospholipids have been separated into many subfractions. So far all these derivatives have been obtained by removing the polar groups of the phosphatides, which has limited the value of the approach. The present report shows that ethanolamine glycerophosphatides converted into nonpolar form by dinitrophenylation and methylation, *i.e.* by "masking" the polar groups instead of removing them, are also well resolved by silica gel chromatography.

Glyceryl-phosphoryl-ethanolamine lipids (GPE-lipids) were isolated from eggs by chromatography on silicic acid. The sample was treated with 1-fluoro-2,4-dinitrobenzene and the reaction product was methylated with diazomethane as described by Collins.[7] Thin layer chromatography (TLC) of the resulting lipid revealed a yellow double-spot. The two components were isolated (Fig. 1), and the slower moving fraction (92 %) was identified as N-dinitrophenyl-O-methyl-phosphatidyl-ethanolamine (EE-GPME-DNP), whereas the faster component (8 %) consisted mainly of the corresponding 1-alkyl-2-acyl derivative (AE-GPME-DNP). These identifications are based on the following observations: 1) Synthetic samples of EE-GPME-DNP and AE-GPME-DNP were obtained by dinitrophenylating and meth-

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