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THE EFFECT OF DIFFERENT INCUBATION SYSTEMS ON THE *IN VITRO* METABOLISM OF NORETHYNODREL*

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SUMMARY

The *in vitro* metabolism of norethynodrel by male rat liver has been studied under a number of different incubation conditions. Both the postmitochondrial supernatant and the microsomal pellet were used as the enzyme source. Optimum incubation conditions for norethynodrel metabolism have been determined with respect to cofactor requirements.

The presence of either sucrose, EDTA, Mg^{2+} or nicotinamide alone or in combination in the incubation medium did not increase substrate metabolism. Maximum product formation occurred using a simple potassium phosphate buffer and a NADPH regenerating system.

THERE is an increasing number of *in vitro* steroid metabolism studies reported in the literature. These studies use as their enzyme source either the postmitochondrial supernatant or the microsomal pellet. The incubation conditions used in these studies differ with respect to the number, type and concentration of cofactor. These differences include the use of an NADPH regenerating system [1–5], the direct addition of NADPH [4, 7, 8], the presence of nicotinamide [3–5], KCl [2, 5], $MgSO_4$ or $MgCl_2$ [1, 4, 5], EDTA [6], sucrose [6], ATP [5] and the type of buffer salt used (phosphate, tris, citrate). The great variation in incubation conditions makes it difficult to correlate metabolic studies carried out in different laboratories.

A study was undertaken to determine optimum conditions for the *in vitro* metabolism of norethynodrel.† We are defining the optimum conditions as those allowing the formation of the greatest amount of the relatively polar products represented in Fig. 1 as peaks E and F. In particular we were interested in obtaining maximum biosynthesis of polyhydroxylated metabolites of norethynodrel so that sufficient quantities could be obtained to permit rigid structural identification. Recent studies in this laboratory [9] have demonstrated that norethynodrel is readily metabolized by the postmitochondrial supernatant of several species, including rat, rabbit, guinea pig and man. Major metabolites identified include the reduction products, 3α,17β-dihydroxy-5(10)-estren-17α-ethynyl and 3β,17β-dihydroxy-5(10)-estren-17α-ethynyl. In addition a number of minor polyhydroxy compounds were isolated and some of the structures completely or partially established [10].

MATERIALS AND METHODS

The sources of the chemicals used in this study were as follows: NADP,

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†Norethynodrel = 17β-hydroxy-5(10)-estren-17α-ethynyl-3-one; norethindrone = 17β-hydroxy-4-estren-17α-ethynyl-3-one.

NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV) and dithioerythritol (DTE) from Sigma Chemical Co. Norethynodrel (Lot No. V-55) was a gift from G. D. Searle & Co. The rat livers were from male Charles River rats, CD strain. Norethynodrel-6,7-^{3}H was synthesized under the supervision of Dr. J. A. Kepler, of this laboratory's radiochemical synthesis group.

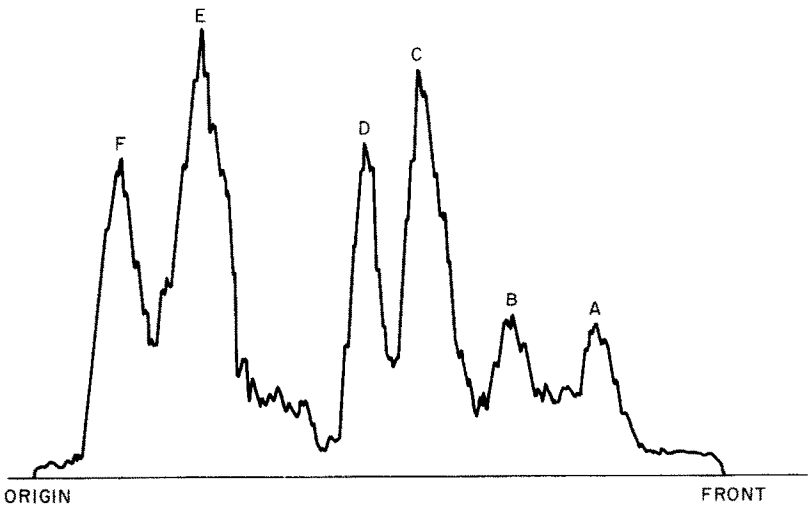

Fig. 1. Radiochromatogram scan of a 5 × 20 cm silica gel plate containing metabolites extracted after a 60 min norethynodrel incubation with rat liver microsomal pellet from 10 g liver and chromatographed in a 25 per cent ethyl acetate in chloroform solvent system. The incubation mixture containing 10 mg norethynodrel (3 μCi), NADPH regenerating system and 0·1 M potassium phosphate buffer, pH 7·4. Peaks A, B, C, D correspond in R_F to norethynodrel, norethindrone, 3α,17β-dihydroxy-5(10)-estren-17α-ethynyl and 3β,17β-dihydroxy-5(10)-estren-17α-ethynyl, respectively. The identification of the more polar compounds resulting in peaks E and F is in progress.

Isolation of liver fraction. Ten grams of male rat liver was homogenized for 3 min on ice in 50 ml buffer using a Virtis "45" homogenizer. The buffers varied with respect to pH, cofactors and gaseous atmosphere as seen in Table 1. The homogenized tissue was centrifuged at 10,000 g for 20 min. The supernatant obtained by centrifugation was transferred to a 250 ml Erlenmeyer flask in which the drug incubation was performed.

When the microsomal pellet was used as the enzyme source, the following modifications were made. The supernatant from the 10,000 g centrifugation was recentrifuged at 105,000 g for 1 hr at 4°C. The resulting supernatant was discarded. The microsomal pellet was combined with 50 ml buffer, dispersed with a glass rod and placed in a 250 ml Erlenmeyer flask, in which the incubation was performed.

Incubation procedure. All incubations were performed for 60 min. When NADPH was used, it was added directly to the incubate, followed by the addition of 10 mg norethynodrel (3 μCi). The flask was then immediately placed in a shaking water bath which had been equilibrated at 37°C.

When the regenerating system was used to produce NADPH, glucose-6-phosphate (2×10^{-3} M), glucose-6-phosphate dehydrogenase (10 units) and NADP (1×10^{-3} M) were added to the incubation flask containing either the

Table 1. Constituents in the potassium phosphate buffer*

Incubation No.	pH	Sucrose (0·25 M)	$MgSO_4$ (1×10^{-4} M)	NADPH (1×10^{-3} M)	Regenerating system†	Nicotinamide (1×10^{-3} M)	EDTA (1×10^{-3} M)
1	7·4			+			
2	7·4	+	+	+		+	+
3	7·4	+		+			
4	7·4	+	+	+			
5	7·4	+	+	+		+	
6	7·4	+	+		+		
7	7·4	+	+		+	+	
8‡	7·4	+	+		+		
9	8·2	+	+		+		
10	6·0	+	+		+		
11	7·4				+		
12§	7·4	+	+		+		

*0·1 M potassium phosphate buffer.

†NADPH regenerating system: glucose-6-phosphate, 2×10^{-3} M, glucose-6-phosphate dehydrogenase, 10 units, NADP, 1×10^{-3} M.

‡Performed in an atmosphere of nitrogen.

§Nitrogen bubbled into liver incubate before addition of cofactors and drug.

postmitochondrial supernatant or the microsomal pellet and incubated for 3 min prior to the addition of substrate.

After 60 min the reaction was stopped by the addition of 100 ml redistilled ethyl acetate to each flask.

Extraction of metabolites. The incubate was extracted 4 times with ethyl acetate, the extracts combined and evaporated to dryness. To each round bottom flask containing the dry extract was added equal parts of previously equilibrated hexane and aqueous (10 per cent) methanol. The aqueous methanol fraction which contained the metabolites was evaporated to dryness and stored as such. This entire procedure is carried out in one day.

Thin layer chromatography. The dried methanol extract was brought into solution with 1·0 ml redistilled ethyl acetate. A 150 μl aliquot of the solvated extract was applied to precoated glass plates (Brinkmann Instruments, Silica Gel F-254) and then eluted with a solvent system containing 25 per cent ethyl acetate in chloroform. The completed chromatographic plates were scanned with a radiochromatogram scanner (Packard Instrument Co., model 7201).

Quantitation of chromatographic bands. Each thin layer chromatographic plate was carefully aligned with its radiochromatograph scan, the radioactive bands marked and the bands then scraped into scintillation vials. The amount of radiolabeled product in each vial was determined and listed in Table 2 as a per cent of the total count on the corresponding plate.

Metabolite identification. The metabolites were subjected to gas–liquid chromatography (GLC) on a column of 3·8 per cent OV-17 on acid-washed, silanized Chromosorb W at 210–240° in an F & M Model 402 flame ionization chromatograph. GLC-mass spectrometry was carried out on a 1 per cent OV-17 column at 210–220° in an LKB model 900 instrument. The 3α- and 3β-hydroxy metabolites were separated and identified by GLC after first preparing their trimethylsilyl ethers by reacting the steroids with hexamethyldisilazane and trimethylchlorosilane in pyridine at 100°. Norethynodrel and norethindrone were identified by GLC and GLC-MS using appropriate reference standards.

Table 2. Results of norethynodrel metabolism using different cofactors and the 10,000 g supernatant from male rat liver

	Radiochromatogram scan peaks*						
Incubation No.	A	B	C	D	E	F	Total E+F
1	1	3	29	10	36	22	58
2	4	4	28	20	25	19	43
3	1	3	25	14	44	13	57
4	1	4	26	15	33	21	54
5	1	4	32	13	32	18	50
6	1	3	32	10	33	20	53
7	1	2	28	23	29	16	45
8	1	3	29	25	30	12	42
9	2	7	32	8	41	10	51
10	1	7	34	18	33	7	40
11	0	2	31	6	38	26	61
12	31	4	29	22	9	5	14

*Expressed as per cent of total radioactivity on the thin layer plate.

RESULTS

The success of an incubation was measured by the total amount of product comprising radiochromatogram peaks E and F. The compounds representing peaks A, B, C and D were identified using previously synthesized steroid standards (Fig. 1) as described in the methods section.

Incubations number 1 and 11 (Table 1) containing only potassium phosphate buffer (0·1 M, pH 7·4) and either NADPH or the NADPH regenerating system were able to biosynthesize as many reaction products and more of the relatively polar products than any other incubation conditions (Table 2). With the 10,000 g supernatant as with the microsomal pellet, the presence of either Mg, EDTA, or nicotinamide or a combination of these agents resulted in decreased polar product formation. Maximum product formation occurred when the regenerating system was used in place of the direct addition of NADPH.

When the air in the incubation flask was replaced by nitrogen, extensive product formation still occurred. However, when nitrogen was bubbled directly into the incubation medium thereby replacing the available soluble oxygen and the reaction then run in an atmosphere of nitrogen, a very significant decrease in product formation was seen.

The possible requirement by the microsomal pellet for the presence of the regenerating system, sucrose and magnesium was determined. Incubations were performed as previously described using a number of different incubation conditions (Table 3). In all incubations using the regenerating system a trace amount of

Table 3. Results of norethynodrel metabolism using different cofactors and the microsomal pellet from male rat liver

Incubation No.	Buffer*	Radiochromatogram scan peaks A	B	C	D	E	F	Total E+F
1	$PO_4^=$, NADPH	10	15	5	6	38	26	64
2	$PO_4^=$, regenerating system	9	10	6	6	49	20	68
3	$PO_4^=$, NADPH, sucrose, $MgSO_4$	13	16	8	9	38	16	54
4	$PO_4^=$, regenerating system sucrose, $MgSO_4$	15	14	11	10	36	13	49
5	Same as 4 plus nicotinamide	7	18	15	13	39	8	47

*Potassium phosphate, 0·1 M, pH 7·4. When applicable, sucrose, 0·25 M; $MgSO_4$, 1×10^{-4} M; Nicotinamide, 1×10^{-3} M.

magnesium was present in the form of magnesium acetate, a component of Sigma's type XV glucose-6-phosphate dehydrogenase preparation.

Figure 1 is a radiochromatogram scan of incubation No. 2 (Table 3). Peaks A, B, C and D correspond in R_F to norethynodrel, norethindrone, 3α,17β-dihydroxy-5(10)-estren-17α-ethynyl, and 3β,17β-dihydroxy-5(10)-estren-17α-ethynyl, respectively. Peaks E and F, containing the polyhydroxy fraction, undoubtedly contain a number of components. Their complete resolution and structural identification are a subject of continuing research in this laboratory [10].

DISCUSSION

It has been found that the progestational steroid, norethynodrel, was most efficiently metabolized *in vitro* when a relatively simple potassium phosphate

buffer was used in conjunction with a NADPH regenerating system and the microsomal pellet. The addition of EDTA, Mg^{2+}, nicotinamide or sucrose did not increase the rate of product formation. In fact the presence of a combination of these chemicals resulted in a decrease in norethynodrel metabolism.

The addition of nicotinamide alone to the incubation mixture resulted in a decrease in polar product formation. If a comparison is made between incubations 4 and 5 and 6 and 7 (Tables 1 and 2) a shift is seen from hydroylation to ketone reduction in the presence of nicotinamide. This is observed as a decrease in peaks E and F with a concomitant increase in peaks C and D. The presence of nicotinamide during a microsomal pellet incubation of norethynodrel decreased by 5 per cent the amount of polar product formed when compared to an identical incubation containing no nicotinamide (Table 3). However, in this case, addition of $MgSO_4$ and sucrose gave a more striking change. Lowering the pH to 6·0 also caused some decrease in the amount of polar product (Incubation No. 10, Tables 1 and 2). None of the above changes, however, gave the marked change observed when oxygen in the incubation system was replaced with nitrogen (Incubation No. 12). Both consumption of norethynodrel and production of polar substances were greatly reduced, thus indicating a requirement for oxygen to obtain complete metabolic conversion.

The presence of a smaller amount of the 3β-hydroxy product in all incubations compared to the amount of the 3α-hydroxy isomer present, indicates either that the rate of formation of the 3β-hydroxy product is slower than that for the 3α-hydroxy isomer or that subsequent conversions selectively deplete the existing concentration of this isomer. This latter idea suggests that both isomers are actually intermediates in the formation of the more polar products.

The optimal incubation conditions for norethynodrel metabolism determined in this study and discussed above can most probably be used for *in vitro* assays in which similar steroids are used as substrate, providing male rat liver is used as the enzyme source.

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