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EFFECT OF STORAGE OF FROZEN LIVER MICROSOMAL PREPARATIONS ON THE HYDROXYLATION OF TESTOSTERONE AND PENTOBARBITAL AND THE *N*-DEMETHYLATION OF ETHYLMORPHINE*

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Abstract—The activity of the liver microsomal enzyme systems responsible for the hydroxylation of testosterone in the 7α-, 16α- and 6β-positions is differentially affected by storage of microsomal preparations at −15°. The 16α-hydroxylase activity decreased upon storage of microsomal suspensions or lyophilized preparations for 20 days, but was not affected when the microsomes were stored as a pellet. In contrast, the 7α-hydroxylase activity remained essentially unchanged while the 6β-hydroxylase activity decreased in all three microsomal preparations. The stability of pentobarbital hydroxylase and ethylmorphine *N*-demethylase was similar to that obtained for testosterone 16α-hydroxylase. The differential effects of storage of liver microsomal preparations on the hydroxylation of testosterone in the 6β-, 7α- and 16α-positions suggest that a single cytochrome cannot alone determine the specificity of the three hydroxylation reactions. In addition, the results suggest that when storage of microsomes for more than 1 day is absolutely necessary, microsomes should be stored as a pellet rather than as a suspension or as a lyophilized powder.

RECENT studies on the hydroxylation of testosterone *in vitro* have shown that the rate of 6β-, 7α- and 16α-hydroxylation can be independently altered by drug treatment.[1–3] Pretreatment of rats with phenobarbital (PB) stimulated the hydroxylation of testosterone in the 7α-, 6β- and 16α-positions to different extents, whereas treatment with 3-methylcholanthrene (MC) stimulated 7α-hydroxylation and inhibited 16α-hydroxylation. The 6β-hydroxylation of testosterone was either stimulated (immature rat) or inhibited (adult rat) by pretreatment with MC.[3] Not only were differences observed between PB and MC pretreatment on the hydroxylation of testosterone, but differences have also been observed on the induction of microsomal cytochromes. Pretreatment of rats with MC has been shown to alter the spectral characteristics of the microsomal hemoprotein, detectable when the reduced hemoprotein combines with ethyl isocyanide[4] or with carbon monoxide.[3, 5, 6] Alteration in the spectral properties of the hemoprotein induced by MC is accompanied by changes in the kinetic properties of the enzyme system hydroxylating 3,4-benzpyrene, indicating that microsomal enzyme systems may be influenced qualitatively or quantitatively or in both ways by drug treatment.[7]

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Since different enzyme systems may function in the hydroxylation of testosterone at three different positions on the steroid nucleus, the effect of prolonged storage of microsomal preparations on the rate of testosterone hydroxylation was studied to determine whether a differential stability for the 6β-, 7α- and 16α-hydroxylation reactions could be demonstrated. Since many similarities exist between drug and steroid hydroxylases in liver microsomes,[8] the effect of the various storage conditions on the metabolism of pentobarbital and ethylmorphine was also determined. The effect of pretreatment with PB or MC on the stability of the various microsomal enzyme systems studied was also determined.

MATERIALS AND METHODS

Adult (240–260 g) and immature (40–60 g) male rats of the Sprague–Dawley strain (Blue Spruce Farms, Altamont, N.Y.) were maintained on a commercial diet (Rockland Farm Rat Diet) and water *ad libitum*. Phenobarbital sodium (37 mg/kg twice daily) or MC (25 mg/kg once daily) was administered intraperitoneally for 3 days. Control animals received corn oil and saline. The animals were killed 18 hr after the last injection, and liver microsomes were prepared from 33 per cent homogenates in 0·25 M sucrose solution as previously described.[9] The microsomal pellet was washed and resuspended in 0·15 M KCl twice to ensure complete removal of the soluble fraction and hemoglobin. The final microsomal pellet was resuspended in 0·1 M KH_2PO_4–K_2HPO_4 buffer, pH 7·4, to a final concentration equivalent to 200 mg of wet weight liver per ml or left as a pellet which was layered with 2 ml phosphate buffer. Other microsomal pellets were resuspended in buffer to a final concentration equivalent to 1·0 g of wet weight liver per ml and lyophilized. All three preparations were stored frozen at −15° for varying periods of time. The storage of the various microsomal preparations for 1 day did not lead to decreased enzymatic activity when compared to preparations assayed immediately.

Liver microsomes, equivalent to 100–200 mg liver, were incubated with testosterone-4-^{14}C or pentobarbital-2-^{14}C in the presence of an NADPH-generating system as previously described.[9–11] The incubation, extraction, chromatography and quantification of radioactive metabolites formed were determined as previously described.[9–11] The composition of the incubation mixture for the determination of ethylmorphine *N*-demethylase has been described previously.[12] Ethylmorphine, at a concentration of 2×10^{-3} M was incubated at 37° for 15 min. Formaldehyde formed from the *N*-demethylation of ethylmorphine was measured by the procedure of Nash as modified by Anders and Mannering.[13] Protein was determined by the method of Sutherland *et al.*[14]

RESULTS

The enzyme system in microsomal preparations obtained from adult or immature rats responsible for the 7α-hydroxylation of testosterone showed little or no decrease in activity during the first 20 days of storage at −15°, regardless of the method of storage of microsomal preparations (Tables 1 and 2). Between 20 and 48 days of storage, the 7α-hydroxylase activity in the lyophilized preparation decreased to 45–50 per cent of its original value, while a considerably smaller decline in activity was found in the suspensions and pellets (Table 1). In contrast, the 16α-hydroxylase activity decreased substantially in the microsomal preparations which were stored

either lyophilized or as a suspension for 20 days, while this enzyme activity remained essentially unchanged when the microsomes were stored as a pellet (Tables 1 and 2). In the lyophilized preparation, over 90 per cent of the original 16α-hydroxylase activity was lost upon storage for 48 days as compared to the 7α-hydroxylase activity

TABLE 1. EFFECT OF STORAGE OF FROZEN LIVER MICROSOMAL PREPARATIONS FROM ADULT MALE RATS ON THE HYDROXYLATION OF TESTOSTERONE*

Pretreatment	Storage condition	Reaction studied	Activity on day 1 (mμmoles formed/mg protein)	Activity on	
				Day 20	Day 48
				(% of activity on day 1)	
Adult C	pellets	7α-OH	5·6	98·2	76·3
PB			8·9	112·7	83·6
MC			9·6	103·9	82·4
C	suspensions	7α-OH	4·1	128·3	82·3
PB			8·8	116·7	86·4
MC			9·3	114·8	81·7
C	lyophilized	7α-OH	3·4	80·4	48·2
PB			8·1	93·8	44·6
MC			9·5	98·5	45·9
C	pellets	16α-OH	12·3	93·1	67·4
PB			17·3	101·5	72·9
MC			1·4	- - -	- - -
C	suspensions	16α-OH	11·0	74·0	32·0
PB			15·7	66·0	25·8
MC			1·8	- - -	- - -
C	lyophilized	16α-OH	10·9	79·7	7·9
PB			14·9	37·7	6·7
MC			1·0	- - -	- - -
C	pellets	6β-OH	11·9	80·0	49·7
PB			25·1	90·0	70·4
MC			10·4	78·7	59·5
C	suspensions	6β-OH	10·4	56·7	41·7
PB			30·2	56·7	32·6
MC			10·7	60·5	42·2
C	lyophilized	6β-OH	8·9	85·5	10·2
PB			26·3	48·8	9·8
MC			9·2	67·7	13·0

* Adult male rats were treated with phenobarbital (PB) (37 mg/kg twice daily) or 3-methylcholanthrene (MC) (25 mg/kg once daily) for 3 days. Liver microsomes were prepared and assayed after storage frozen overnight (day 1) or for 20–48 days as a suspension in 0·1 M KH_2PO_4-K_2HPO_4, pH 7·4, as a pellet layered with 2 ml phosphate buffer or as a lyophilized preparation. Microsomes equivalent to 100 mg liver were incubated with 900 mμmoles testosterone-4-^{14}C for 15 min at 25° in the presence of an NADPH-generating system. Values for day 20 represent the average of 3 values from 2 experiments, where each value was obtained with the pooled livers from 4 rats. Values for day 48 represent a single determination of 6 pooled livers. The dashed lines denote little or no enzyme activity on day 1 of assay and stability measurements were not possible.

which decreased 50–55 per cent over the same period (Table 1). Although the 16α-hydroxylase activity also decreased both in suspensions and pellets which were stored for 48 days, the decrease in activity in the suspensions was greater. The ability of all three microsomal preparations to 6β-hydroxylate testosterone decreased over

TABLE 2. EFFECT OF STORAGE OF FROZEN LIVER MICROSOMAL PREPARATIONS FROM IMMATURE MALE RATS ON THE HYDROXYLATION OF TESTOSTERONE*

Pretreatment	Storage condition	Reaction studied	Activity on day 1 (mμmoles formed/mg protein)	Activity on	
				Day 20	Day 48
				(% of activity on day 1)	
Immature					
C	pellets	7α-OH	12·4	90·6	73·4
PB			27·2	86·7	65·5
MC			31·1	94·2	75·3
C	suspensions	7α-OH	12·4	97·9	65·8
PB			23·2	101·3	87·7
MC			27·4	106·6	83·7
C	pellets	16α-OH	1·0	---	---
PB			10·4	98·6	63·5
MC			0·7	---	---
C	suspensions	16α-OH	0·8	---	---
PB			13·3	61·6	32·2
MC			0·8	---	---
C	pellets	6β-OH	6·8	84·8	40·2
PB			14·1	71·5	51·5
MC			9·7	68·3	48·6
C	suspensions	6β-OH	7·6	47·3	53·6
PB			16·2	37·6	25·7
MC			9·4	57·4	47·6

* Immature male rats were treated with PB (75 mg/kg in divided daily doses) or MC (25 mg/kg once daily) for 3 days. Liver microsomes were prepared and assayed after storage frozen overnight (day 1) or for 20–48 days as a suspension in 0·1 M KH_2PO_4–K_2HPO_4, pH 7·4, or as a pellet layered with 2 ml phosphate buffer. Microsomes equivalent to 100 mg liver were incubated with 900 mμmoles testosterone-4-^{14}C for 15 min at 25° in the presence of an NADPH-generating system. Values for day 20 represent the average of 3 values from 2 experiments, where each value was obtained with the pooled livers from 4 rats. Values for day 48 represent a single determination of 6 pooled livers. The dashed lines denote little or no enzyme activity on day 1 of assay and stability measurements were not possible.

the first 20 days of storage while, after 48 days of storage, the activity in the lyophilized preparation had decreased much more than that in the pellets or suspensions.

Tables 3 and 4 demonstrate the effect of storage for 20 days on the hydroxylation *in vitro* of pentobarbital-2-^{14}C and the *N*-demethylation of ethylmorphine. The effect of storage for 20 days on the metabolism of these substrates from both immature and adult rats is similar to that found for testosterone 16α-hydroxylase; that is, storage of microsomes as a suspension led to a decrease in activity, while the activity of microsomes stored as a pellet for 20 days remained essentially unchanged.

As reported previously by Kuntzman *et al.*,[11] chronic treatment of rats with PB stimulates the liver microsomal metabolism of pentobarbital *in vitro*. The present studies show that treatment of rats with MC results in a marked inhibition of pentobarbital metabolism by microsomes from immature and adult rats (Table 3). This inhibition by MC treatment is similar to that observed for the 16α-hydroxylation of testosterone (Tables 1 and 2). Gram *et al.*[15] have shown that MC treatment to rabbits inhibits the metabolism of codeine, hexobarbital, amphetamine and aminopyrine

TABLE 3. EFFECT OF STORAGE OF FROZEN LIVER MICROSOMAL PREPARATIONS FROM IMMATURE AND ADULT RATS ON THE HYDROXYLATION OF PENTOBARBITAL*

Pretreatment	Storage condition	Pentobarbital hydroxylase activity on day 1 (mμmoles formed/mg protein)	Activity on day 20 (% of activity on day 1)
Immature			
C	pellets	0·61 (0·60, 0·62)	106·9 (104·2, 109·6)
PB		3·36 (3·50, 3·23)	108·8 (116·1, 101·4)
MC		0·29 (0·27, 0·31)	104·3 (95·2, 113·4)
C	suspensions	0·56 (0·48, 0·64)	64·8 (67·1, 62·5)
PB		3·57 (3·25, 3·90)	67·3 (47·7, 86·9)
MC		0·35 (0·29, 0·42)	95·0 (89·3, 100·7)
Adult			
C	pellets	1·59 (1·49, 1·69)	105·4 (93·2, 117·6)
PB		2·73 (2·70, 2·76)	114·3 (109·9, 118·7)
MC		0·55 (0·58, 0·52)	116·8 (110·1, 123·5)
C	suspensions	1·57 (1·40, 1·75)	56·2 (67·1, 45·3)
PB		3·07 (3·18, 2·96)	63·1 (55·5, 70·7)
MC		0·60 (0·64, 0·56)	85·1 (82·2, 88·0)

* Immature and adult male rats were treated with PB (37 mg/kg twice daily) or MC (25 mg/kg once daily) for 3 days. Liver microsomes were prepared and assayed after freezing and storage overnight (day 1) or for 20 days as a suspension in 0·1 M KH_2PO_4–K_2HPO_4, pH 7·4, or as a pellet layered with 2 ml phosphate buffer. Incubation and assay conditions were as described under Methods. Values represent the mean of 2 determinations using livers from 3 rats for each determination. The numbers in parentheses are the values for individual determinations.

TABLE 4. EFFECT OF STORAGE OF FROZEN LIVER MICROSOMAL PREPARATIONS FROM IMMATURE AND ADULT RATS ON THE *N*-DEMETHYLATION OF ETHYLMORPHINE*

Pretreatment	Storage condition	Ethylmorphine *N*-demethylase activity on day 1 (mμmoles formaldehyde formed/mg protein)	Activity on day 20 (% of activity on day 1)
Immature			
C	pellets	58·1 (54·0, 62·3)	92·9 (88·4, 97·4)
PB		136·2 (148·7, 123·8)	109·5 (115·8, 103·2)
MC		61·8 (70·0, 53·6)	107·1 (98·4, 115·8)
C	suspensions	60·5 (55·5, 65·6)	61·6 (60·5, 62·7)
PB		172·0 (163·5, 180·6)	57·0 (48·8, 65·2)
MC		65·1 (71·3, 58·9)	69·4 (76·0, 62·8)
Adult			
C	pellets	81·5 (75·5, 87·5)	87·6 (98·8, 76·4)
PB		134·3 (138·9, 129·7)	118·9 (117·4, 120·4)
MC		62·6 (57·6, 67·7)	106·2 (118·6, 93·8)
C	suspensions	87·6 (76·2, 99·1)	48·4 (34·0, 62·8)
PB		165·9 (160·0, 171·9)	58·9 (66·4, 51·4)
MC		79·4 (80·8, 78·0)	73·5 (69·1, 77·9)

* Immature and adult male rats were treated with PB (37 mg/kg twice daily) or MC (25 mg/kg) for 3 days. Liver microsomes were prepared and assayed after freezing and storage overnight (day 1) or for 20 days as a suspension in 0·1 M KH_2PO_4–K_2HPO_4, pH 7·4, or as a pellet layered with 2 ml phosphate buffer. Incubation and assay conditions were as described under Methods. Values represent the mean of 2 determinations using livers from 3 rats for each determination. The numbers in parentheses are the values for individual determinations.

by the smooth endoplasmic reticulum. In confirmation of previous results,[4] PB treatment increased the metabolism of ethylmorphine *in vitro* and treatment of rats with MC had essentially no effect on ethylmorphine *N*-demethylation (Table 4).

It appears from the results presented in Tables 3 and 4 that MC pretreatment may lead to an increased stability of microsomal enzymes stored as a suspension in contrast to PB-treated and control animals, but further studies to establish this definitively would be needed.

DISCUSSION

The results presented in this paper demonstrate that the activities of the enzyme systems responsible for the hydroxylation of testosterone in the 7α-, 16α- and 6β-positions are differentially affected by the prolonged storage of microsomes in various ways. The ability to hydroxylate testosterone in the 7α-position was only minimally affected upon storage of various microsomal preparations for 20 days. In contrast, the ability to hydroxylate testosterone in the 16α-position decreased upon storage of microsomal suspensions or lyophilized preparations for 20 days, but was not affected when the microsomes were stored as a pellet. The testosterone 6β-hydroxylase activity decreased in all three microsomal preparations upon storage for 20 days. The ability to hydroxylate pentobarbital and to *N*-demethylate ethylmorphine decreased markedly upon storage of microsomal suspensions for 20 days, but was relatively unchanged when the microsomes were stored as a pellet. These results resembled those obtained for the 16-α-hydroxylation of testosterone.

The studies presented here demonstrate that storage of microsomal suspensions results in differential changes in the rate of testosterone 6β-, 7α- and 16α-hydroxylation and suggest, therefore, that more than one component with differing stabilities are responsible for the regulation of microsomal hydroxylations. The results also indicate that, in general, when storage of liver microsomal preparations for longer than 1 day is contemplated, the microsomes should be stored as a pellet at $-15°$ rather than as a suspension or as a lyopnilized powder, but microsomes should not be stored unless necessary. During the past few years, several papers have appeared on the stability of liver microsomal drug-metabolizing enzymes when stored frozen under various conditions.[9, 16–20]

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