EFFECTS OF LYOPHILIZATION AND STORAGE OF RAT LIVER MICROSOMES ON ACTIVITY OF ANILINE HYDROXYLASE, CONTENTS OF CYTOCHROME b_5 AND CYTOCHROME P-450 AND ANILINE-INDUCED P-450 DIFFERENCE SPECTRUM

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Abstract—Effects of lyophilization and storage of rat liver $9,000\times g$ supernatant fraction or microsomes on activity of aniline hydroxylase, contents of cyt. P-450 and cyt. b_5 and aniline-induced P-450 difference spectrum were studied. The lyophilization of rat liver $9,000\times g$ supernatant fraction or microsomes produced about 20% decrease in the activity of aniline hydroxylase. Aniline hydroxylase was stably stored for 6 months if lyophilized $9,000\times g$ supernatant fraction of rat liver had been stored in an atmosphere of nitrogen or in a low pressure of air. Activity of aniline hydroxylase and contents of cyt. P-450 and cyt. b_5 were not reduced during aerobical storage for a week. When lyophilized microsomes were stored for 6 months anaerobically, aniline hydroxylase, cyt. P-450 and cyt. b_5 were more stable as compared to aerobical storage. However, activity of aniline hydroxylase was reduced approx. 40% by the storage. The reduction of aniline hydroxylase activity was more significant than that of contents of cyt. P-450 and cyt. b_5 . Reduction of either activity of aniline hydroxylase or contents of cyt. P-450 and cyt. b_5 was more marked when lyophilized microsomes were stored with glycerol. Aniline hydroxylase in lyophilized $9,000\times g$ supernatant fraction from phenobarbital treated rabbit was stably stored, and aerobically little activity was lost during storage for 7 months.

There have been reports on the stability of drug metabolizing enzymes in liver microsomes (1-6). Gram and Fouts (6) reported that there was a marked species difference in stability of drug metabolizing enzymes between mice, rats and rabbits using various substrates, and that enzymes in rat liver were in most cases more unstable than those in rabbit liver. In addition, Levin *et al.* (5) reported that liver microsomes kept in a pellet form tended to lose activities less than those in a suspension or lyophilized form. In some experiments, investigators have used microsomes kept in a pellet form. Moreover, Ichikawa and Yamano (7) demonstrated that liver microsomal cyt. P-450 was stably stored aerobically by addition of glycerol even at 16°C.

It was of interest for the authors to investigate the effects of lyophilization and storage of microsomes for long period on the liver microsomal enzymes, as these effects have not yet been established. For this purpose, the authors compared the stabilities of aniline hydroxylase, cyt. P-450, cyt. b_5 and aniline-induced P-450 difference spectrum using rat liver $9,000\times g$ supernatant fraction or microsomal fraction lyophilized and stored in various environments.

MATERIALS AND METHODS

Animals: Male rats of Wistar strain weighing 100 to 150 g and male albino rabbits weighing about 2.7 kg were used. The animals were fasted for 18 hr prior to sacrifice but were given water *ad libitum*. Phenobarbital pretreatment comprised five consecutive daily s.c. injections of phenobarbital sodium (100 mg/kg in the rabbit), and the last injection was given approx. 48 hr prior to sacrifice. In the rat, 8 to 10 livers were pooled and used.

Preparation of liver microsomes: The animals were stunned by a blow on the head, and decapitated. The livers were immediately perfused with 1.15% KCl solution from *inferior vena cava* to portal vein. This procedure was carried out at room temp., since cold KCl solution could contract the blood capillaries in the liver and all blood cannot be quickly removed. The perfusion from *inferior vena cava* to portal vein also improved with a rapid removal of the blood. If one employs this method to remove blood from liver, the process can be completed in only one or two min. After perfusion, the livers were quickly removed and placed in 1.15% KCl solution on ice. All remaining procedures were carried out at a temp. under 4°C. After pouring off the KCl solution through a layer of paper, the livers were minced with scissors, then 20% homogenate of the livers in 1.15% KCl solution was prepared with a Potter-Elvehjem homogenizer which had a teflon pestle. The homogenate was centrifuged at $9,000 \times g$ for 20 min in a TOMINAGA refrigerated centrifuge, Model S-62. The supernatant fluid was re-centrifuged at $105,000 \times g$ for 60 min in a HITACHI ultracentrifuge, Model 65 P. All g values were calculated for the center of the centrifuge tube. The resulting microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.5) with a Potter-Elvehjem homogenizer having a teflon pestle. Protein was assayed by a method of Lowry *et al.* (8).

Lyophilization and storage of $9,000 \times g$ supernatant fraction, microsomes and soluble fraction: The $9,000 \times g$ supernatant fraction, microsomes and soluble fraction were frozen in a deep freezer for 2 to 3.4 hr, then lyophilized in a KYOWA lyophilizer, Model RL-300S. The final atmospheric pressure in the sample chamber was below 80 μ Hg. Displacement of the gas phase in a vessel was made by the vacuum-flash method. When storage of the preparations lasted for 6 months, the gas phase was displaced by the vacuum-flash method, then stored in a refrigerator at -20°C . At the end of 3 months, the vessel was re-flashed with the gas, re-sealed and returned to the refrigerator. Moreover, in the case of storage under low atmospheric pressure of air, the air in the vessel was removed by an aspirator to 24 mmHg of pressure, and the vessel was placed in a deep freezer. At 3 months, the air was removed again and the vessel was placed in the deep freezer. In the case of addition of glycerol, 0.7 ml of glycerol was added into a vessel containing lyophilized microsomes being equivalent to 3 ml of $9,000 \times g$ supernatant fluid. At time intervals after lyophilization, re-distilled water was added in the lyophilized samples, then homogenized with a homogenizer and resultant microsomal suspensions were used for the enzyme assays and spectrophotometric measurements.

Assay of aniline hydroxylase activity: Incubations were carried out at 37°C aerobic-

ally for 30 min. Incubation media contained glucose-6-phosphate (20.0 μ moles), NADP (0.4 μ mole), $MgCl_2$ (37.5 μ moles), nicotinamide (75.0 μ moles), aniline (5 μ moles), 1.4 ml of 0.2 M Na-K phosphate buffer (pH 7.4) and 3.0 ml of enzyme preparation in a final volume of 5.0 ml. Aromatic hydroxylation of aniline was determined by measuring *p*-aminophenol according to the method of Kato and Gillette (9).

Measurement of difference spectra of microsomal cyt. P-450: Difference spectra were recorded using a HITACHI recording spectrophotometer, Model EPS-3T, with an integrating sphere accessory. The carbon monoxide-induced difference spectrum of dithionite-reduced microsomes was obtained by the method essentially as described by Omura and Sato (10). Cyt. P-450 and cyt. P-420 were determined from CO difference spectra of dithionite-reduced samples using a value of $91\text{ cm}^{-1}\text{mM}^{-1}$ for the molar extinction increment between 450 and 490 $m\mu$ for cyt. P-450, and a value of $110\text{ cm}^{-1}\text{mM}^{-1}$ between 420 and 490 $m\mu$ for cyt. P-420 (11). Cyt. P-450 difference spectrum induced by aniline was obtained using oxidized microsomes. A 0.2 ml of substrate solution was added to 3.8 ml of the microsomal suspension. With each addition to the sample cuvette, a corresponding volume of water was added to the reference cuvette. Cyt. b_5 content in microsomes was determined as described by Omura and Sato (10). The difference in absorbance between 424 and 409 $m\mu$ was measured, and an extinction coefficient of $185\text{ cm}^{-1}\text{mM}^{-1}$ was used to convert the data from absorbancy to $m\mu$ moles of cyt. b_5 .

RESULTS

1. Effect of storage of lyophilized 9,000 $\times$ g supernatant fraction for 6 months on activity of aniline hydroxylase in the rat

The lyophilization of 9,000 $\times$ g supernatant fraction produced about 20% loss of the activity. During storage of lyophilized samples for 6 months, varying loss of the activity was seen with the conditions. When they were stored in a low pressure of air or in an atmosphere of nitrogen, they did not lose their activity during a 6 months storage, whereas they lost about 20% of their activity by storage in the atmosphere of air. Accordingly, the best result for storage of aniline hydroxylase was obtained if lyophilized 9,000 $\times$ g supernatant fraction was stored anaerobically.

TABLE 1. Effects of six months storage of lyophilized 9,000 $\times$ g supernatant fraction on activity of aniline hydroxylase in rats

	Aniline hydroxylase activity $m\mu$ moles/mg*/hr (range)**	% of control
Fresh 9,000 $\times$ g sup. f (control)	56.9 (61.2, 52.5)	100
After lyophilization	45.2 (45.2, 45.2)	79.4
Lyophilized, stored in N_2	44.2 (43.9, 44.5)	77.7
Lyophilized, stored in pure air	35.2 (35.2, 35.2)	61.9
Lyophilized, stored in low air pressure	49.2 (49.0, 49.4)	86.5

*Microsomal protein

**Range in duplication

2. *Effects of storage of lyophilized microsomes and soluble fraction for 1 week in air on activity of aniline hydroxylase and contents of cyt. P-450 and cyt. b₅ in the rat*

As shown in Table 2, about 20% loss of the activity of aniline hydroxylase was seen with lyophilization. With storage of lyophilized microsomes and soluble fraction for 1 week in air, further loss of the activity of aniline hydroxylase did not occur. Similar effect of lyophilization and storage was observed in content of cyt. P-450, whereas, unexpectedly, content of cyt. b₅ was apparently increased by the storage (Table 3).

TABLE 2. Effects of one week storage of lyophilized microsomes and soluble fraction on the activity of aniline hydroxylase in the rat.

Microsomes	Sol. f.	Activity of aniline hydroxylase m μ mole/mg/hr (range)**	% of control
Fresh (control)	Fresh	40.7 (40.7, 40.7)	100
	Fresh	33.1 (33.1, 33.1)	81.3
Lyophilized	Lyophilized	33.5 (33.8, 33.1)	82.3
	Stored at 8°C	33.5 (34.2, 32.8)	82.3
Lyophilized, stored for	Fresh	34.5 (37.4, 31.5)	84.8
1 week	Lyophilized	32.9 (33.0, 32.7)	80.8
	Stored at 8°C	30.9 (31.5, 30.3)	75.9

* Soluble fraction stored at 8°C aerobically for 1 week without lyophilization.

** Range in duplication

TABLE 3. Effects of storage for 1 week of lyophilized microsomes on the contents of cyt. P-450 and cyt. b₅.

Microsomes	Cyt. P-450 (m μ mole/mg protein)	Cyt. b ₅ (m μ mole/mg protein)
Fresh	0.956	0.324
Lyophilized	0.804	0.303
Lyophilized, stored for 1 week	0.804	0.363

3. *Effects of conditions of storage for 6 months of lyophilized microsomes and soluble fraction on activity of aniline hydroxylase in the rat*

Effects of long term storage (6 months) of microsomes and soluble fraction of rat liver on activity of aniline hydroxylase are shown in Table 4 to Table 7.

The activities just after lyophilization were $84.8 \pm 6.1\%$ (S.E., $n=6$) of those in fresh samples. The activity of aniline hydroxylase appeared to be dependent not only on the activity of microsomal enzyme but also on the enzymes in the soluble fraction. This fact may suggest that enzymes localized in soluble fraction such as glucose-6-phosphate dehydrogenase lose the activities if the soluble fraction is stored in aerobic conditions. Microsomes stored in an atmosphere of low pressure of air or in nitrogen lost relatively small activity of aniline hydroxylase, but about 60 to 70% of the activity was lost when they were kept in an atmosphere of oxygen or of air. Practically most of the activity was lost when lyophilized microsomes were stored with glycerol, ethylene glycol or propylene glycol.

TABLE 4. Effects of storage for 6 months of lyophilized microsomes (in air) and soluble fraction on the activity of aniline hydroxylase.

Microsomes	Sol. fraction	Activity of aniline hydroxylase m μ moles/mg.hr (range)*	% of control
Lyophilized (control)	Fresh	26.7 (26.0, 27.4)	100
	Fresh	9.6 (9.6, 9.6)	36.0
	Lyophilized, stored in N ₂	9.7 (9.7, 9.7)	36.3
Lyophilized, stored for 6 months in pure air	Lyophilized, stored in O ₂	12.4 (12.7, 12.0)	46.4
	Lyophilized, stored in pure air	8.7 (9.0, 8.3)	32.6
	Lyophilized, stored in low air pressure	9.8 (9.9, 9.6)	36.7

*Range in duplication

TABLE 5. Effects of storage for 6 months of lyophilized microsomes (in low pressure of air) and soluble fraction on the activity of aniline hydroxylase.

Microsomes	Sol. fraction	Activity of aniline hydroxylase m μ moles/mg*/hr (range)**	% of control
Lyophilized (control)	Fresh	26.7 (26.0, 27.4)	100
	Fresh	17.8 (17.8, 17.8)	66.7
	Lyophilized, stored in N ₂	15.7 (16.3, 15.0)	58.8
Lyophilized, stored for 6 months in low air pressure	Lyophilized, stored in O ₂	11.4 (11.1, 11.7)	42.7
	Lyophilized, stored in pure air	10.5 (10.5, 10.5)	39.3
	Lyophilized, stored in low air pressure	16.9 (17.1, 16.7)	63.3

*Microsomal protein,

**Range in duplication

TABLE 6. Effects of storage for 6 months of lyophilized microsomes (in N₂) and soluble fraction on the activity of aniline hydroxylase.

Microsomes	Sol. fraction	Activity of aniline hydroxylase m μ moles/mg/hr (range)*	% of control
Lyophilized (control)	Fresh	28.1 (28.1, 28.1)	100
	Fresh	12.5 (12.5, 12.5)	44.5
	Lyophilized, stored in N ₂	16.7 (16.1, 17.3)	59.4
Lyophilized, stored for 6 months in N ₂	Lyophilized, stored in O ₂	14.4 (14.2, 14.5)	51.2
	Lyophilized, stored in pure air	15.6 (15.9, 15.2)	55.5
	Lyophilized, stored in low pressure	18.2 (18.0, 18.3)	64.8

*Range in duplication

TABLE 7. Effects of storage for 6 months of lyophilized microsomes (in O₂) and soluble fraction on the activity of aniline hydroxylase.

Microsomes	Sol. fraction	Activity of aniline hydroxylase m μ mole/mg/hr (range)*	% of control
Lyophilized (control)	Fresh	28.1 (28.1, 28.1)	100
	Fresh	10.8 (10.8, 10.8)	38.4
	Lyophilized, stored in N ₂	9.4 (8.8, 10.0)	33.5
Lyophilized, stored for 6 months in O ₂	Lyophilized, stored in O ₂	7.7 (7.2, 8.2)	27.4
	Lyophilized, stored in pure air	10.2 (10.5, 9.9)	36.3
	Lyophilized, stored in low air pressure	13.2 (12.9, 13.5)	47.0

* Range in duplication

TABLE 8. Effects of six months storage of lyophilized microsomes on the content of cyt. P-450.

Condition of storage of microsomes	P-450 (m μ mole/mg protein)			Stored Lyophilized $\times 100$	P-420 m μ mole/mg protein
	Fresh	Lyophilized	Stored		
in low pressure of air	0.65	0.62	0.57	91.9	—
in N ₂	0.66	0.61	0.59	96.7	—
in O ₂	0.66	0.61	0.47	77.0	—
in air	0.65	0.62	0.40	64.5	—
in low pressure of air + glycerol	0.93	0.84	0.19	22.6	0.47
in N ₂ + glycerol	0.86	0.91	0.04	4.4	0.41
in O ₂ + glycerol	0.86	0.91	0.13	14.3	0.29
in air + glycerol	0.84	0.79	0.11	19.9	0.32

TABLE 9. Effect of storage for 6 months of lyophilized microsomes on the content of cyt. b₅.

Condition of storage of microsomes	Fresh	Lyophilized	Stored	Stored Lyophilized	× 100
	(mμ mole/mg protein)				
in low pressure of air	0.36	0.32	0.25	78.1	
in N ₂	0.34	0.31	0.24	77.4	
in O ₂	0.34	0.31	0.25	80.6	
in pure air	0.36	0.32	0.30	93.8	
in low air pressure + glycerol	0.25	0.27	0.29	107.4	
in N ₂ + glycerol	0.39	0.41	0.20	48.8	
in O ₂ + glycerol	0.39	0.41	0.18	43.9	
in pure air + glycerol	0.27	0.30	0.19	63.3	

TABLE 10. Effect of storage for 6 months of lyophilized microsomes on the aniline-P-450 binding difference spectrum.

Condition of storage of microsomes	Amount bound to cyt. P-450			Stored Lyophilized $\times 100$
	Fresh	Lyophilized	Stored	
in low pressure of air	$1.16 \times 10^{-2*}$	$1.12 \times 10^{-2*}$	$0.77 \times 10^{-2*}$	68.8
in N ₂	1.24	1.20	0.84	70.0
in O ₂	1.24	1.20	0.67	55.8
in pure air	1.16	1.12	0.69	61.6
in low air pressure + glycerol	1.57	1.80	0.60	33.3
in N ₂ + glycerol	1.65	1.62	0.40	24.7
in O ₂ + glycerol	1.65	1.62	0.23	14.2
in pure air + glycerol	0.90	0.82	0.48	58.5

Aniline : 1 mM

*OD peak-trough correlated with baseline/protein concentration.

4. *Effects of various conditions of storage for 6 months of lyophilized microsomes on cyt. P-450 content, cyt. b₅ content and aniline-induced difference spectrum of cyt. P-450*

In microsomes stored in an atmosphere of low pressure of air and in an atmosphere of nitrogen, cyt. P-450, cyt. b₅ and aniline-P-450 binding spectrum tended to be more stably stored than those kept in other conditions. It was found that microsomes stored with glycerol were kept less stably than those stored without glycerol. In the case of glycerol, cyt. P-420 appeared instead of cyt. P-450, and less cyt. b₅ and aniline-P-450 binding spectrum remained.

FIG. 1. Periodical changes in the activity of aniline hydroxylase in lyophilized 9,000 $\times$ g supernatant fraction of phenobarbital treated rabbit liver.

5. *Effects of storage of 9,000×g supernatant fraction on the activity of aniline hydroxylase in the rabbit*

In preliminary experiments, microsomes and soluble fraction from rabbit liver were lyophilized and stored in an atmosphere of air, after which the periodical change in the activity of aniline hydroxylase was observed. The lyophilization produced about 20% loss of activity, but little loss of the activity was observed during storage for 120 days (data not shown). To investigate the stability of aniline hydroxylase in the 9,000×g supernatant fraction, 9,000×g supernatant fraction from phenobarbital treated rabbits was employed, since the enzyme from phenobarbital treated rabbits was assumed to be unstable compared to that from control rabbits (Fig. 1).

Unexpectedly, the activity of aniline hydroxylase in 9,000×g supernatant fraction from phenobarbital treated rabbits was apparently increased by the lyophilization and the activity decreased slowly during storage for 210 days, however, the activity at 210 days was not lower than that before lyophilization. Thus, it can be concluded that drug metabolizing enzymes of rabbit liver are more stable than those of rat liver from the findings presented here and as described by Gram and Fouts (6).

DISCUSSION

In the rat liver, 9,000×g supernatant fraction was more stably stored than microsomes. Recently, the authors found that heat labile factor in the soluble fraction inhibits formation of lipid peroxides of liver microsomes and then stabilizes microsomal N-demethylase of ethylmorphine (12). If the inactivation of aniline hydroxylase is caused by the lipid peroxidation in the microsomes, the stability of 9,000×g supernatant fraction may be depending upon the inhibitory factor of lipid peroxidation in the soluble fraction. In most cases, disappearance of aniline hydroxylase during storage of microsomes was more than that of magnitude of aniline-induced P-450 difference spectrum and cyt. P-450 content. This finding is supported by a finding that activity of ethylmorphine N-demethylase is rapidly lost without significant loss of cyt. P-450 content (at most 10%) when microsomes are aged at 45°C aerobically for 30 min (12). Since activity of aniline hydroxylase was not recovered even when fresh soluble fraction was added, the loss of the activity of aniline hydroxylase during storage might be accompanied either with inactivation of NADPH-cyt.c reductase or with change in membrane structure of endoplasmic reticulum. Recent observations of Wills (13) and the authors (14) that NADPH-linked lipid peroxidation in liver microsomes produced an inactivation of drug metabolizing enzymes, and another one by Gram and Fouts (15) that significant lipid peroxidation was seen in the rat but negligible peroxidation occurred in the rabbit, may suggest that drug metabolizing enzymes in the rabbit liver are more stable than those in the rat. As previously described (14), addition of EDTA, a potent inhibitor of lipid peroxidation (16), prevented inactivation of ethylmorphine N-demethylase which occurs during incubation. Accordingly, the fact that rabbit liver was stably stored is confirmed as being due to lack of lipid peroxidase in rabbit liver, however, the reason why apparent activation of aniline

hydroxylation is observed with the storage of lyophilized $9,000\times g$ supernatant fraction of rabbit liver is yet to be determined.

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