Biochemical Pharmacology, Vol. 23, pp. 2332–2334. Pergamon Press, 1974. Printed in Great Britain.

Stability of suspensions of rat liver microsomes quick-frozen with liquid nitrogen and stored at −85°

(*Received* 4 *September* 1973; *accepted* 18 *January* 1974)

THE NEED to store suspensions of microsomes for short or prolonged time periods is often encountered in the course of normal laboratory work that involves the assay of the drug-metabolizing enzymes of endoplasmic reticulum. This laboratory has examined a method to achieve reliable storage of microsomal suspensions. The purpose of the procedure was to be able to stop assay work when necessary and to freeze the remaining portions of the microsomal suspensions being assayed. This would avoid an ultracentrifugation step to form a pellet of the unused microsomal suspension. Such suspensions could later be unfrozen and assays resumed.

While reports by several previous workers[1–7] indicated the feasibility of such a method, additional information was needed on the effects of long-term storage, at lower temperatures than those previously reported, on both cytochrome P-450 stability and typical enzymic activities. This laboratory has found that quick freezing of microsomal suspensions with liquid nitrogen and subsequent storage at −85° offers a reliable method for storing hepatic microsomal suspensions for periods up to 3–6 weeks.

Sprague–Dawley male rats averaging 350 g in body wt with free access to food and water were sacrificed by cervical dislocation. The livers were immediately removed, washed and placed in ice-cold 1·15% KCl solution. The livers were minced and homogenized in 2 vol. of cold 1·15% KCl solution with a glass tube and Teflon pestle. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°. The supernatant was decanted and centrifuged at 160,000 *g* for 1 hr. The microsomal pellet was washed by resuspension in cold 1·15% KCl solution and recentrifuged in an identical manner. The pellet was resuspended in cold 1·15% KCl solution to a final microsomal suspension volume of 1 ml/g fresh liver weight.

The microsomal suspensions to be frozen were placed in Erlenmeyer flasks and put in about 2 in. of liquid nitrogen for 2–4 min until frozen. The frozen suspensions were then stored in a −85° deep freeze. For assay, frozen microsomes were partially thawed at room temperature and then were placed on ice. Once completely thawed, these samples were resuspended by transfer to tubes and with four gentle strokes of a Teflon pestle.

The following assays were done on the freshly prepared microsomes and on microsomes maintained frozen for 1, 3 or 6 weeks: protein, cytochromes b_5 and P-450, NADPH oxidase, nitroreductase, aniline hydroxylase and *N*-demethylation of ethylmorphine (EM). Each assay included four sets of duplicates for statistical analysis with an Olivetti Underwood Programmer 101 program. The data are expressed as the mean values ± estimated standard deviation of the population mean. Student's *t*-test was used to make statistical comparisons between the fresh and frozen microsomes. When an NADPH generating system was used, the system was mixed together and allowed to remain at room temperature for 15 min. Reactions were started by the addition of the generating system.

Protein concentration of the microsomal fractions was determined by the method of Lowry *et al.*[8] and the cytochrome b_5 and P-450 contents were determined spectrophotometrically at room temperature by the $(\Delta E_{427}–E_{410})$ and the $(\Delta E_{450}–E_{490})$ according to the methods of Smuckler *et al.*[9]

NADPH oxidase was determined by the method of Gillette *et al.*,[10] which measures the rate of disappearance of the reduced pyridine nucleotide by the ΔO.D. for 5 min at 340 nm.

The reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid was based on a modified method of Smith and Van Loon.[11] Incubation mixtures in 10-ml flasks contained 8 mg protein, 1 μmole NADP, 25 μmoles glucose 6-phosphate, 1·2 units glucose 6-phosphate dehydrogenase, 25 μmoles $MgCl_2$, 28 μmoles nicotinamide, 12 μmoles *p*-nitrobenzoic acid and were adjusted to a final volume of 2·85 ml with 0·1 M, pH 7·4 phosphate buffer. The samples were capped with serum vial stoppers, flushed for 2 min with nitrogen gas and then incubated for 20 min at 37° in a shaking waterbath. The reaction was stopped with 2·5 ml of 15% trichloroacetic acid and the mixture was centrifuged at room temperature. Three ml of supernatant was removed and treated as follows: 0·5 ml of 0·2% sodium nitrite was added and, after 3 min, 0·5 ml of 1% ammonium sulfamate was added. Three min later, 0·5 ml of 0·2% *N*-(1-naphthyl) ethylenediamine dihydrochloride was added and, after 15 min, 2 ml of 95% ethanol was added to stabilize the color.

The hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol according to the method of Kato and Gillette.[12] In our assay, 20 μmoles of redistilled aniline was incubated with the reaction mixture for 20 min at 37° in a shaking waterbath. The apparent K_m values for aniline were determined from reciprocal plots of data obtained with the same assay procedure, except that the aniline concentrations were 1·0, 0·10, 0·04 and 0·03 mM.

The *N*-demethylation of ethylmorphine was determined by measuring formaldehyde formation. The incubation mixture consisted of 2 mg protein, 2 μmoles NADP, 50 μmoles glucose 6-phosphate, 2 units glucose 6-phosphate dehydrogenase, 25 μmoles $MgCl_2$, 3 μmoles ethylmorphine and was adjusted to a final volume of 3 ml with 0·1 M, pH 7·4 phosphate buffer. The mixtures were incubated for 10 min at 37° and analyzed for formaldehyde with a modified Nash reagent according to the method of Davis *et al.*[13] The apparent K_m values for ethylmorphine were determined from reciprocal plots of data obtained with the same assay procedure, except that the ethylmorphine concentrations were 1·0, 0·17, 0·08 and 0·06 mM.

The data in Table 1 show that rat liver microsomes quick-frozen in liquid nitrogen can be stored at −85° for 1 week and in most instances for 3 and 6 weeks without significant ($P < 0.005$) losses in content of cytochromes P-450 and b_5, and without loss of enzymic activities when compared to freshly prepared microsomes. Only NADPH oxidase showed a steady and significant drop in activity with increasing storage time. Activity of this enzyme dropped 13 per cent after 1 week of storage, 25 per cent after 2 weeks and 40 per cent after 6 weeks. Thus, NADPH oxidase assays should be done within 1 week after being frozen in this manner. Nitroreductase activity was consistently stable over 6 weeks of storage time.

Cytochromes P-450 and b_5, aniline hydroxylase and EM *N*-demethylase remained stable for 3 weeks, but showed greater than a 10 per cent loss of activity after being frozen 6 weeks. Cytochrome P-450 content decreased 9 per cent in 3 weeks, while EM *N*-demethylase activity decreased 14 per cent after 6 weeks of storage. Aniline hydroxylase activity decreased 11 per cent from the control at 1 week, but did not change further at 3 weeks (−8 per cent) and decreased only slightly more after 6 weeks (−14 per cent). Since the activity after 1 week of storage is nearly the average of 3 and 6 weeks, it can be assumed that the slight decrease occurred at the time of freezing the microsomes.

The apparent K_m values for the drug substrates with aniline hydroxylase and EM *N*-demethylase activities remained the same over the 6-week period, indicating that the affinity of the enzymes for their respective substrates had not changed after being frozen 6 weeks.

Leadbeater and Davies[1] in a similar study used washed microsomes as microsomal suspensions, which were placed in a −40° deep freeze where they froze solid within 1 hr. At their[1] initial observation time of 3–4 days after freezing, 20–25 per cent activity was lost and about 50 per cent loss occurred in 30 days in the *N*-demethylation of codeine, the *N*-demethylation of morphine, the *O*-demethylation of codeine and the hydroxylation of diethyl tryptamine. The data indicated that when microsomal suspensions are frozen at −40° activity is partially lost as a result of a slow freezing rate. However, Leadbeater and Davies[1] found no loss of activity in three of four enzymic reactions over 30 days when the combined microsome plus soluble fraction was stored at −40° either as the suspension or freeze dried. Storage at 0° of the combined suspension resulted in loss of all activity within 7 days.

Hewick and Fouts[5] studied the effect of storage at 1° under nitrogen gas of a washed microsomal suspension prepared from phenobarbital-induced rats and similarly found a significant loss in activity. The

TABLE 1. EFFECTS OF FREEZING MICROSOMAL SUSPENSIONS WITH LIQUID NITROGEN AND STORAGE AT −85° ON CYTOCHROMES b_5 AND P-450 CONTENT AND ENZYMIC ACTIVITY OF RAT LIVER MICROSOMES

Weeks frozen (−85°)	Microsomal protein (mg/g liver)	Microsomal cytochromes (nmoles/mg protein) P-450	b_5	Apparent K_m (mM) Aniline hydroxylase	EM *N*-demethylase
Control (fresh)	17·1 ± 0·4	0·59 ± 0·01	0·88 ± 0·02	0·04 ± 0·01	0·13 ± 0·03
1	16·2 ± 0·4	0·66 ± 0·03	0·87 ± 0·01	0·04*	0·13*
3	17·0 ± 0·1	0·63 ± 0·02	0·87 ± 0·01	0·04*	0·12*
6	17·1 ± 0·4	0·54 ± 0·01†	0·77 ± 0·00†	0·04*	0·14*

	NADPH oxidase	Aniline hydroxylase	Ethylmorphine *N*-demethylase	Nitroreductase
	(nmoles product/min/mg microsomal protein)			
Control (fresh)	4·0 ± 0·3	0·37 ± 0·01	8·8 ± 0·3	0·55 ± 0·08
1	3·5 ± 0·1	0·33 ± 0·01	9·0 ± 0·3	0·59 ± 0·04
3	3·0 ± 0·0†	0·34 ± 0·07	8·6 ± 0·6	0·53 ± 0·01
6	2·4 ± 0·3†	0·32 ± 0·03	7·6 ± 0·9	0·58 ± 0·04

* Data are averaged duplicates.

† Significant difference at $P < 0.005$ for the comparison of 1, 3 and 6 week frozen microsomes with fresh microsomes.

N-demethylation of (+) benzphetamine decreased 70 per cent and the *p*-hydroxylation of aniline decreased 50 per cent in 96 hr of storage. However, cytochrome b_5 increased about 10 per cent, while cytochrome P-450 decreased 10 per cent in 96 hr. Levin *et al.*[3] suggest that when liver microsomal preparations must be stored for longer than 1 day the microsomes should be stored as a pellet at $-15°$ rather than as a suspension or as a lyophilized powder. Leber and Bousquet[6] found that an acetone powder preparation of rat hepatic microsomes was active in the oxidative metabolism of hexobarbital, aminopyrine, *p*-nitroanisole and the reductive metabolism of *p*-nitrobenzoic acid. Kuntzman *et al.*[2] reported the effects of storage on hepatic microsomes of excised whole liver which was frozen overnight.[2] Jondorf and Donahue[4] reported that the decline of microsomal *N*-demethylation of aminopyrine can be prevented when the rat carcass or excised livers are stored at 0–4° and assayed within 6 hr. Wade *et al.*[7] observed that rat and rabbit microsomal cytochrome P-450 is more stable when stored as the microsomal pellet at $-15°$ than when stored either as the pellet at 0° or as a suspension at $-15°$ or 0°. While commenting that sample storage at $-15°$ should allow maintenance of microsomes for at least 8 days after re-ultracentrifugation, without significant loss of cytochrome P-450 content, these authors did not determine the possible changes in enzymic activity of microsomes that may have occurred during storage.

Data presented in this paper indicate that microsomal suspensions, when quick-frozen with liquid nitrogen and stored at $-85°$, do not display loss of stability as reported by other workers. No significant losses of enzymic activities were observed at the end of a 1-week storage when compared to those of fresh microsomes, except a gradual decrease in NADPH oxidase activity.

Acknowledgement—The authors gratefully acknowledge that this investigation was supported by United States Public Health Service Grant CA-034808 from the National Cancer Institute.

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Biochemical Pharmacology, Vol. 23, pp. 2234–2337. Pergamon Press, 1974. Printed in Great Britain.

Activation of ethanol metabolism by 2,4-dinitrophenol in the isolated perfused rat liver

(*Received* 29 *October* 1973; *accepted* 21 *January* 1974)

IT HAS PREVIOUSLY been proposed that the mitochondrial reoxidation of NADH normally constitutes the rate-limiting step of alcohol metabolism in the liver, because uncoupling agents are able to increase the rate of alcohol metabolism in liver slices[1] and in the intact animal.[2,3] It can be argued, however, that