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EFFECT OF STORAGE ON MICROSOMAL MIXED FUNCTION OXIDASE ACTIVITY IN MOUSE LIVER

CHARLES L. LITTERST, EDWARD G. MIMNAUGH, REGINALD L. REAGAN and THEODORE E. GRAM

Laboratory of Toxicology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014, U.S.A.

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Abstract—The stability of mixed function oxidase components from hepatic microsomes of mice was studied. Microsomal pellets were overlaid with isotonic, buffered KCl and frozen at −20° for up to 20 days. Ethyl morphine *N*-demethylase and aniline hydroxylase were the least stable, showing significant decreases in activity after 3 and 5 days of storage respectively. NADPH cytochrome *c* reductase and cytochrome P-450 were both stable for at least 10 days. Electron micrographs of resuspended pellets after storage showed an increasing tendency toward aggregation of microsomal vesicles as the length of storage increased.

IT HAS been shown that storage of liver microsomes under various conditions for various periods brings about decreases in the amounts of electron transfer components such as cytochrome P-450 and cytochrome b_5 and decreases in the activity of the associated drug-metabolizing enzymes. In general, it appears as if the rate and extent of loss of activity are dependent upon the specific storage conditions and also upon the component or substrate studied. Thus Leadbeater and Davies[1] showed that stability of frozen microsomal preparations differed, depending on whether the microsomes were frozen as a suspension, as a pellet overlaid with buffer, or as a lyophilized powder, with stability decreasing in the order listed. Hewick and Fouts[2] demonstrated that the type I spectral change in rat liver microsomes was more labile than the type II binding spectra during 96 hr of storage. In addition, Leadbeater and Davies[1] have shown no loss of codeine *N*-demethylase activity for up to 30 days, while the codeine *O*-demethylase activity decayed rapidly in a biphasic way. This instability of microsomal mixed function oxidase (MFO) activity has been shown primarily in rat and rabbit, although one report utilizing the hamster has also appeared.[3] No information is available for the mouse, however, a species utilized widely in the chemotherapy program of the National Cancer Institute. Therefore, we have conducted preliminary experiments designed to elucidate the effect of storage on the activity of the microsomal MFO system and its components from the livers of mice.

METHODS

Enzyme preparation. Livers from male CDF_1 mice were pooled prior to homogenization in 2 vol. of KCl–Tris buffer (0·15 M KCl + 0·05 M Tris–HCl, pH 7·4). Homogenization was conducted in a Potter-type homogenizer with a motor-driven Teflon pestle. The homogenate was diluted to contain 250 mg liver/ml and centrifuged at

9000 *g* for 20 min in a refrigerated Sorvall centrifuge. The resulting floating fat layer was carefully removed and discarded and the supernatant drawn off with a syringe. Microsomes were sedimented by centrifuging the 9000 *g* supernatant for 60 min at 105,000 *g* in a refrigerated Beckman L3-50 ultracentrifuge. After aspirating and discarding the 105,000 *g* supernatant, the microsomal pellet was overlaid with KCl–Tris buffer and placed in a −20° freezer for 1, 3, 5, 7, 10 or 20 days. The samples were thawed on ice and resuspended using a Teflon–glass Potter–Elvehjem homogenizer. Fresh or frozen microsomal pellets were resuspended by hand using 11 full strokes of the pestle.

Analytical procedures. Microsomal protein was estimated as described by Lowry *et al.*[4] Microsomal cytochrome P-450 content was determined by a dithionite difference spectrum[5] at a protein concentration of 3 mg/ml in a Shimadzu MPS-50L multipurpose recording spectrophotometer.

The activity of NADPH cytochrome *c* reductase was determined at 37° by measuring the rate of appearance of reduced cytochrome *c*[6] on a Gilford model 300N spectrophotometer equipped with a model 4008 digital data lister. The incubation mixture contained cytochrome *c* (0·05 mM), NADPH (0·9 mM), microsomal protein (0·2 mg), and KCN (1 mM) in a total vol. of 3 ml. An extinction coefficient of 18·7 cm^{-1} mM^{-1} was used.

Ethylmorphine *N*-demethylase and aniline 4-hydroxylase activities were determined after aerobic incubation at 37° in a Dubnoff metabolic shaker. Incubation mixtures consisted of an NADPH generating system composed of NADP (1 mM), glucose 6-phosphate (10 mM), $MgCl_2$ (5 mM), 2 units glucose 6-phosphate dehydrogenase, Tris–HCl buffer (50 mM, pH 7·4), microsomal protein (1 mg/ml), and either ethylmorphine (10 mM) or aniline hydrochloride (5 mM) in a final vol. of 3 ml. After

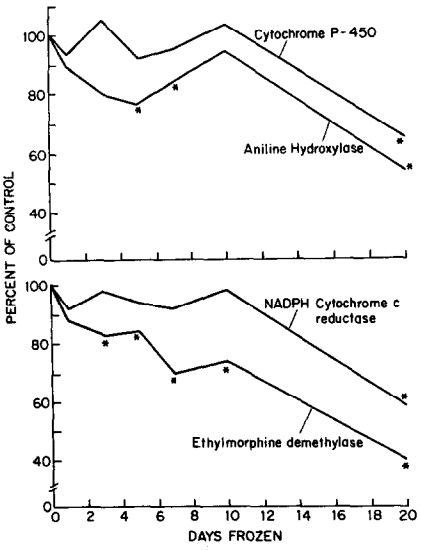

FIG. 1. Effect of increasing length of storage on presence and activity of the microsomal drug-metabolizing enzyme system from mouse liver. Data are presented as per cent of control and starred points represent values significantly different from control values ($P \leq 0{\cdot}05$). Control values were: cytochrome P-450, 0·105 ± 0·008 ΔO.D./mg; NADPH cytochrome *c* reductase, 0·111 ± 0·015 nmole/min/mg; ethylmorphine *N*-demethylase, 9·84 ± 1·00 nmoles/min/mg; aniline hydroxylase, 1·29 ± 0·15 nmoles/min/mg.

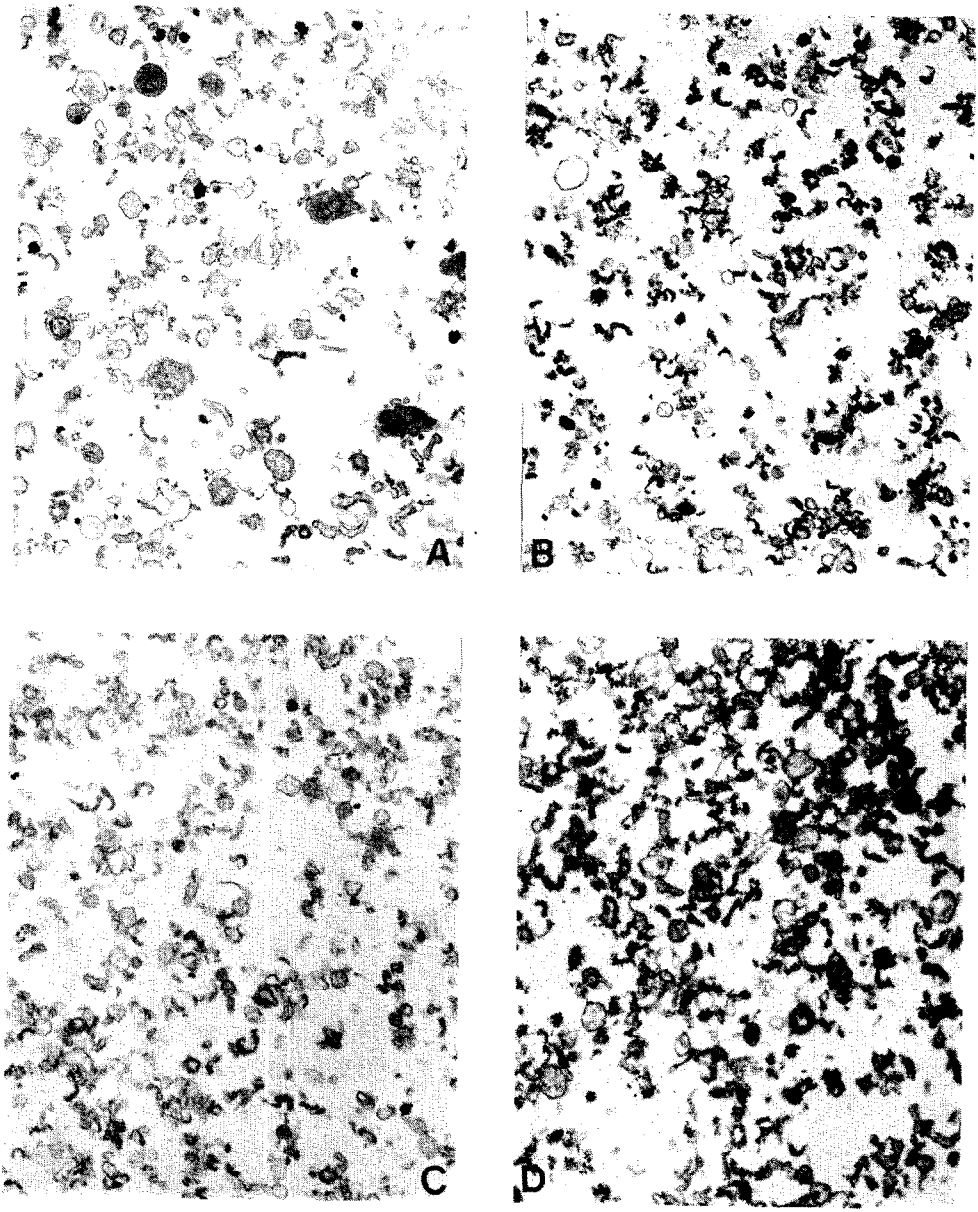

FIG. 2. Electron micrographs (× 25,000) of resuspended mouse liver microsomes after 0 (A), 1 (B), 3 (C) and 5 (D) days of storage at −20°.

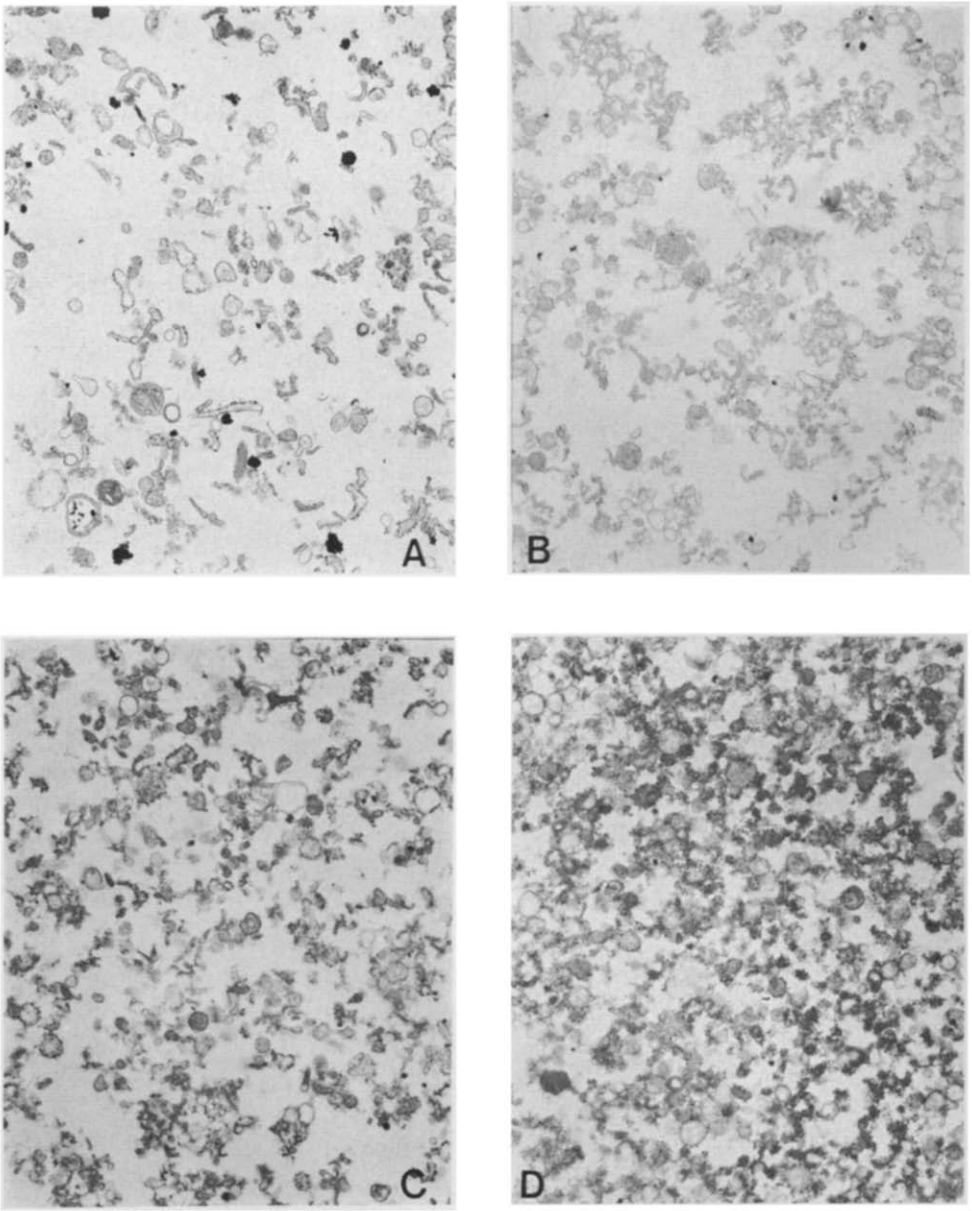

FIG. 3. Electron micrographs (× 25,000) of resuspended mouse liver microsomes after 0 (A), 7 (B), 10 (C) and 20 (D) days of storage at −20°. Note increasing extent of aggregation of microsomal vesicles, particularly in plates C and D.

incubation for 15 min, ethylmorphine demethylase activity was determined by assay of formaldehyde using the Nash reaction as described by Cochin and Axelrod.[7] Aniline 4-hydroxylation was estimated by the amount of *p*-aminophenol formed by the method of Imai *et al.*[8]

Electron microscopy. For electron microscopy, aliquots of the microsomal suspensions were fixed in ice-cold Dalton's Chrome Osmium solution[9] for 3 hr at 0–4°. The samples were centrifuged for 20 min at 2500 rev/min and then placed in a refrigerator for 48 hr. The samples were then allowed to stand overnight in 10% formalin containing 0·25% uranyl acetate prior to dehydration in graded solutions of ethyl alcohol and propylene oxide. The tissue was embedded in Epon-Araldite after being in Epon-Araldite–propylene oxide (1:1) for 16 hr. Thin sections were cut with a diamond knife and an LKB ultramicrotome, placed on Collodion-carbon coated copper grids and stained with uranyl acetate and lead citrate for 15 min.[10] Sections were examined with a JEOL JEM-100B electron microscope at an accelerating voltage of 80 kV and an objective aperture of 50 μm.

RESULTS

The effect of storage on the activity of some components of the hepatic microsomal MFO system is shown in Fig. 1. It can be seen that all parameters studied are stable for at least 24 hr and that only ethylmorphine demethylase was significantly decreased after 72 hr of storage. Microsomal protein content after storage was comparable at all time points with freshly prepared microsomes. Figures 2 and 3 show electron micrographs of microsomal pellets at the various times of storage and show a progressive increase in the amount of microsomal clumping.

DISCUSSION

Little work has been done to define the stability of the mixed function oxidase components in the mouse and similar reports utilizing other species are rare. Hewick and Fouts[2] showed that rat liver microsomes lose up to 30 per cent of their cytochrome P-450 content after 90 hr of storage at 1° under nitrogen. By contrast, our results indicate no significant loss of cytochrome P-450 from mouse liver microsomes after at least 10 days of storage and these findings are in accord with those of Burke and Bridges,[3] who demonstrated the almost total stability of cytochrome P-450 in hamster liver microsomes after 72 hr of storage at either 2° or 20°. In addition, Wade *et al.*[11] have shown that cytochrome P-450 from phenobarbital-induced rats is stable for up to 8 days at 15°. NADPH cytochrome c reductase showed a stability similar to that of cytochrome P-450 with little loss in activity after 10 days of storage.

Bauer and Kiese[12] showed that the ability of rabbit liver microsomes to hydroxylate aniline declined by a maximum of 30 per cent after 2 weeks of storage at 2°. Our results show mouse liver microsomal aniline hydroxylase activity to be less stable than that of rabbit liver as reported by Bauer and Kiese, with a decrease of about 20 per cent after 1 week and 50 per cent after 20 days. The only report of ethylmorphine *N*-demethylase activity for other species is that of Levin *et al.*[13] Although in their work the mean of two determinations showed no change after 20 days of storage as a pellet, the two individual values for both control and frozen samples were greatly different and make it difficult to accurately evaluate their data. With the

mouse system utilized in the present study, ethylmorphine *N*-demethylase activity was the least stable of our parameters; a 15 per cent decline in activity was observed by day 3 and a small change noticed after 24 hr of storage.

The importance of the physical conditions during storage has been reported by Levin *et al.*[13] and by Burke and Bridges.[3] These authors both showed that the microsomal pellet overlaid with buffer maintained considerably more activity than was true if the microsomes were resuspended in buffer and then frozen. Our present results confirm the stability of several microsomal components and drug-metabolizing enzyme activity when stored as a pellet.

The electron microscope data show an increased aggregation of microsomal vesicles with increasing time of storage. Upon resuspension, this aggregation was evident as an obvious and in some cases rapid formation of sediment in the bottom of the tubes containing the resuspension. Such sediments are not seen with resuspensions of freshly prepared microsomes. Even though the degree of aggregation seen in Figs. 2 and 3 appears to correlate well with the decline in enzyme activity, previous work has shown that microsomal vesicles can aggregate independently of a decrease in enzyme activity,[14] and it has also been shown that sucrose solutions of high molarity will clump microsomal membranes without producing a concomitant decrease in enzyme activity.[15]

The results presented in this paper indicate that the activity of the microsomal mixed function oxidase system isolated from livers of mice has satisfactory stability when frozen and suggest the usefulness of freezing these preparations for later use.

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