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ALDRIN EPOXIDASE ACTIVITY OF RAT LIVER AND RAT LIVER MICROSOMES UNDER VARIOUS CONDITIONS OF STORAGE*

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Abstract—The stability of rat hepatic microsomal mixed function oxidation systems stored under various conditions was assessed by a measurement of aldrin epoxidase, NADPH cytochrome *c* reductase, NADPH neotetrazolium reductase activities and cytochrome P-450 levels. Male rat livers stored at −20° for 30 days showed little loss of the two NADPH-dependent microsomal reductase activities and cytochrome P-450. Microsomal suspensions from these livers showed higher protein content, increased aldrin epoxidase activity, decreased endogenous as well as NADPH-dependent lipid peroxidation and higher stability during storage at 4°. The microsomal epoxidase systems of liver were less stable when stored as microsomal pellets than as whole liver at either −10° or −20°.

Enzymes in microsomal suspensions prepared from fresh liver were unstable at 4°, but retained full activity if the suspensions contained 10^{-2} M KCN or were prepared from prefrozen livers.

These results indicate that freezing of the liver causes a structural change in microsomes resulting in an increase in epoxidase activity.

ATTEMPTS to improve the storage stability of microsomal enzyme systems have been reported by several laboratories. Leadbeater and Davis[1] found that the enzymes involved in the *N*-demethylation of codeine and morphine retained full activity after 30 days storage of microsomal preparations at −40°. Under the same conditions, however, the enzymes for *O*-demethylation of codeine were unstable. Another report[2] states that codeine and aminopyrine demethylase activity decreases to less than 20 per cent after 24 hr storage of microsomes at 0–2°. Freeze-dried, washed microsomes suffered substantial losses of morphine *N*-demethylase and ethyl-tryptamine hydroxylase activity after 2 weeks storage at −40°. In contrast to this, lyopholized microsome suspensions were found to have "appreciable anthranilic acid hydroxylating activity" after several weeks storage at −20°.[3] Schonbrod and Terriere[4] stabilized naphthalene hydroxylase of house fly microsomes by storage in dilute suspension in Tris buffer at 0°.

A 17 per cent increase in 3,4-benzpyrene hydroxylase activity was noted by Kuntzman *et al.*[5] when rat livers were frozen overnight. The freezing of microsomal pellets improved the storage stability of carbaryl-metabolizing systems[6] and stabilized androgen hydroxylation[7] while frozen microsomal suspensions remained active in the desaturation of fatty acids for an unspecified period.[8]

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These reports show that the microsomal enzymes have rather specific storage requirements and that studies in which the quantitative aspects of microsomal metabolism are involved should be preceded by a study of the stability of the enzymes. In the work reported here we have made such a study of the microsomal enzymes which convert the insecticide aldrin to its epoxide, dieldrin.

METHODS

Male rats (8–12-week-old) of the Oregon State Wistar strain were anesthetized with ether and sacrificed. Livers were immediately removed, rinsed thoroughly with cold isotonic KCl and chilled. In storage experiments, several livers were diced and mixed thoroughly and divided into equal portions. Samples to be frozen were placed in 20-ml screw cap vials and stored immediately at the desired temperature. At the end of storage, frozen samples were thawed for 15 min at 0° before homogenization. Procedure for isolation of microsomes has been described.[9] Control microsomes from fresh, unfrozen liver were prepared in each experiment. Microsomal pellets which were to be frozen were rinsed briefly with cold 0·15 M KCl, drained carefully and stored in 15-ml centrifuge tubes covered with parafilm. At the end of the storage period the pellets were resuspended in 0·05 M Tris buffer, pH 8·0, so that 1 ml of suspension was equivalent to 1 g of liver. This pH is optimum for microsomal aldrin epoxidase.[9] The protein content of the resuspension was determined by the biuret method.[10]

Assay of aldrin epoxidase. Aldrin epoxidation by microsomes was determined by incubation of 0·05 ml of the microsomal suspension which contained from 14 to 20 mg of protein per ml, with aldrin, in the presence of an NADPH-generating system and oxygen.[9] Activity was determined by the formation of aldrin's 6,7-epoxide, dieldrin, which was measured by gas chromatography.

Assay of microsomal components. The reduction of neotetrazolum chloride (NT) and cytochrome *c* in the presence of NADPH,[11] and the level of cytochrome P-450-CO complex[12] was measured spectrophotometrically in a Cary spectrophotometer, model 11, with cuvettes of 1-cm optical path.

Determination of lipid peroxidation. Microsomal lipid peroxidation was measured by thiobarbiturate (TBA) chromogen formation. Incubations were performed at 30° for 5 min according to the method of Lewis *et al.*[13] The diene conjugate method[14] was also used as a measure of peroxidation. Microsomal suspensions were extracted with 10 vol. of a chloroform:methanol mixture (2:1) and the extract was evaporated to dryness under nitrogen. The recovered lipid material was redissolved in redistilled methanol. The absorption of the solution at 233 mμ was measured in a Beckman DU spectrophotometer or in a Cary model 11 spectrophotometer.

In studying the effect of the extracted lipid material on aldrin epoxidation by fresh microsomes, an aliquot of the methanol solution was transferred to a 50-ml Erlenmeyer flask and evaporated under nitrogen prior to the addition of the incubation mixture.

RESULTS

Temperature of storage and aldrin epoxidase activity. Aldrin epoxidase prepared from livers stored 28 days at −20° was as active as the enzyme from fresh liver, but storage at −10° resulted in a 60 per cent loss of epoxidase activity (Table 1). Enzyme activities

in Table 1 are based on microsomal protein and on liver weight. The latter is preferred because of the effect of storage on the yield of sedimented protein, as will be discussed later.

Another effect of freezing on the enzyme activity of stored livers was noted in experiments in which subsamples of pooled livers were assayed at intervals over a

TABLE 1. STABILITY OF MICROSOMAL ALDRIN EPOXIDASE IN LIVERS STORED AT SUBZERO TEMPERATURES*

Storage conditions	Activity/mg protein†	Activity/g liver†
No storage‡	6·80 ± 0·69	119·0 ± 8·5
−10°	2·85 ± 1·24	60·5 ± 7·3
−20°	7·16 ± 0·51	123·1 ± 6·6

* Livers from four male rats were diced, pooled, divided into equal portions and stored at indicated temperatures for 28 days.

† mμmoles Dieldrin produced in 15 min. Each value is mean of 4 separate assays.

‡ Microsomes prepared from fresh, unfrozen portions of the pooled livers.

5-week storage period (Fig. 1). For 3 weeks both storage conditions resulted in microsomes of greater activity than those prepared from fresh liver. Factorial analysis of variance showed a significant time–temperature interaction ($P < 0{\cdot}05$). However, the activating effect of temperature was more pronounced, especially at the lower temperature (−20°). The activating effect of freeze-storage was seen in another experiment in which aldrin epoxidase, NADPH cytochrome *c* reductase, NADPH NT reductase activities, and the cytochrome P-450 level were assayed at intervals over a 45-day storage period (Fig. 2). In this case there was a slight increase in P-450 levels during the first 2 weeks while epoxidase activity was almost double the original value on the tenth day.

When microsomes prepared from freshly excised livers were stored at −10° and −20° (Fig. 3), epoxidase activity patterns were somewhat different than those seen when liver tissue was stored. The higher storage temperature appeared to be more favorable, although the increase in enzyme activity was not as pronounced as in the previous experiment. Pellets stored at −20° showed a gradual decline in activity, while the −10° pellets retained nearly full activity for 20 days. The aldrin epoxidase system of pellets and livers was also stable after 24 hr storage at −72°. The enzyme was not assayed at longer intervals.

Stability of microsomal suspensions. Freezing also retards the loss of epoxidase activity by microsomal suspensions prepared from frozen livers. The retention of epoxidase activity by suspensions of microsomes previously stored as pellets and those prepared from frozen and fresh liver is shown in Fig. 4. Microsomal suspensions prepared from nonfrozen livers and stored at 4° lost their aldrin epoxidase activity much faster than those from frozen livers. Aldrin epoxidase from frozen pellets was also more stable than that of fresh liver. Assays of NADPH cytochrome c reductase, NADPH NT reductase, cytochrome P-450, and aldrin epoxidase (Fig. 5) showed that all four are considerably more stable in microsomes from frozen livers than those from fresh liver. It can be seen that aldrin epoxidase is the least stable of the four components.

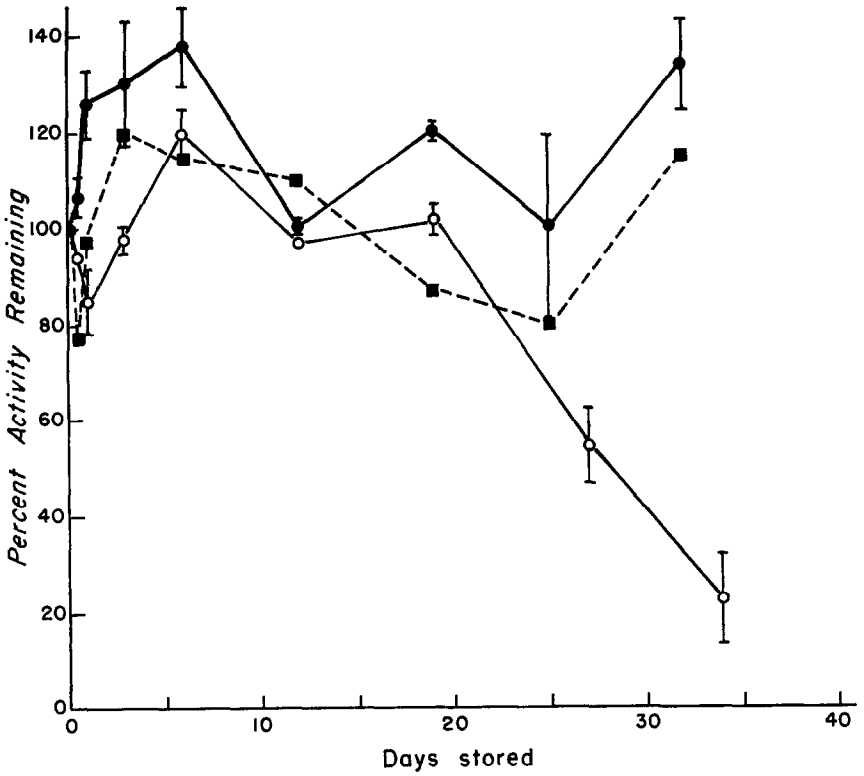

FIG. 1. Stability of aldrin epoxidase in rat liver stored at −10° and −20°. Results are expressed as per cent of the specific activity of microsomes prepared from fresh, nonfrozen samples and are based on liver weight, except as indicated. Each plot is the average of two experiments using a pool of six livers. Each point represents four assays with the range shown by the vertical lines. Least significant difference = 25·11 ($P < 0·05$). Liver stored at −20°, ●—●; liver stored at −10°, ○—○; liver stored at −20° but activity based on microsomal protein, ■—■.

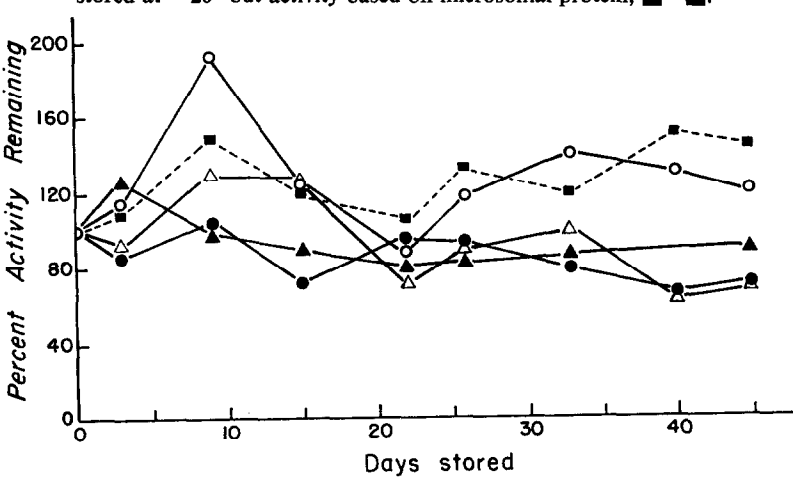

FIG. 2. Stability of microsomal components in rat liver stored at −20°. Results of one experiment with pooled livers of four animals; each assay in duplicate. Results expressed on liver weight basis. Aldrin epoxidase, ○—○; cytochrome P-450, △—△; NADPH NT reductase, ●—●; NADPH cytochrome *c* reductase, ▲—▲; microsomal protein per cent of 0 dry value, ■—■.

Role of endogenous inhibitors. Inhibitors or destructive enzymes in fresh liver could have caused the loss of aldrin epoxidase activity seen in Fig. 5. This possibility was investigated by measuring epoxidase activities in 1:1 mixtures of freshly prepared microsomes (from frozen livers) and aged microsomes (prepared from fresh livers but stored 12 days at 4°). There was no reduction in epoxidase activity or stability in the

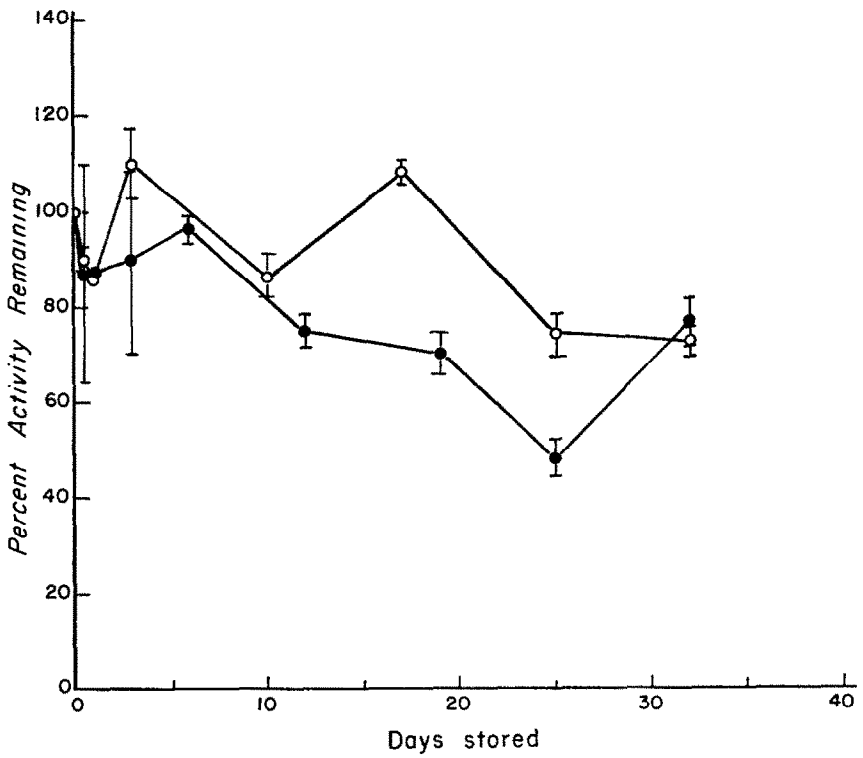

FIG. 3. Stability of aldrin epoxidase in microsomal pellets stored at −10° and −20°. Microsomes were isolated from fresh, nonfrozen livers. Results are expressed as per cent of specific activity of fresh microsomes from the same batch and are based on liver weight. Each plot is the average of two experiments using a pool of six livers. Each point represents four assays with the range shown by the vertical lines. Least significant difference = 14·54 ($P < 0·05$). Pellets stored at −10°, ○—○; pellets stored at −20°, ●—●.

mixture. In another experiment, aged microsomes were extracted with 2:1 chloroform: methanol and the extracted substance incubated with fresh liver microsomes. Only after 20 days storage at 4° did the aged microsomes contain extractives which inhibited the epoxidase system. In this case there was a 50–80 per cent inhibition of the enzyme in fresh microsomes equivalent to approximately 0·07 g liver by an extract of aged microsomes equivalent to 2 g of liver.

The substantial loss of epoxidase activity by microsomal suspensions prepared from fresh liver could be reduced if the buffered suspensions contained 10^{-2} M cyanide (Fig. 6). This preparation had 50 per cent of its original activity after 2 weeks storage,

while microsomes from perfused or control livers, suspended in buffer only, lost more than 40 per cent of their activity in 24 hr. Microsomes from prefrozen liver were again more stable than those from fresh liver.

Role of lipid peroxidation. Slater[15] has reported that during the early stage of induction *in vivo* of lipid peroxidation by CCl_4 there was no decrease in NADPH-dependent microsomal enzymes. However, the inhibition of lipid peroxidation in

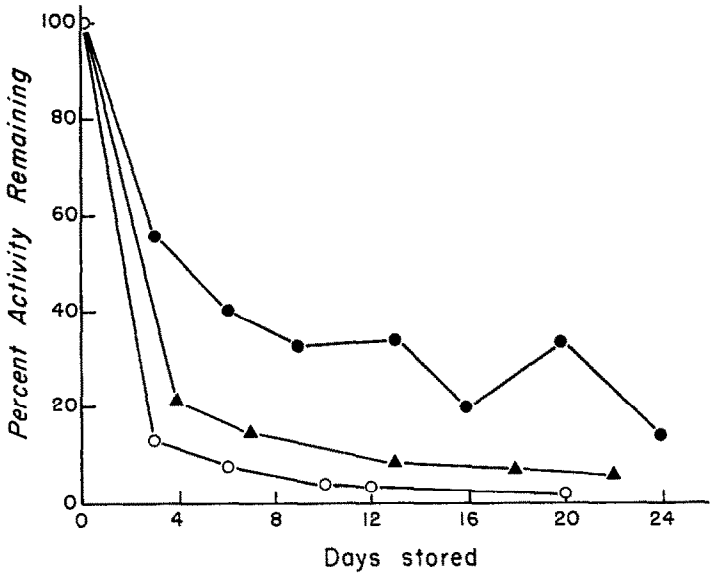

FIG. 4. Stability of aldrin epoxidase in microsomal suspensions stored at 4°. Each plot based on duplicate assays from a pool of four livers; results expressed on liver weight basis. Microsomes from nonfrozen liver, ○—○; microsomes from frozen pellets previously stored at −20° for 12 days, ▲—▲; microsomes from livers previously stored at −20° for 12 days, ●—●.

microsomes has been found to stimulate aldrin epoxidation.[13] Microsomal lipid peroxidation has been found to be catalyzed by free Fe(II) and possibly by protein-bound iron.[16] EDTA and α,α′-dipyridyl, which probably inhibit lipid peroxidation by chelating the iron, increased dieldrin production.[13] Furthermore, the damage of membranes,[17] proteins, enzymes and amino acids[18] by peroxidation is well known.

These reports and the similarity of freezing and cyanide effects in the present study (Fig. 6) suggested an explanation for the effect of prefreezing of liver on the storage stability of the epoxidase. Microsomes from frozen and nonfrozen livers were assayed for peroxidation immediately after preparation and after 3 days storage using the TBA and diene conjugate methods described earlier. Results are summarized in Table 2. Substantial quantities of the TBA-reacting substances were found in the microsomes from fresh livers after 3 days storage at 4° and these increased on incubation with NADPH. TBA-reactive compounds were about one-tenth as great in the microsomes from frozen livers. Diene conjugation was also higher in the microsomes from fresh liver.

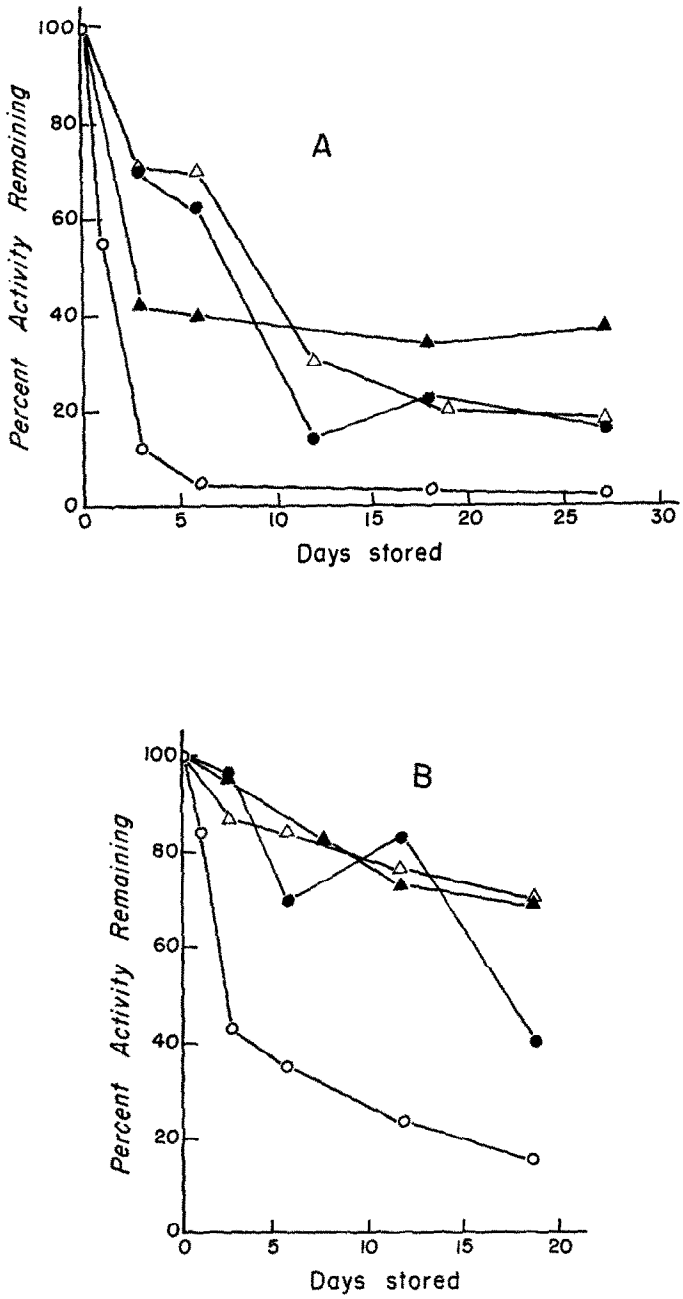

FIG. 5. Stability of microsomal components in microsomes stored as suspensions at 4°. A, microsomes from nonfrozen liver; B, microsomes from livers previously stored for 12 days at −20°. Each plot based on duplicate assays from a pool of four livers; results expressed on liver weight basis. Aldrin epoxidase, ○—○; cytochrome P-450, ▲—▲; NADPH NT reductase, ●—●; NADPH cytochrome *c* reductase, △—△.

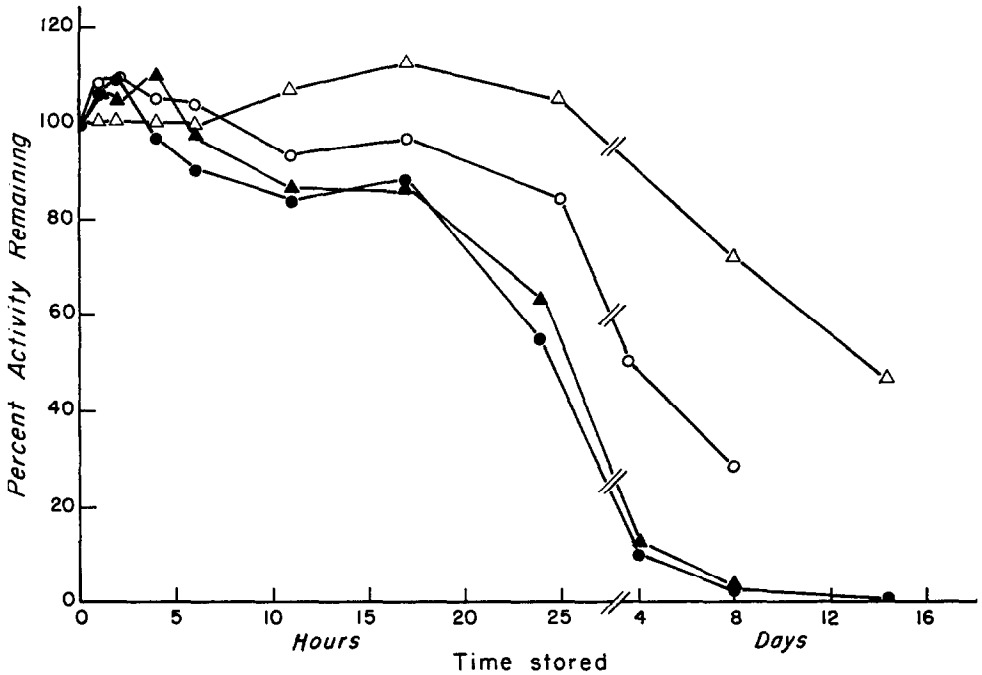

FIG. 6. Effect of prefreezing, perfusion, or cyanide on the stability of aldrin epoxidase in microsomal suspensions stored at 4°. Microsomes were suspended in 0·05 M Tris buffer, pH 8·0, unless otherwise stated. Each plot based on duplicate assays from a pool of four livers; results expressed on liver weight basis. Microsomes from nonfrozen livers, ●—●; microsomes from livers perfused with cold, isotonic KCl, ▲—▲; microsomes from livers frozen at −20° for 24 hr, ○—○; microsomes from nonfrozen livers suspended in 0·05 M Tris buffer, pH 8·0, containing 10^{-2} M cyanide, △—△.

TABLE 2. LIPID PEROXIDATION IN RAT LIVER MICROSOMES AS INDICATED BY TBA REACTION AND BY DIENE CONJUGATE ABSORPTION*

Source of microsomes	Days stored at 4°	Incubation mixture, in the order of addition	TBA reaction†	Diene conjugate‡ absorption
Fresh livers	0	NADPH + TCA§ + ms‖	0	—
	0	NADPH + ms	0·16	—
	3	NADPH + TCA + ms	0·18	—
	3	NADPH + ms	0·25	—
	3	—	—	3·85
Livers at −20° for 30 days	0	NADPH + TCA + ms	0	—
	0	NADPH + ms	0·02	—
	3	NADPH + TCA + ms	0	—
	3	NADPH + ms	0·02	—
	3	—	—	1·15

* Microsomes equivalent to 0·2 g liver were assayed for TBA chromagen by the method of Lewis *et al.*[13] A $CHCl_3$:MeOH (2:1) extract of microsomes equivalent to 2 g liver was assayed for diene conjugates by the method of Recknagel and Ghoshal.[14]

† O.D./min/g liver (535 mμ).

‡ O.D./min/g liver (233 mμ).

§ Trichloroacetic acid.

‖ Microsomes.

DISCUSSION

The results of the experiments described here indicate that the enzymes involved in the epoxidation of aldrin are subjected to both activating and inactivating processes during storage. Freezing of the intact organ favors increased epoxidase activity while storage after fragmentation of the hepatic cell has the opposite effect, especially when storage is at prefreezing temperatures. Furthermore, the improved storage stability, which is the net effect after both activating and inactivating processes, is greater at $-20°$ than at $-10°$.

Activation of aldrin epoxidase at subzero temperatures was accompanied by greater yields of microsomal protein per unit weight of fresh liver (Fig. 2). This suggests some physical change in tissue structure so that a more complete fragmentation was achieved during grinding and centrifugation. The increased activity of the epoxidase system was evident, whether calculated on the basis of microsomal protein or liver weight, although the latter resulted in a greater increase (Fig. 1). It, thus, appears that at least part of the additional protein was involved in the increased activity. Three explanations can be offered. One is that components required for microsomal activity were released from other cell constituents, such as the mitochondria. Another likelihood is that freezing of hepatic tissue altered the microsomal structure in such a way as to reveal additional active sites. Lastly, physical changes in liver tissues caused by freezing may have resulted in a higher recovery of active microsomes. While structural changes due to freezing are a plausible explanation of the increased activity, such changes apparently did not result in increased activities of NADPH cytochrome *c* reductase or NADPH NT reductase or increased levels of cytochrome P-450 (Fig. 2).

In addition to aldrin epoxidase stimulation, freezing of livers at $-20°$ decreases the rate of deterioration of microsomes prepared from frozen livers and stored as suspension at 4° (Fig. 4). This treatment also retards lipid peroxidation (Table 2), However, microsomes prepared from fresh liver and mixed, after several days storage with active microsomes prepared from frozen livers did not inhibit aldrin epoxidase activity in the latter and did not increase their rate of deterioration. These results indicate that the deleterious effect of lipid peroxidation on microsomal enzymes which occurs during storage results more from free radical intermediates from peroxidizing lipids[18, 19] than from the accumulation of peroxides. Otherwise, peroxides accumulated during storage would have inhibited the aldrin epoxidase reaction.

Utley *et al.*[16] have observed that increased availability of microsomal Fe_x occurring after heating of microsomes enhanced peroxidation. This suggests an explanation for the lower levels of peroxidation after freezing. These iron-containing sites may become stabilized and not available as catalysts for peroxidation.

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