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# The Effect of Diethanolamine on Hepatic and Renal Phospholipid Metabolism in the Rat[1]

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The Effect of Diethanolamine on Hepatic and Renal Phospholipid Metabolism in the Rat. BARBEE, S. J., AND HARTUNG, R. (1979). *Toxicol. Appl. Pharmacol.* **47**, 421–430. DEA inhibited the *in vitro* synthesis of both phosphatidyl choline and phosphatidyl ethanolamine in liver tissue. In each case the $K_I$ was approximately 3 mM DEA. DEA inhibited the formation of phosphatidyl choline competitively, and produced a mixed type of inhibition for the synthesis of phosphatidyl ethanolamine. Administration of a single dose of 250 mg/kg DEA failed to produce inhibition of synthesis, but 330 mg/kg/day caused significant inhibition when given repeatedly. The most notable reduction of choline and ethanolamine incorporation occurred in the liver. The synthesis of ethnolamine phosphoglycerides declined to 27% of the control value after 1 week of DEA administration; no further reduction was seen during the remainder of the 3-week dosing regimen. Choline incorporation fell to 82% of the control value after 1 week, and to 47% and 41% after 2 and 3 weeks of DEA administration, respectively. The incorporation of these endogenous bases in renal tissue was also decreased. Ethanolamine phosphoglyceride synthesis declined steadily throughout the dosing regimen reaching a level 41% of control. Choline incorporation declined to 71% or control by the end of the third week. The kinetics of synthesis of the phospholipid derivatives of choline, ethanolamine, and DEA proved that the former two compounds were synthesized at a faster rate and in greater quantities. They were also catabolized at a slightly faster rate than the derivatives of DEA. The biological half-life of the phospholipid derivatives of DEA is longer than that of similar derivatives of choline and ethanolamine. This may favor accumulation of the DEA-containing phospholipid during chronic exposure.

Diethanolamine (DEA) is a common substance used in the chemical and pharmaceutical industries as an intermediate for the production of detergents, solubilizers, cosmetics, drugs, and textile finishing agents, and as an absorbent for acidic gases (Sutton, 1963). Studies have shown that several biologically exogenous alkanolamines are capable of interacting in phospholipid metabolism by competing with choline and ethanolamine (Welch and Landau, 1942; Wells and Remy, 1961; Chojnacki and Korzybski, 1963; Morin, 1969). Artom *et al.* (1958) noted that DEA can undergo incorporation into a phospholipid derivative *in vivo*. However, little was known regarding the effect of DEA on phospholipid metabolism *in vivo*, and no information existed evaluating its

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effect on phospholipid metabolsim *in vitro*. This study was undertaken to determine whether DEA produces inhibition of the synthesis of phospholipids both *in vitro* and *in vivo* in hepatic and renal tissues from the rat.

## METHODS

Male albino rats of the Sprague–Dawley strain were used throughout this investigation. The animals were housed in pairs in stainless-steel cages. They were maintained in air-conditioned quarters and supplied with food and water *ad libitum*. All animals were allowed to acclimate for at least 3 days. Animal sacrifice was accomplished by decapitation.

Diethanolamine (DEA) was purchased from the J. T. Baker Chemical Company. [1,2-$^{14}$C]Diethanolamine was obtained from ICN Tracer Lab with a specific activity of 0.306 mCi/nmol. Ethanolamine was purchased from Eastman Organic Chemical and [2-$^{3}$H]ethan-1-ol-2-amine hydrochloride from Amersham/Searle with a specific activity of 3.80 Ci/nmol. Choline chloride was purchase from Merck and Company and [*methyl*-$^{3}$]choline chloride from New England Nuclear Corporation with a specific activity of 4.2 Ci/nmol. DEA was analyzed for purity by thin-layer chromatography in three solvent systems: (1) ethyl alcohol : ammonium hydroxide (39%), (80 : 20); (2) methyl alcohol : ammonium hydroxide (39%), (80 : 20); and *n*-butyl alcohol : ammonium hydroxide (39%), (70 : 30), using aluminium oxide F-254 neutral type E, layer thickness 0.20 mm, from Brinkmann Instruments, Inc. DEA was visualized on the thin-layer plate using iodine vapor. By this procedure, only one spot was observed on plates from the three solvent systems. The purity of the [$^{14}$C]DEA was checked by thin-layer chromatography in solvent system one. Greater than 99% of the radioactivity was located at $\pm 0.20$ $R_f$ units from the $R_f$ of the nonlabeled DEA.

All labeled samples were placed in scintillation vials together with a scintillation cocktail consisting of 20 g 2,5-diphenyloxazole (PPO) and 0.4 g 1,4-di(2,5-diphenyloxazolyl)benzene (POPOP) dissolved in 4 liters of toluene. The samples were counted using a Nuclear Chicago Mark II liquid scintillation counter. The counting efficiency was determined by the use of internal standards.

The effects of DEA on *in vitro* phospholipid synthesis were determined using the livers from adult rats. The animals were not deprived of food before use. Immediately after sacrifice, the liver was quickly removed and approximately 5 g was placed in an isolation medium at 0°C. This medium consisted of 0.25 M special enzyme grade sucrose, 1 mM EDTA (acid form), 10 mM KCl, and 10 mM Tris base adjusted to pH 6.6 at 25°C with HCl (Brabec *et al.*, 1974). The pH of Tris base is temperature dependent, and the pH of the solution was 7.0 at 0°C. The liver was minced and washed once with the isolation medium. The sample was transferred to a glass tissue grinding vessel containing approximately 20 ml of isolation medium and homogenized by three strokes of a Teflon pestle fitted to an electric motor, driven at 600 rpm. The homogenate was equally divided between two 30-ml glass centrifuge tubes and spun at 1000*g* for 10 min in a refrigerated centrifuge at 2–4°C. Upon completion, the supernatant fractions from each tube were combined in a 30-ml glass centrifuge tube and placed on ice.

Incorporation of radioactive ethanolamine or choline into phosphatidyl ethanolamine or phosphatidyl choline was used as a measure of phospholipid synthesis. Additionally, labeled DEA was used to determine the *in vivo* synthesis of its phospholipid derivative. The incubation medium was a modification of that used by Weiss *et al.* (1958) and Roberts and Bygrave (1973). It contained 50 mM Tris buffer (pH 7.5 at 30°C), 10 mM magnesium chloride, 0.5 mM CTP, 10 mM ATP, 1 $\mu$M diolein, and approximately 2 mg of 1000*g* fraction protein all in a total volume of 0.5 ml. The diolein solution was prepared by placing 2 mg diolein along with 100 mg of Tween-20 to act as solubilizer in 100 ml of water. The concentrations of ethanolamine and choline were varied between 25 and 200 $\mu$M with a specific activity of 1.0 $\mu$Ci/50 nmol. DEA concentration was varied between 100 and 3000 $\mu$M. To determine the kinetic constants for the synthesis of the phospholipid derivative of DEA, the concentration of labeled precursor was varied from 5 to 20 mM with a specific activity of 0.05 $\mu$Ci/$\mu$mol. Incubation tubes (125 × 10 mm with screw caps) were used for all *in vitro* assays. Tubes were prepared by adding distilled water, incubation medium, labeled precursor, and inhibitor (DEA) and placing them on ice. The reaction was started by addition of 1000*g* fraction protein. The tubes were immediately mixed with a Vortex mixer and incubated in a Dubnoff metabolic shaker for 1 hr at 37°C in duplicate. The rate of the *in vitro* incorporation of choline, ethanolamine, and diethanolamine into respective phospholipid derivatives was investigated and found to be linear for at least 60 min. Zero-timed blanks were used to determine the amount of radioactivity carried over in the extraction process. The reaction was stopped by the addition of 1.5 ml of chloroform : methanol (1 : 2). This addition also served to start the phospholipid extraction. The phospholipid extraction is a modification of the method of Bligh and Dyer (1959). Following the chloroform : methanol, 0.5 ml of 2 M KCl and 1.0 ml chloroform were added and the tubes were thoroughly mixed with a Vortex mixer. The tubes were

then centrifuged at 1000*g* to achieve phase separation. The lower phase was transferred to a clean tube. The upper phase was washed once with 1.0 ml chloroform, mixed, and spun at 1000*g*. The lower phase was removed and added to the first extraction. The pooled lower phases were washed with 2.0 ml of a solution consisting of 47 parts 0.9% sodium chloride, 48 parts methanol, and 3 parts chloroform. The tubes were mixed, spun at 1000*g*, and then the upper phase was aspirated. The lower phase was taken to dryness under a stream of nitrogen in a water bath at 40°C. The phospholipids were redissolved in 1.0 ml of chloroform/methanol (1 : 2). An aliquot of 0.2 ml from each tube was transferred to scintillation vials and 10 ml scintillation cocktail added. The samples were counted as previously described.

In order to assess the extent of inhibition of the synthesis of the phospholipid derivatives of choline and ethanolamine in hepatic and renal tissue by DEA *in vivo*, three groups of four animals each were dosed for 1, 2, or 3 weeks with 2 mg/ml DEA in the drinking water. One group of four animals served as control. All animals were selected to be the same age at time of sacrifice. At the completion of the DEA dosing regimen, animals were injected ip with either 5 $\mu$Ci of choline or ethanolamine containing no added carrier. They were placed in 200-mm desiccators in pairs and sacrificed 6 hr postinjection.

The second part of the *in vivo* investigation was designed to determine the biological half-life of the phospholipid derivatives of DEA and to compare the kinetics of the phospholipid derivatives of choline and ethanolamine to those of DEA in hepatic and renal tissue. Sixty-four animals were used in this study; 32 received 10 $\mu$Ci of [$^{14}$C]DEA at a dose of 250 mg/kg and 5 $\mu$Ci of [$^{3}$H]choline (no added carrier) ip while the other group received a similar amount of [$^{14}$C]DEA and 5 $\mu$Ci of [$^{3}$H]ethanolamine (no added carrier) via the same route. Four animals from each group were sacrificed at 6 hr, and 1, 2, 4, 5, 6, 7, and 9 days postinjection.

Immediately after sacrifice, the liver and kidneys from each animal were removed and placed in separate glass vessels with a volume of 5% trichloroacetic acid sufficient to give a protein concentration of approximately 10 mg/aliquot of sample taken for phospholipid extraction. The tissues were homogenized with a Brinkmann Polytron tissue homogenizer for 30 sec. An aliquot was taken for phospholipid extraction as described above.

The protein concentration was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used in the preparation of protein standards.

Data were analyzed statistically by analysis of variance and by linear and nonlinear regression analysis. Kinetic constants were determined by the method of Lineweaver and Burk (1934) and inhibitor constants were found according to the method of Dixon and Webb (1964).

## RESULTS

The effect of increasing inhibitor (DEA) concentration on phosphatidyl choline syn-

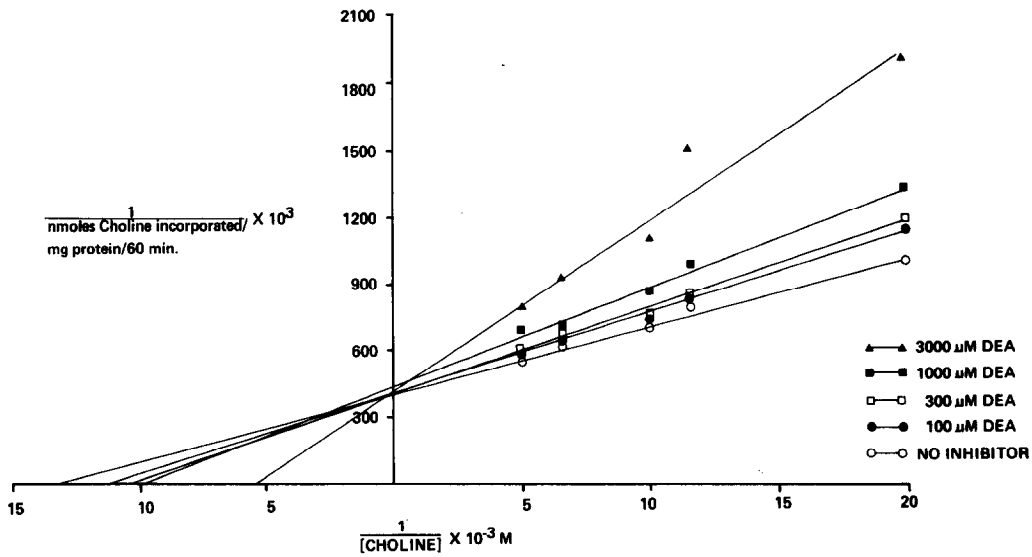


FIG. 1. Lineweaver–Burk plot of the incorporation of choline into phosphatidyl choline in the presence of varying concentrations of diethanolamine.

thesis is summarized in the Lineweaver–Burk plots in Fig. 1. Regression lines indicate competitive inhibition of the synthesis of this compound by DEA, which is not surprising in view of the structural similarity between choline and DEA.

The results obtained from the investigation of the inhibition of phosphatidyl ethanolamine synthesis by DEA are not as distinct as those for phosphatidyl choline. These data indicate, as illustrated in Fig. 2, that DEA produces significant inhibition of synthesis, but the Lineweaver–Burk plots indicate that it is not solely a competitive type of inhibition as was observed for phosphatidyl choline synthesis. The $K_i$ for DEA-inhibited synthesis of phosphatidyl choline synthesis is 2.6–2.9 mM while that of phosphatidyl ethanolamine synthesis is also 2.6–2.9 mM.

The next step was to investigate whether

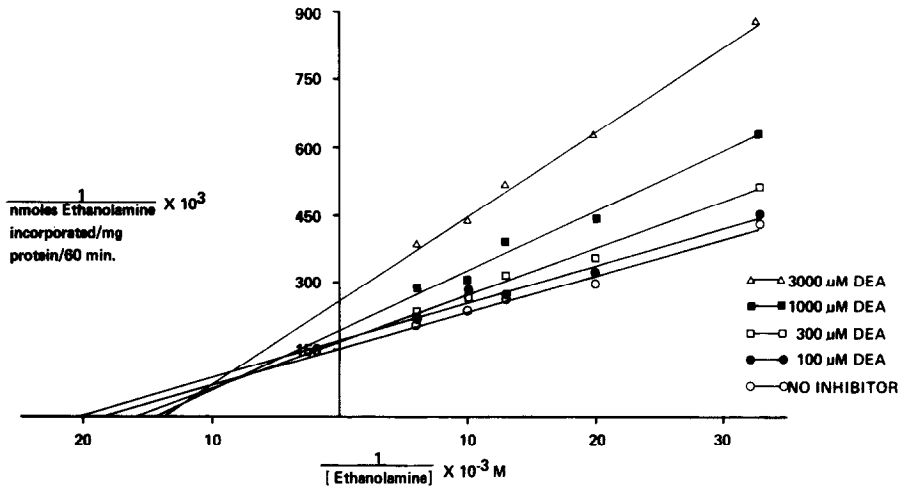


FIG. 2. Lineweaver–Burk plot of the incorporation of ethanolamine into phosphatidyl ethanolamine in the presence of varying concentrations of diethanolamine.

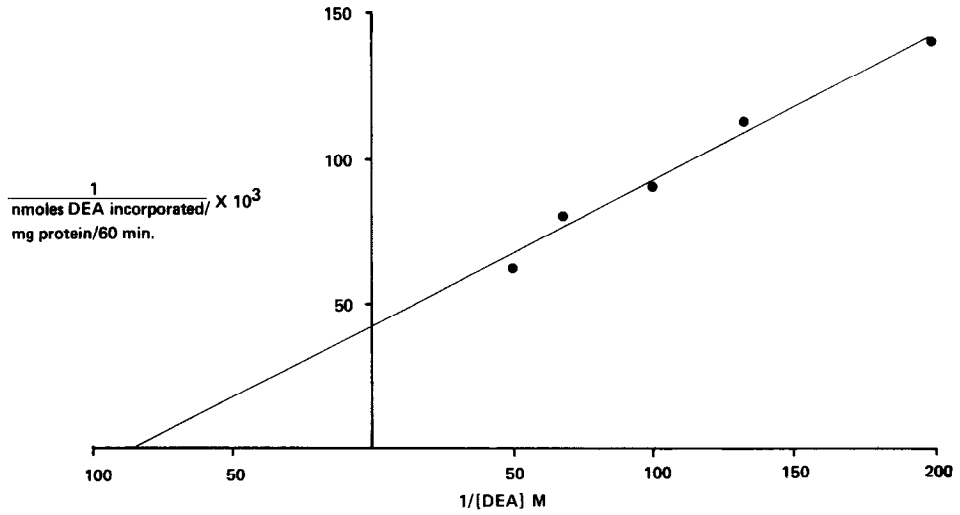


FIG. 3. Lineweaver–Burk plot of the incorporation of diethanolamine into a phospholipid derivative.

TABLE 1

LACK OF INHIBITION OF THE *in Vivo* SYNTHESIS OF THE PHOSPHOLIPID DERIVATIVES OF CHOLINE AND ETHANOLAMINE[a]

| Nitrogenous base precursor | Counts per minute/mg protein | | | |
|---|---|---|---|---|
| | Hepatic tissue | | Renal tissue | |
| | Control | DEA treated | Control | DEA treated |
| Choline | 1031 ± 99 | 1057 ± 70 | 382 ± 35 | 393 ± 17 |
| Ethanolamine | 1918 ± 98 | 2019 ± 96 | 236 ± 6 | 304 ± 15[b] |

[a] Each value represents the mean ± SE of data from four animals. Animals were administered 5 μCi of [$^3$H]ethanolamine or [$^3$H]choline ip combined with 250 mg/kg DEA and sacrificed 6 hr following injection.

[b] $p < 0.05$.

DEA could be incorporated into a phospholipid *in vitro* via this enzymatic pathway. Figure 3 is a Lineweaver–Burk plot of the incorporation of DEA into a phospholipid derivative. From the graph, the apparent $K_m$ and $V_{max}$ were calculated to be 11.6 mM and 21.0 nmol/mg protein/60 min, respectively. Compared to the apparent $K_m$ for phosphatidyl choline and phosphatidyl ethanolamine synthesis of 75.5 and 53.5 μM, respectively, DEA turns out to be a less effective substrate for this reaction.

Table 1 summarizes an evaluation of the acute effects of DEA on the *in vivo* synthesis of phospholipid derivatives of choline and ethanolamine. The results from this experiment are at variance with those from *in vitro* experimentation. There is no evidence of inhibition of the incorporation of either base tested. In fact, there appears to be mild stimulation of synthesis in all samples.

When DEA is administered for up to 3 weeks, the results differ from those found after a single dose of DEA. The incorporation of [$^3$H]ethanolamine and [$^3$H]choline into hepatic and renal phospholipids is inhibited. The data illustrating this difference are presented in Figs. 4 and 5. The most significant inhibition of the incorporation of exogenous tritiated ethanolamine was observed in hepatic tissue where it dropped to 27% of the control value after only 1 week of DEA administration. This level was essentially maintained through the 3 weeks of testing. Choline incorporation exhibited a more gradual decrease; a decline to 82% of the control value was observed after 1 week. After 2 and 3 weeks of DEA administration, the incorporation of choline into phosphatidyl choline declined to 47 and 41% that of

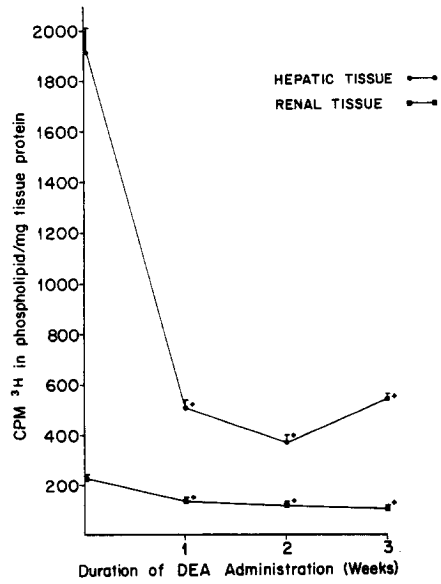


FIG. 4. The effect of subacute administration of diethanolamine on the *in vivo* synthesis of phospholipid derivatives of ethanolamine. Diethanolamine was administered orally in drinking water at 2.0 mg/ml (320 mg/kg/day). Each point represents the mean ± SE of data from four animals. A plus sign (+) denotes values which are statistically significant ($p < 0.05$).

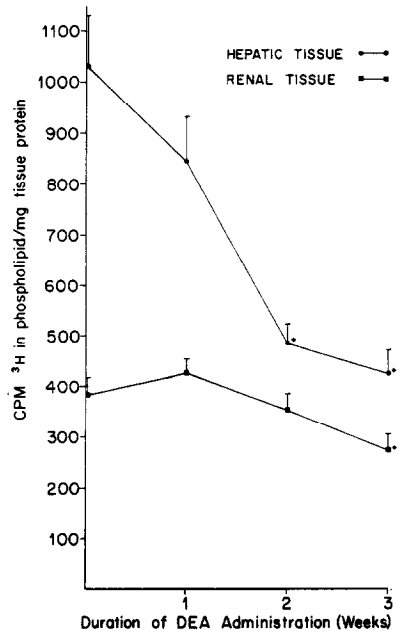


FIG. 5. The effect of subacute administration of diethanolamine on the *in vivo* synthesis of phospholipid derivatives of choline. Diethanolamine was administered orally in drinking water at 2.0 mg/ml (320 mg/kg/day). Each point represents the mean ±SE of data from four animals. A plus sign (+) denotes values which are statistically significant ($p < 0.05$).

control, respectively. Decreases in incorporation of these two bases into renal tissue were also seen, although not to the degree observed in liver. [$^3$H]Ethanolamine incorporation declined throughout the dosing regimen to 47% of the control value after 3 weeks of DEA administration. [$^3$H]Choline incorporation actually increased after 1 week, but by the end of the third week the incorporation of [$^3$H]choline into phospholipid derivatives had fallen to 71% of the control value.

The elimination kinetics of the labeled phospholipid derivative of DEA compared to those of choline and ethanolamine is presented in Figs. 6–8. One day following the administration of [$^3$H]choline, [$^3$H]ethanolamine, and [$^4$C]DEA, significantly greater incorporation into phospholipid derivatives was noted for the former two compounds. Only 2.02% of the DEA administered was incorporated into phospholipid derivatives in liver and 0.23% in kidney; 7.20% of the administered choline and 19.8% of the administered ethanolamine were incorporated into these lipid derivatives from hepatic tissue; in contrast, the kidney incorporated 0.75% of the administered choline and 2.69% of the administered ethanolamine. This finding is to be expected; choline and ethanolamine are natural substrates and the $K_m$ value for DEA incorporation is roughly 150 times that of choline and ethanolamine.

The half-life for the disappearance of the phospholipid derivatives of DEA, from liver tissue, is approximately 3.5 days, and 4.2 days from the kidneys. The half-life for labeled choline is approximately 1.7 days in hepatic tissue and about 2.1 days in the kidney. The values for ethanolamine are approximately 1.6 days for liver and 2.3 days for kidney.

## DISCUSSION

The apparent variation in the type of inhibition of the synthesis of phosphatidyl choline and phosphatidyl ethanolamine produced by DEA raises the interesting question of whether separate enzymes are involved in part or all of the synthesis of these two compounds. From the differences observed of the *in vitro* inhibition of phosphatidyl choline and phosphatidyl ethanolamine synthesis by DEA, it would appear that either these enzymes are the same but that each compound has its own particular active site, or that separate enzymes are involved. There is evidence to suggest that separate enzymes exist for a portion of the pathway for the *de novo* synthesis of phosphatidyl choline and phosphatidyl ethanolamine (Sung and Johnstone, 1967; Ramasarma and Wetter, 1957; Broad and Dawson, 1974; Sundler, 1975).

Acute administration of DEA failed to inhibit the *in vivo* synthesis of hepatic phospholipid derivatives of choline and ethanolamine. This observation is anomalous in view of the *in vitro* findings. DEA is con-

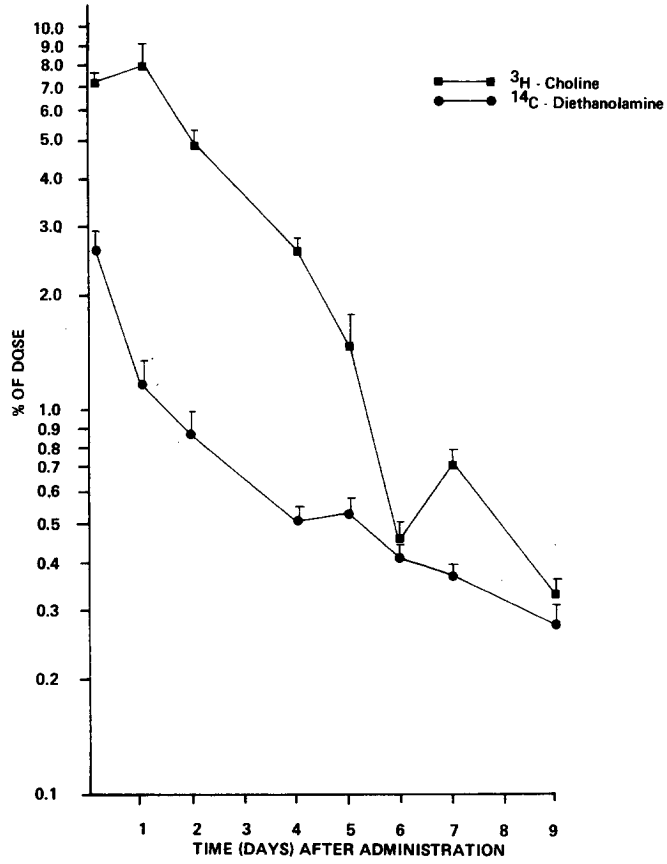


FIG. 6. *In vivo* elimination kinetics of the hepatic phospholipid derivatives of [$^{3}$H]choline and [$^{14}$C]diethanolamine.

verted to a phospholipid derivative *in vivo* and, under such conditions, would appear to have access to the enzymes which synthesize phospholipid derivatives of choline and ethanolamine.

The inhibition of [$^{3}$H]choline and [$^{3}$H]ethanolamine incorporation *in vivo* occurs only after repeated administration of DEA. The primary site of phospholipid synthesis is located on the endoplasmic reticulum (Wilgram and Kennedy, 1963; Schneider, 1963; McMurray and Dawson, 1969; Jungalwala and Dawson, 1970a,b). In 1971, Foster found that repeated dosing with DEA inhibited hepatic microsomal drug-metabolizing activity. He also found that the activity of this system was unaffected by *in vitro* exposure to DEA, even at concentrations of 20 mM. The mixed function oxidase system was also unaffected by acute *in vivo* administration. The pattern of inhibition of phospholipid synthesis by DEA, found in this study, correlates well with the results of inhibition of hepatic microsomal enzyme activity obtained by Foster (1971). It is likely that the *in vivo* incorporation of DEA into hepatic phospholipids plays a direct role in the inhibition of both phospholipid synthesis and drug metabolism. Ellingson and Lands (1968) and Strobel *et al.* (1970) have found that specific phospholipids perform specific functions in cellular metabolism. The insertion of a different phospholipid into a site requiring a phospholipid of specific structure would alter, in all likelihood, the function of that particular site. Following the inter-

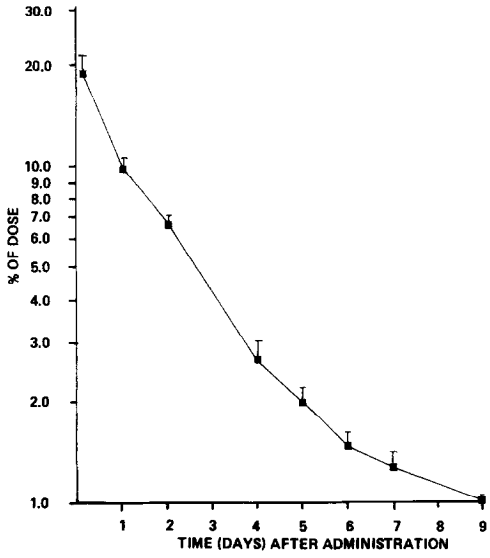


FIG. 7. *In vivo* elimination kinetics of the hepatic phospholipid derivatives of [$^3$H]ethanolamine.

pretation of Ellingson and Lands (1968), the effect could be a reduction in the activity of certain enzymes located on the endoplasmic reticulum.

Since DEA appears not to affect synthesis of these compounds after acute administration, thus ruling out direct involvement, the alternative is one of indirect action. The synthesis of DEA to a phospholipid derivative probably results in its incorporation into membrane constituents of the endoplasmic reticulum, and likely would change the activity of enzymes dependent upon these membranes. The observed reduction in the rates of phospholipid synthesis may be due to reduction of the activity of enzymes located on the endoplasmic reticulum.

A second observation from these data which deserves attention is the increased

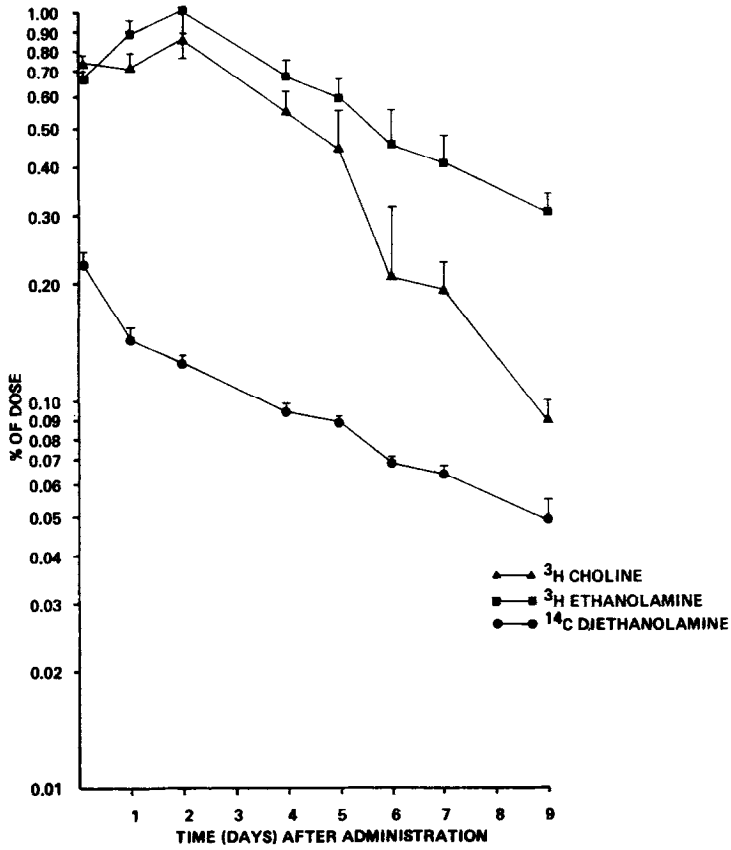


FIG. 8. *In vivo* elimination kinetics of the renal phospholipid derivatives of [$^3$H]choline, [$^3$H]ethanolamine, and [$^{14}$C]diethanolamine.

inhibition of *in vivo* phospholipid synthesis in the liver in comparison to the kidney. In this particular experiment, DEA was administered via the drinking water. Compounds given by the oral route are absorbed from the gastrointestinal tract and enter the hepatic portal circulation which initially transports them to the liver before entering the systemic circulation. Consequently, hepatic tissue has the first opportunity to handle metabolically orally administered substances. It is possible, through absorption and metabolism by the liver, that the concentration of DEA presented to the kidney was significantly less per gram of tissue than for hepatic tissue.

Incorporation into phospholipid derivatives of atypical molecules with subsequent insertion into membrane structure could act in two ways to inhibit microsomal synthesis of necessary cellular constituents, i.e., alteration of the enzymes themselves as well as reduction of synthesis of essential components of the membrane. The phosphoglycerides of DEA appear to be catabolized at a slower rate than those of choline and ethanolamine, thus confirming the prediction of Artom *et al.* (1958). The reduced rate of degradation may promote accumulation of such atypical phospholipids in body tissues.

## ACKNOWLEDGMENTS

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