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REVERSIBLE, HEPATIC, LYSOSOMAL PHOSPHOLIPIDOSIS IN RAT INDUCED BY SUBCHRONIC DAILY ADMINISTRATION OF TROSPECTOMYCIN SULFATE

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Abstract—Trospectomycin sulfate is an experimental aminocyclitol antibiotic which has been shown previously to induce the formation of cytoplasmic lamellar bodies in rat and dog liver in subchronic experiments. The effect of repeated daily administration of trospectomycin sulfate on hepatic phospholipid levels and activities of marker enzymes for subcellular organelles was examined. Rats were treated for 30 or 90 days with 0, 50, or 250 mg/kg/day of trospectomycin sulfate prior to being killed, and another group was dosed for 90 days and then allowed to recover for 79 days prior to sacrifice. Transmission electron microscopy showed the presence of lamellar bodies in hepatocytes in both 50 and 250 mg/kg groups at 90 days but no other apparent changes in cellular morphology. Total phospholipids were increased significantly (1.6-fold) only at 90 days ($P < 0.01$) and only in the 250 mg/kg group. Phosphatidylcholine, phosphatidylinositol, and two acidic lysosomal phospholipids, bis(monoacylglycero)phosphate and acylphosphatidylglycerol, accounted for 42, 35, and 21% of the increase in total phospholipids. Changes in the activities of marker enzymes were generally confined to the 250 mg/kg group at 90 days, with the largest and most significant increases being in the lysosomal enzymes acid phosphatase and hexosaminidase ($P < 0.01$). Levels of all phospholipids and marker enzymes, with the exception of succinate dehydrogenase, were not significantly different from controls 79 days after cessation of dosing, and lamellar bodies had disappeared. We conclude that repeated trospectomycin sulfate treatment in rat induces a reversible, dose- and time-dependent lysosomal phospholipidosis in liver which is characterized by an increase in lysosomal enzymes and selected anionic phospholipids.

Numerous cationic amphiphilic chemicals have been shown to induce phospholipidosis accompanied by cytoplasmic lamellar body formation in a variety of animal species [reviewed in Ref. 1]. More hydrophilic amines, such as the freely water soluble antibiotic gentamicin [2–4], an aminoglycoside, also have been shown to elicit lamellar body formation. The lamellar bodies constitute an intracellular membrane storage compartment that in many cases has been shown to contain acid phosphatase and consequently, they are thought to be derived from or related to the lysosome [1].

Trospectomycin sulfate (Fig. 1) is a water-soluble aminocyclitol antibiotic that has been shown to induce lamellar body formation in the perfused rat liver and cultured rat hepatocytes within hours of dosing [5, 6]. In this case, as in others where chemical agents have been shown to cause a rapid onset of lamellar body formation [7–10], the effect does not appear to be linked with acute cytotoxicity. Sustained cellular phospholidosis can be injurious, however, since subacute administration of several phospholipidosis inducers to animals, including humans, has been associated with cellular dysfunction and

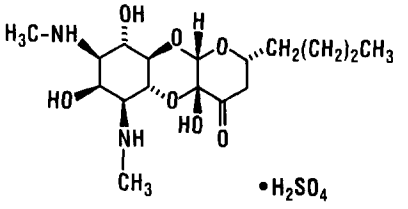

Fig. 1. Chemical structure of trospectomycin sulfate.

degeneration [1]. For example, subacute gentamicin administration to rats evokes renal tubule cell necrosis characterized by cellular phospholipidosis [2–4, 9, 11]. In contrast to this behavior, 30-day, high-dose administration of trospectomycin sulfate to the rat or dog elicits lamellar body formation principally in the liver with no associated hepatic necrosis [12]. A modest, dose- and time-dependent increase in serum transaminases was observed, but no histological changes other than foamy cytoplasmic vacuolation of centrilobular hepatocytes were noted.

The absence of frank hepatic necrosis during subacute trospectomycin treatment was consistent with the *in vitro* finding that the drug was noncytotoxic, but prompted us to investigate the phenomenon further in order to explain the seemingly benign nature of the lamellar body accumulation. Like gentamicin, trospectomycin is excreted primarily in urine as unchanged drug by the rat [13]. Unlike gentamicin, however, a minor portion of the dose is

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sequestered without metabolism in the hepatocyte for eventual biliary excretion, and this property probably accounts for its selective effect on the liver [13]. Whereas gentamicin is concentrated in the tubule cell lysosomal compartment where it is thought to inhibit phospholipid catabolism [14, 15], trospectomycin is more widely distributed among organelles in the hepatocyte [13], and it may act by a different mechanism. The present study was conducted to define quantitatively the phospholipidosis produced by repeated trospectomycin administration in the rat, and to determine, using marker enzymes, which organelles were affected. The doses selected for this study were 50 and 250 mg/kg, which are 1.7 and 8.3 times the maximum anticipated human clinical dosage rate of 30 mg/kg/day.*

MATERIALS AND METHODS

Chemicals. All solvents were reagent grade, glass distilled from Burdick & Jackson (Muskegon, MI). Materials for marker enzyme assays were purchased from the Sigma Chemical Co. (St Louis, MO), and phospholipid standards were from Serdary Research Laboratories (New London, Ontario). Boron trifluoride in methanol (14%) was from Alltech/Applied Science (Deerfield, IL). Trospectomycin sulfate (6′-*n*-propylspectinomycin hydrogen sulfate, pentahydrate, formula weight 562.6) was supplied by The Upjohn Co. (Kalamazoo, MI).

Animal model. Male and female Sprague–Dawley rats (216–270 g for males and 144–193 g for females at start of study) were obtained from Charles River Laboratories, Inc. (Portage, MI). They were housed individually in stainless steel cages with wire mesh floors over adsorbent paper. Food (Purina Lab Chow®; Ralston Purina Co., St Louis, MO) and water were provided *ad lib.*, and the animal rooms were maintained on a 12-hr light/dark cycle. Half doses of trospectomycin sulfate dissolved in sterile water for injection with 0.9% benzyl alcohol were given twice daily, separated by 6 hr, in the dorsal region of the neck and thorax at levels of 0, 50, and 250 mg/kg/day. Control animals received the benzyl alcohol vehicle only. An interval of 6 hr elapsed between doses. Two males and two females at each dose level were killed, at 30 and 90 days of dosing, by carbon dioxide anesthesia followed by exsanguination. Reversibility animals (two animals per sex per dose group) were dosed for 90 days and killed 79 days after discontinuation of dosing. The length of the recovery period was determined by the length of time required for serum transaminase levels to return to normal values.

Electron microscopy. Rat liver was minced into 1 mm^3 pieces and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde [16] in 0.1 M sodium cacodylate buffer, pH 7.2. Following 3 × 5 min washes in buffer, samples were post-fixed in 1.0% osmium tetroxide in buffer for 1 hr. Samples were then rinsed three times in buffer, dehydrated with graded ethanols through 100%, cleared with propylene oxide, and imbedded in Polybed 812 (Polysciences, Warrington, PA). Thin sections were stained with uranyl acetate and Reynolds lead citrate [16] and viewed and photographed with a JEOL 1200 EX electron microscope.

Marker enzymes. After sacrifice, approximately 1 g of liver was removed from each animal, blotted dry, weighed, and minced. Each sample was homogenized in 10 ml of cold 0.9% NaCl with a Potter–Elvehjem homogenizer and teflon pestle, and the homogenate was stored at −20°. The concentration of protein [17] and the activities of acid phosphatase [18], hexosaminidase [19], caproyl esterase (pH 5.0) [19], cytochrome P-450 reductase [20]; α-mannosidase (pH 5.5) [21], succinate dehydrogenase [22], 5′-nucleoside phosphodiesterase [23], catalase [24], and tyrosine aminotransferase [25] were determined.

Lipid extraction and analysis. Approximately 1 g of liver was removed from each rat, blotted dry, weighed, and homogenized on ice in 20 ml chloroform/methanol (2:1, v/v) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was filtered through Whatman No. 1 paper, and the filter paper and retentate were rehomogenized with 20 ml chloroform/methanol (2:1, v/v) and filtered as described above. The combined filtrates were transferred to a 125-ml separatory funnel and shaken vigorously with 8 ml of 0.9% NaCl. The lower phase was collected and the upper phase washed with a replacement volume of Folch theoretical lower phase (chloroform/methanol/0.9% NaCl, 86:14:1, by vol.). The combined lower phases were taken to dryness by rotary evaporation at 30° using absolute ethanol to ensure complete removal of water. The residue was quantitatively transferred to a 10-ml volumetric flask with chloroform/methanol (5:1, v/v) containing 0.1% (w/v) butylated hydroxytoluene and stored at −20°. Total phospholipids were quantified by phosphorous analysis using a modified Bartlett procedure [26]. For analysis of the distribution of esterified fatty acids in the phospholipids, phospholipid fractions were isolated using aminopropyl bonded-phase columns [27], and methyl esters were prepared using boron trifluoride [28]. The phospholipid composition of individual rat livers was determined by the high performance liquid chromatography method of Christie [29] except that a Varex laser light scattering detector was used. The results were expressed as area percents, and the method was validated on selected samples by comparing with the phospholipid composition determined by two-dimensional thin-layer chromatography with phosphorus analysis. Synthetic standards of BMP and APG† were chemically and biologically synthesized [30].

Statistical analysis. There were no apparent differences in liver ultrastructure, marker enzyme activities, or phospholipid contents between male

* Throughout this paper, doses are expressed in terms of trospectomycin sulfate pentahydrate bulk drug weight. To convert to free base equivalents, multiply the dose by 0.67, which is the number of grams of trospectomycin base per gram of trospectomycin sulfate pentahydrate.

† Abbreviations: BMP, bis(monoacylglycero)phosphate; APG, acylphosphatidylglycerol; PI, phosphatidyl inositol; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; SM, sphingomyelin; PS, phosphatidyl serine; and CL, cardiolipin.

and female rats within treatment groups (N = two per sex), so the data from both sexes were pooled for statistical analysis of treatment effects. Equality of variances among treatment groups for a given parameter (e.g. total phospholipid content) was tested according to Hartley's F_{max} variance ratio test [31]. If variances were not significantly different ($P > 0.05$), then treatment effects were tested by one-way analysis of variance (ANOVA). When the ANOVA probability of a larger F value was less than 0.05, individual pairwise comparisons of group means were made by Student's *t*-test. If the data failed Hartley's test, then treatment effects on parameters were examined with Box's means test with corrected degrees of freedom [31]. In the special case of phospholipids which were only detected in drug treatment groups, the statistical significance of their presence was tested by determining whether the 95% confidence interval for the treatment group included zero concentration.

RESULTS

Electron microscopy. Intracellular lamellar bodies were observed in hepatocytes of high- and mid-dose groups (90 days dosing). The inclusions consisted of several concentric layers of electron-dense, membrane-like material surrounded by a limiting membrane, and ranged in diameter from 0.5 to 2.0 μm (Fig. 2). There were no other obvious treatment effects on cellular morphology. There was a dose-dependent, subjective increase in the number of lamellar bodies in hepatocytes at 90 days which was not evident after the recovery period. Seventy-nine days after withdrawal of the drug, the hepatocellular morphology of treated animals was similar to controls.

Marker enzyme activities. Marker enzymes for lysosomes, the Golgi apparatus, mitochondria, and cytoplasm were affected significantly by drug treatment (Fig. 3A). After 90 days of dosing, both acid phosphatase and hexosaminidase were increased significantly (1.65 times and 2.44 times higher than control respectively) in the high dose group (250 mg/kg). In contrast, caproyl esterase, another lysosomal enzyme, was decreased significantly in both the 50 and the 250 mg/kg dose groups (approximately 0.75 times the control). The Golgi apparatus marker enzyme α-mannosidase was increased significantly in the 250 mg/kg group (1.45 times the control respectively), and the mitochondrial enzyme succinate dehydrogenase was also increased significantly in the 250 mg/kg group (1.27 times the control). Tyrosine aminotransferase, a cytoplasmic marker, was reduced significantly in both the 50 and 250 mg/kg dose groups. Marker enzymes for peroxisomes (catalase) and endoplasmic reticulum (cytochrome P-450 reductase) both decreased in activity in response to dose, but the changes were not significant. The plasma membrane enzyme 5′-nucleotide phosphodiesterase showed no apparent dose response.

With the exception that succinate dehydrogenase remained significantly elevated, no significant differences between the treatment groups for any of the marker enzymes were detected 79 days after cessation of dosing (Fig. 3B). The high dose acid phosphatase and hexosaminidase were still higher and the esterase lower than the controls, but the differences were smaller and not statistically significant.

Phospholipids. The hepatic total phospholipid concentration per gram wet weight was increased significantly in the 250 mg/kg dose group after 90 days (1.6 times the control) but not after 30 days of dosing (Fig. 4). There was no significant treatment effect on total phospholipids at either 30 or 90 days in the 50 mg/kg group, nor was there a significant effect 79 days after discontinuation of dosing. Examination of the phospholipid profile of individual rats revealed significant changes in the relative concentration of several phospholipids at both 30 and 90 days in the 250 mg/kg dose group (Fig. 5, A and B). Comparing percentages of total HPLC peak areas, PI was increased and PE, PC, and SM were all decreased, and two unusual acidic phospholipids, BMP and APG, were observed. The decreased relative concentrations of PE, PC, and SM in the 250 mg/kg group at 90 days were offset by the increased total phospholipid content, so that the actual concentration of all phospholipids was increased. Approximately 42% of the total phospholipid increase was due to PC, 35% was due to PI, and 21% was due to BMP + APG. The distribution of fatty acids in each phospholipid fraction was not altered significantly except for small increases and decreases in the relative amounts of arachidonic and stearic acids respectively (data not shown). By 79 days post-dose, no significant treatment effects were detected and neither APG nor BMP was detected (Fig. 5C).

DISCUSSION

The objective of the present study was to evaluate the effect of repeated daily administration of trospectomycin sulfate to rats on liver phospholipids and marker enzymes for subcellular organelles. Previous studies in the rat and dog have shown drug-related increases in serum transaminases as well as in the formation of cytoplasmic lamellar bodies [12]. We found that, on average, 90-day drug treatment with 250 mg/kg/day but not 50 mg/kg significantly increased the phospholipid concentration in rat liver per gram wet weight and altered the phospholipid composition. Consistent with the previous observation that the number of lamellar bodies in hepatocytes of drug-treated animals was variable within a dose group [12], the hepatic phospholipid concentration of individual rats was variable. The mean increase in total phospholipid concentration per gram wet weight in the 90-day 250 mg/kg group was 1.6-fold, which is similar to the increase in rat liver phospholipids caused by subchronic administration of various cationic amphiphilic amines such as chloroquine and 4,4′-diethylaminoethoxyhexestrol (4,4′-DH) [1]. Both the concentration of total phospholipids and their composition reverted to normal upon discontinuance of dosing.

The most notable changes in the hepatic phospholipid composition of drug-treated rats were the increase in the relative concentration of PI to 230%

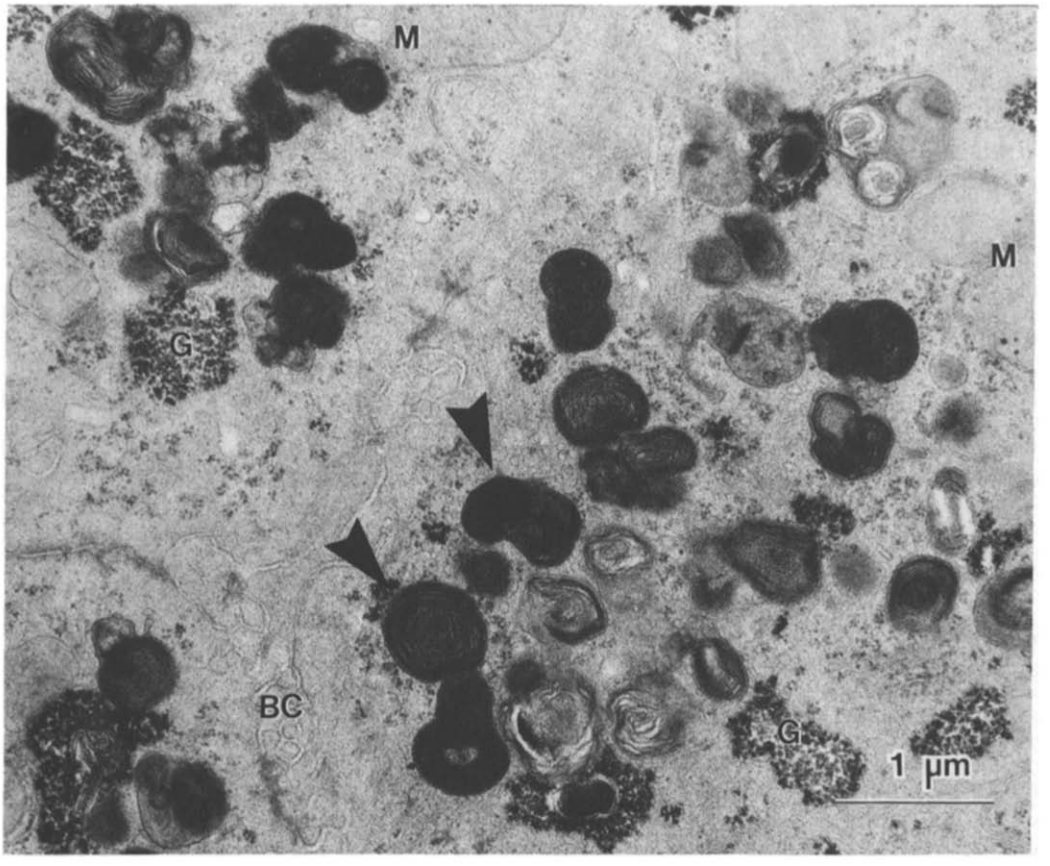

Fig. 2. Transmission electron microscopy of the liver following 90 days of dosing with trospectomycin sulfate (250 mg/kg/day). Cytoplasmic lamellar bodies (arrows) in rat hepatocytes are frequently clustered near the biliary canaliculus. Abbreviations: BC, biliary canaliculus; M, mitochondria; and G, glycogen.

of control and the appearance of two unusual phospholipids associated with lysosomal phospholipid storage disorders, BMP and APG [32–34]. PC, PI, and BMP + APG, in decreasing order, were together responsible for greater than 95% of the 1.6-fold increase in total phospholipid. Cardiolipin, which is primarily localized in the inner mitochondrial membrane [35], was increased modestly but not significantly at 90 days. These changes in phospholipid composition of the liver were reflected in the phospholipid composition of purified lamellar bodies*, indicating that, as expected, the lamellar bodies were the organelle responsible for the increase in total hepatic phospholipids.

The principal difference between the composition of accumulated phospholipids induced by the cationic amphiphilic amines compared to trospectomycin was in the relative contribution of PI and PE. In the case of trospectomycin, PI accounted for 35% of the increase in total phospholipid and the contribution of PE was negligible, whereas in the case of chloroquine and 4,4′-DH, PI accounts for 15–20% and PE accounts for 7–15% [36, 37]. The greatest percentage of the increase in all three cases was due to PC, and all three agents caused the formation of BMP and APG. The renal phospholipidosis induced by the aminoglycoside antibiotic gentamicin is more similar to the hepatic phospholipidoses caused by chloroquine and 4,4′-DH than by trospectomycin with regard to the contributions of PI and PE [38, 39]. Both gentamicin and the cationic amphiphilic amines are speculated to cause phospholipidosis by inhibiting lysosomal phospholipases [14, 15].

Besides the presence of unusual lysosomal phospholipids caused by subchronic trospectomycin treatment, the activity pattern of marker enzymes provides further evidence for a trospectomycin-related perturbation of lysosomes. Of the various marker enzymes affected by trospectomycin, the lysosomal enzymes acid phosphatase and hexosaminidase showed the largest and most significant increases ($P < 0.001$ and 0.01 respectively) in the high dose group after 90 days of dosing. The effect on lysosomes was not uniform, however, since caproyl esterase was decreased significantly in the same group ($P < 0.05$). The possibility that esterase activity was decreased due to direct inhibition by trospectomycin in the liver homogenate was ruled out on the basis that a control liver homogenate fortified with drug showed no change in activity (data not shown). Differential changes in the relative activities of lysosomal marker enzymes may be due to enhanced production of certain enzymes or the proliferation of a distinct subpopulation of lyso-

* Cox JW, Ulrich RG and Epps DE, manuscript in preparation.

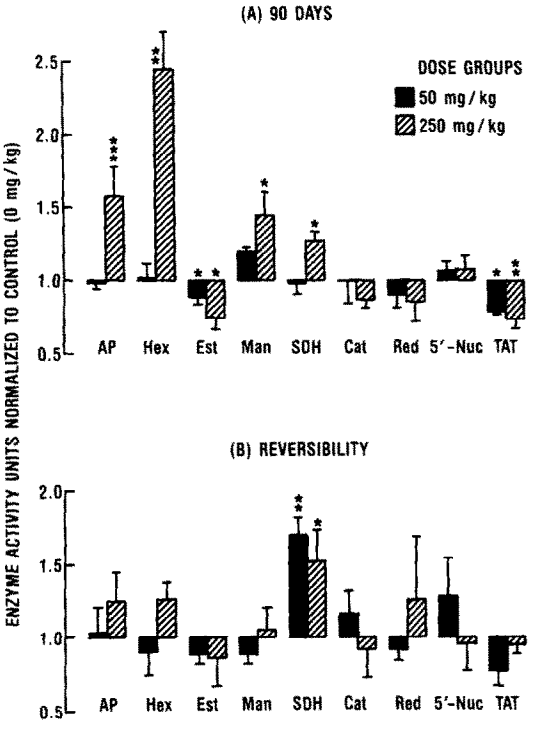

Fig. 3. Levels of marker enzymes in liver homogenates of animals dosed for 90 days (panel A) with trospectomycin sulfate and of animals dosed for 90 days and then allowed a 79-day recovery period (panel B). Animals received 0, 50, or 250 mg/kg/day. Results are normalized to the control and are expressed as the mean ± SE (N = four rats per group). The statistical significance of differences compared to the matched control (0 mg/kg) dose group is indicated as: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$. Abbreviations: AP, acid phosphatase (lysosomes); Hex, hexosaminidase (lysosomes); Est, caproyl esterase (pH 5.0) (lysosomes); Man, α-mannosidase (pH 5.5) (Golgi); SDH, succinate dehydrogenase (mitochondria); Cat, catalase (peroxisomes); Red, cytochrome P-450 reductase (endoplasmic reticulum); 5′-Nuc, 5′-nucleotide phosphodiesterase (plasma membrane); and TAT, tyrosine aminotransferase. Control values, in units of nmol/min/mg protein, at 90 days were: AP, 34.2 ± 2.3; Hex, 7.47 ± 1.1; Est, 41.1 ± 1.9; Man, 0.121 ± 0.003; SDH, 13.7 ± 1.1; Cat, 160 ± 19; Red, 1.62 ± 0.23; 5′-Nuc, 27.4 ± 2.3; and TAT, 7.38 ± 0.49. The reversibility control values were: AP, 22.6 ± 2.7; Hex, 3.70 ± 0.63; Est, 44.8 ± 1.9; Man, 0.062 ± 0.010; SDH, 9.81 ± 0.49; Cat, 139 ± 19; Red, 0.950 ± 0.063; 5′-Nuc, 12.2 ± 1.00; and TAT, 4.32 ± 0.57.

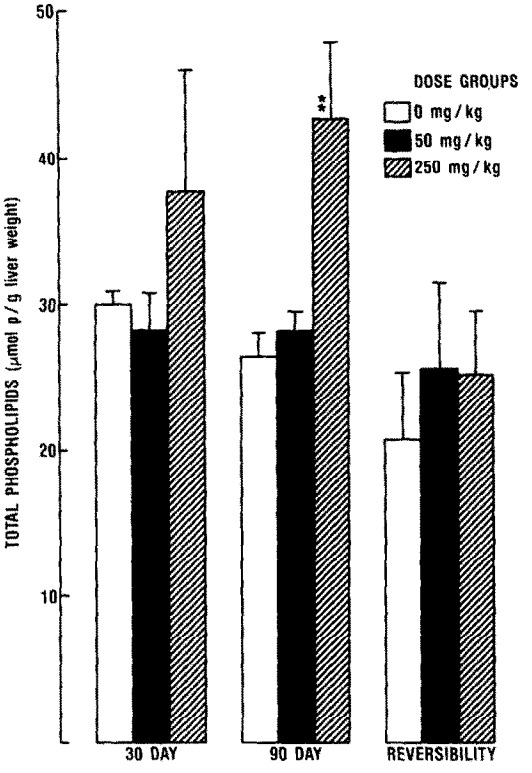

Fig. 4. Total phospholipid levels in livers of rats dosed for 30 days, for 90 days, or for 90 days and then allowed a 79-day recovery period before being killed. Results are expressed as the mean ± SE (N = four rats per group). Key: (**) statistically significant compared to the matched control (0 mg/kg) group, $P < 0.01$.

somes. In this regard, the lamellar bodies induced by trospectomycin have been shown to possess acid phosphatase activity [6] and probably represent an abnormally lipidic lysosomal population.

The cellular effects of trospectomycin, however, were not confined solely to the lysosomal compartment. Marker enzymes for the Golgi apparatus and mitochondria were increased modestly, but significantly ($P < 0.05$), in the high dose group at 90 days, and the cytoplasmic marker enzyme tyrosine aminotransferase was decreased significantly ($P < 0.01$). These effects may be a direct result of drug interaction with the organelle since hepatic subcellular distribution studies have shown that at least 50% of trospectomycin in the depot compartment is distributed evenly among light (endoplasmic reticulum and Golgi) to heavy (mitochondria) membrane fractions [13]. Alternatively, they may be a sequela of lysosomal dysfunction. For example, the cytoplasmic volume of the cell was diminished due to the presence of numerous lamellar bodies, and this may partially explain the decrease in tyrosine aminotransferase activity. All of the phospholipid and marker enzyme changes induced by trospectomycin, with one exception, reversed upon discontinuation of dosing. Only succinate dehydrogenase, a mitochondrial enzyme, remained elevated (Fig. 3B), which may reflect an alteration in the turnover rate of mitochondria due to lysosomal dysfunction.

Taken together, the available phospholipid and marker enzyme data indicate that repeated administration of trospectomycin sulfate at doses of 250 mg/kg/day induced a reversible, lysosomal phospholipidosis which was time dependent, and which was characterized by a selected increase in lysosomal enzymes and anionic phospholipids. It was not associated, however, with lethal cellular injury. There was no significant effect of 90-day drug treatment on either phospholipid concentration, phospholipid composition, or marker enzyme activities at a dose level of 50 mg/kg/day, which is 1.7 times the maximum anticipated human clinical dosage rate (30 mg/kg/day for up to 14 days). Current studies are directed towards elucidating the

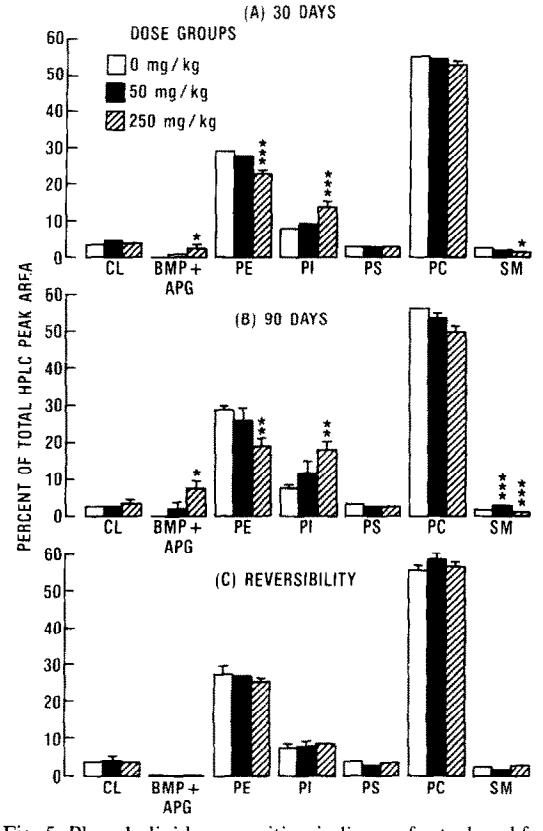

Fig. 5. Phospholipid composition in livers of rats dosed for 30 days (panel A) and 90 days (panel B) and of rats dosed for 90 days and then allowed a 79-day recovery period (panel C). Animals received 0, 50, or 250 mg/kg/day of trospectomycin sulfate. Results are expressed as the mean ± SE (N = four rats per dose group). Where error brackets are not shown, the SE was less than 0.5%. The statistical significance of differences compared to the matched control (0 mg/kg) group is indicated as: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$.

mechanism of hepatic uptake of trospectomycin and understanding its effects on membrane metabolism.

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