Pulmonary Targeting of Liposomal Triamcinolone Acetonide Phosphate

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Purpose. To explore the use of triamcinolone acetonide phosphate liposomes as a pulmonary targeted drug delivery system.

Methods. Triamcinolone acetonide phosphate liposomes composed of 1,2-distearoyl phosphatidylcholine and 1,2-distearoyl phosphatidyl glycerol and triamcinolone acetonide 21-phosphate dipotassium salt were prepared by dispersion and extruded through polycarbonate membranes. Encapsulation efficiency and *in vitro* stability at 37°C were assessed after size exclusion chromatography. TAP liposomes (TAP-lip) or TAP in solution (TAP-sol) were delivered to rats either by intratracheal instillation (IT) or intravenous (IV) administration. Pulmonary targeting was assessed by simultaneous monitoring of glucocorticoid receptor occupancy over time in lung (local organ) and liver (systemic organ) using an ex vivo receptor binding assay as a pharmacodynamic measure of glucocorticoid action.

Results. *In vitro* studies in different fluids over 24 hours, showed that more than 75% of the TAP remained encapsulated in liposomes. Cumulative pulmonary effects after IT administration of TAP-lip were 1.6 times higher than liver receptor occupancy. In contrast, there was no difference in the pulmonary and hepatic receptor occupancy time profiles when TAP was administered intratracheally as a solution. No preferential lung targeting was observed when TAP-lip was administered IV. As indicated by the mean effect times, lung receptor occupancy was sustained only when TAP-lip was administered IT.

Conclusions. Intratracheal administration of TAP-lip provided sustained receptor occupancy, and increased pulmonary targeting which was superior to IT administration of TAP-sol or IV administration of TAP-lip. The use of liposomes may represent a valuable approach to optimize sustained delivery of glucocorticoids to the lungs via topical administration.

KEY WORDS: triamcinolone acetonide; pulmonary targeting; liposomes; glucocorticoid receptors; sustained release.

INTRODUCTION

Glucocorticoids are beneficial in treating various pulmonary diseases, including asthma, sarcoidosis, and other conditions associated with alveolitis. Although systemic glucocorticoid therapy is effective in such conditions, prolonged administration carries the risk of toxicity and side effects (1). In attempts at reducing systemic side effects, several clinically efficacious glucocorticoids, including triamcinolone acetonide (TA), are employed for delivery as aerosols.

We have been interested in optimizing pulmonary targeting of glucocorticoids for inhalation therapy. In a recent study, we showed that lung specificity is achieved when glucocorticoid suspensions are administered intratracheally. In contrast, lung targeting is not observed when a glucocorticoid solution is administered intratracheally, presumably because of the fast absorption of the lipophilic steroid (2). This suggests that pulmonary targeting depends on slow release from the delivery form which results in a prolonged pulmonary residence time.

The use of liposomes has been suggested to provide sustained pulmonary release for various drugs including glucocorticoids such as beclomethasone dipropionate and dexamethasone (3, 4, 5; for a recent review see 6). However, we have found that while liposomes have a high loading capacity for lipophilic glucocorticoids such as TA under equilibrium conditions, TA is rapidly released under non-equilibrium conditions from the liposome matrix upon dilution or administration (7). In retrospect, this is predictable, given the observations of Schanker & co-workers (8) that lipophilic glucocorticoids cross membranes practically unhindered. Our findings question the benefits of achieving sustained pulmonary release from such preparations.

As liposomes seemingly provide no barrier function for such glucocorticoids we hypothesized that by encapsulating water-soluble derivatives of TA rather than the lipophilic parent compounds we could overcome this problem. If the liposomal membrane serves as a rate-limiting barrier for the release of a water-soluble TA derivative, then slow drug release and consequent improvement in pulmonary targeting might be possible.

We therefore selected the water-soluble salt triamcinolone acetonide phosphate (a prodrug of TA) for developing a formulation which captured the negatively charged TAP within liposomes. This formulation was delivered either by intratracheal or intravenous injection to rats and compared with intratracheally administered TAP-sol. Since pharmacodynamic effects of glucocorticoids are receptor mediated, tracking receptor occupancy in lung and liver using the above mentioned animal model allowed for indirect assessment of pulmonary and systemic effects.

MATERIALS AND METHODS

Materials

Analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.); (1,2,4-^{3}H) triamcinolone acetonide (45 Ci/mmol) was purchased from New-England Nuclear (Boston, MA). Lipids were obtained from Avanti Polar Lipids (Alabaster, AL).

Drug Solutions

Triamcinolone acetonide phosphate (TAP provided as a gift from Dr. K. Reininger, Bristol Myers Squibb, Regensburg, Germany) was dissolved in buffered saline to a concentration of 15 mg/ml. Before dosing, the respective stock solutions were diluted with buffered isotonic saline to 0.2 mg/ml of TAP.

Animals

All animal procedures were approved by the Animal Care Committee of the University of Florida, an AALAC approved

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facility. Specific-pathogen-free, non-adrenalectomized male F-344 rats, weighing approximately 250 g were housed in a 12 hr light/dark cycle, in a constant temperature environment. Animals were allowed free access to water and rat chow, but were food-fasted overnight prior to each experiment.

Liposome Preparations

Liposomes composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (89.4 mg) and 1,2-distearoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (10.6 mg) in a 9:1 molar ratio respectively, were dissolved in chloroform. The chloroform was removed by rotary evaporation at 63°C to obtain a dry lipid film. After adding one ml of 100 mg/ml TAP solution in isotonic buffer saline (PBS, Cellgro™, pH 7.5), the lipids were dispersed by shaking at 63°C for 2h. This crude liposome formulation underwent 10 cycles of freezing (in dry ice and methanol) and thawing at 63°C and was then extruded 30 times using a Liposofast® extruder (Avestin Co. Ottawa, Ontario, Canada) through 0.2 mm polycarbonate filters. Liposomes were sized using a submicron particle sizer (Nicomp model 270) to ensure size uniformity of the formulations, and stored under nitrogen at 4°C. On the day of the experiment, 200 μl of liposomal dispersion were passed through 10 ml Sephadex G-75 in a PD-10 column (Pharmacia, Sweden) with a PBS mobile phase. Liposomes were present in the first 4 to 6 ml of eluate (void volume). TAP concentrations were monitored by HPLC (See below.) For animal experiments, liposomes were diluted to a final concentration of TAP of 0.2 mg/ml. Isotonicity of liposomes was confirmed prior to use with a vapor pressure osmometer M 5500 (Wescor, Inc. Logan, UT). Lipid content was determined as previously described (9).

HPLC Method

Liposomal TAP concentration after gel filtration was determined in a 10 μl aliquot of the liposome dispersion by adding 480 μl of a mixture of 80:20 methanol:PBS and 10 μl of methylprednisolone (100 μg/ml ethanol solution) as an internal standard. The mixture was vortexed for 1 min and 70 μl of the solution was injected onto a Nucleosil C_{18} (150 × 4.6 mm) column, using a mixture of acetonitrile:0.05 M sodium phosphate buffer (pH 2) 35:65 v/v, as mobile phase at a flow rate of 1 ml/min. UV detection was performed at 254 nm. The lower limit of detection was 30 μg/ml.

Method for Recovering Lung Lavage Fluids

Rats were anesthetized and lung lavage fluid was obtained after tracheal cannulation with a 21 gauge polyethylene catheter. Approximately 5 ml of sterile isotonic saline were slowly injected to fill the lungs. The fluid was withdrawn by gentle aspiration, and spun at 2000 rpm for ten minutes at 4°C. The cell free supernatant was aspirated and used on the same day to assess the *in vitro* stability of liposomes.

In Vitro Stability

The *in vitro* stability of freshly prepared TAP-lip was tested at 37°C using PBS, rat lung lavage fluid, tissue culture medium RPMI-1640m or tissue culture medium with 10% fetal bovine serum. After gel filtration, an aliquot of the liposome preparation was diluted 5-fold (75-fold from the initial preparation) with the respective fluids (TAP final concentration of 100 μg/ml, a concentration that simulates sink conditions and is high enough to be quantified by the HPLC system employed). After incubation at 37°C, aliquots of 200 μl (in duplicate) were removed at 10, 30 min, 1, 3, 6, 18, and 24 h and the samples were then passed through Sephadex G-50 dry minicolumns of 1 ml bed size (10). Aliquots of 10 μl eluates were analyzed by HPLC.

Administration of Drugs

The animals were anesthetized via intraperitoneal injection and the corresponding TAP-lip or TAP-sol (100 μg/kg of TAP) administered as previously described (2). Animals (one animal per time point) were decapitated at 1, 2, 5, 6, 12, or 18 hours after IT or IV administration of TAP-lip. The lungs and livers were immediately processed for receptor binding studies. A total of 3 (12 and 18 hr) to 6 (0–6 hr) independent experiments were performed for a given time point after IT administration of TAP-lip. For the IT administration of TAP-sol and the IV administration of TAP-lip 4 and 2 animals were used per time point, respectively.

Experiments were performed on different days for every form (IV or IT) of administration and for each type of preparation; e.g. TAP-sol or TAP-lip. A sham animal (receiving either IV or IT buffered saline) was always included on the day of experiment. Each animal represented a single time point with paired data for both liver and lung.

Receptor Binding Assays

Receptor binding assays were performed as previously described (2) with modification. Aliquots of supernatants from tissue homogenates, (150 μl) were transferred into microcentrifugation tubes that contained 25 μl of ^{3}H-triamcinolone acetonide (^{3}H-TA) in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and, 30 nM) and 25 μl of incubation buffer or 25 μl of unlabeled TA (3 mM) to determine total and non-specific binding, respectively. Aliquots of 150 μl of the resultant cytosol preparations were transferred into microcentrifugation tubes containing 25 μl of ^{3}H-TA in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and 30 nM) and 25 μl of incubation buffer to determine the amount of total radioactivity.

Estimates for total bound ^{3}H-TA (TA_T), nonspecifically bound ^{3}H-TA (TA_{NS}) and free ^{3}H-TA (TA_F) were used to determine the number of available binding sites (B_{max}) and the equilibrium binding constant (K_d). The data were fitted to equation 1 using the non-linear curve-fitting program MINSQ (Micromath, Salt Lake City, UT).

$$TA_T = B_{max} * TA_F/(TA_F + K_d) + TA_{NS} \quad (1)$$

In instances where the high in vivo receptor occupancy did not allow for reliable B_{max} determination by the above method, B_{max} was estimated as the difference of TA_T values and TA_{NS} observed for incubation with 30 nM ^{3}H-TA (highest tracer concentration). This was justifiable as the comparison of calculated B_{max} values from both methods resulted in similar values for B_{max} ($R^2 = 0.985$, $n = 45$).

Pulmonary and hepatic B_{max} values were converted into % of control (average values for sham rats). K_d values were

necessary only to calculate B_{max}, and are not given in the result section.

For a given form of administration, differences between the pulmonary and hepatic receptor occupancies were tested by paired student T test. Data analyzed represented the pool of paired (hepatic and pulmonary) receptor occupancies for individual single time points (not AUC's) of all animals included in a given experimental sub-set. Statistical significance was assumed for $p < 0.05$.

For assessing differential receptor occupancy between lung and liver, the cumulative change from baseline (AUC) was calculated for the 6 and 18 hour investigation period by the trapezoidal rule from percent occupied receptor (E_x)-time profiles. The pulmonary targeting factor (T) was defined as

$$T = AUC_{Lung}/AUC_{Liver} \quad (2)$$

A targeting factor of greater than 1 would indicate preferential lung targeting. The area under the first moment curve ($AUMC_\infty$) was calculated by the trapezoidal rule from $E_x^* t_x$ versus t_x-pairs. Effects after the last measurement points were extrapolated for TAP-sol and data of reference 2 assuming a linear decline of the effect over time at late time points (11). These estimates were also used to derive AUC_∞. The mean pulmonary effect times (MET) were calculated consequently from AUC_∞ and $AUMC_\infty$(MET = $AUMC_\infty/AUC_\infty$).

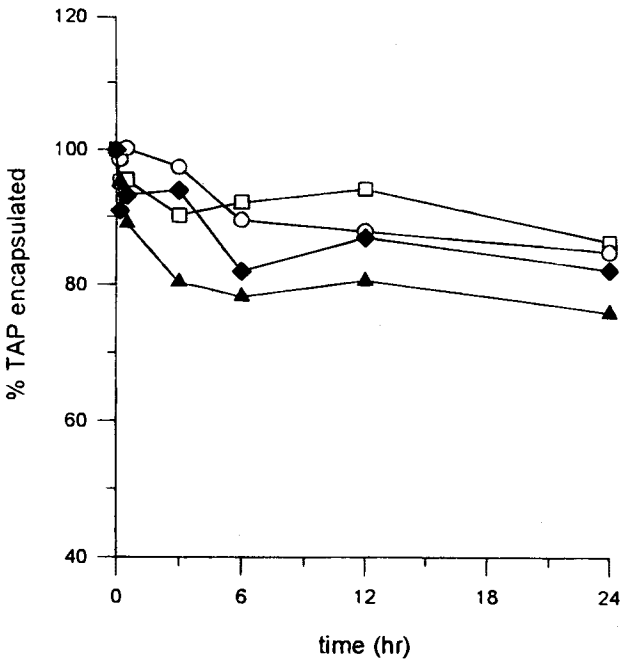

Fig. 1. *In vitro* stability of DSPC:DSP TAP liposomes at 37°C under sink conditions in PBS (◆), culture medium (□), lung lavage fluid (○), and 90% culture medium plus 10% fetal bovine serum (▲). The percentage of TAP remaining encapsulated in liposomes over time of incubation is shown.

RESULTS

Characterization of TAP-lip

The volume weighted mean particle diameter of the liposome obtained using a Gaussian distribution analysis was 207 ± 16 nm. A 40% average loss of lipid resulted after extrusion and gel filtration of liposomes. The encapsulation efficiency varied from 7 to 8.5% of the total amount of TAP, corresponding to a molar drug/lipid ratio of 1:7.

In Vitro Stability in Biological Fluids

Results of the liposome stability studies are shown in Fig. 1. The TAP-lip preparations retained greater than 75–80% of the entrapped drug through 24 hours when incubated in various fluids.

Receptor Binding Studies

To exclude potential assay artifacts (e.g. *ex vivo* release of glucocorticoids from intact liposomes present in resected tissues), experiments were performed in which cytosol from untreated animals was spiked with TAP-lip (TAP 20 μg) and consequently processed as described in Materials and Methods. There was a negligible difference between the % of free receptors of cytosol spiked with liposomes (85% ± 10%, $n = 4$) and control cytosol not spiked with TAP-lip (B_{max} = 100%).

There were statistically significant differences in glucocorticoid receptor occupancy between lung and liver after intratracheal administration of TAP-lip for the 18 h period. Receptor occupancy was more pronounced for lung than for liver (Fig. 2b), reflected also in an AUC based lung targeting factor of 1.6 (Table I). In contrast, there was no difference in these parameters when equivalent doses of TAP were administered IT as a solution, with the lung and liver curves being superimposable (Fig. 2a, Table I).

After IV administration of TAP-lip, no significant differences were observed in receptor occupancy profiles of lung and liver (Fig. 2c, Table 1) for at least the first 6 hours.

The lung receptor occupancy profiles between the various TAP preparations and methods of administration are compared in Fig. 3 together with previously reported data on triamcinolone acetonide solutions (TA-sol) (2). As is shown in this figure, lung receptor occupancy was significantly sustained after IT administration of TAP-lip relative to IT and IV of TAP-sol and TAP-lip, respectively, and to IT or IV administered TA-sol. The distinct sustained receptor occupancy characteristics of TAP-lip can be seen also from the estimates of the mean effect times (Table I). These MET values were 1.5–1.9 times longer than that of TAP-lip administered IV and TAP-sol administered IT (Table I), respectively.

DISCUSSION

TA is a clinically established, potent and inhalable synthetic glucocorticoid which binds to the glucocorticoid receptors. In contrast to TA, TAP (a prodrug of TA) is highly water-soluble (solubility in water > than 50 mg/ml), and although the encapsulation efficiency was substantially less than that of TA (2), we found TAP-lip (unlike TA-lip) to be more stable, even when incubated in buffers or biological fluids at 37°C (Fig. 1).

In the systemic circulation, TAP is cleaved rapidly to the pharmacologically active triamcinolone acetonide (12). Our results are in agreement with prior observations, as the receptor occupancy profiles after administration of the TAP-sol are very

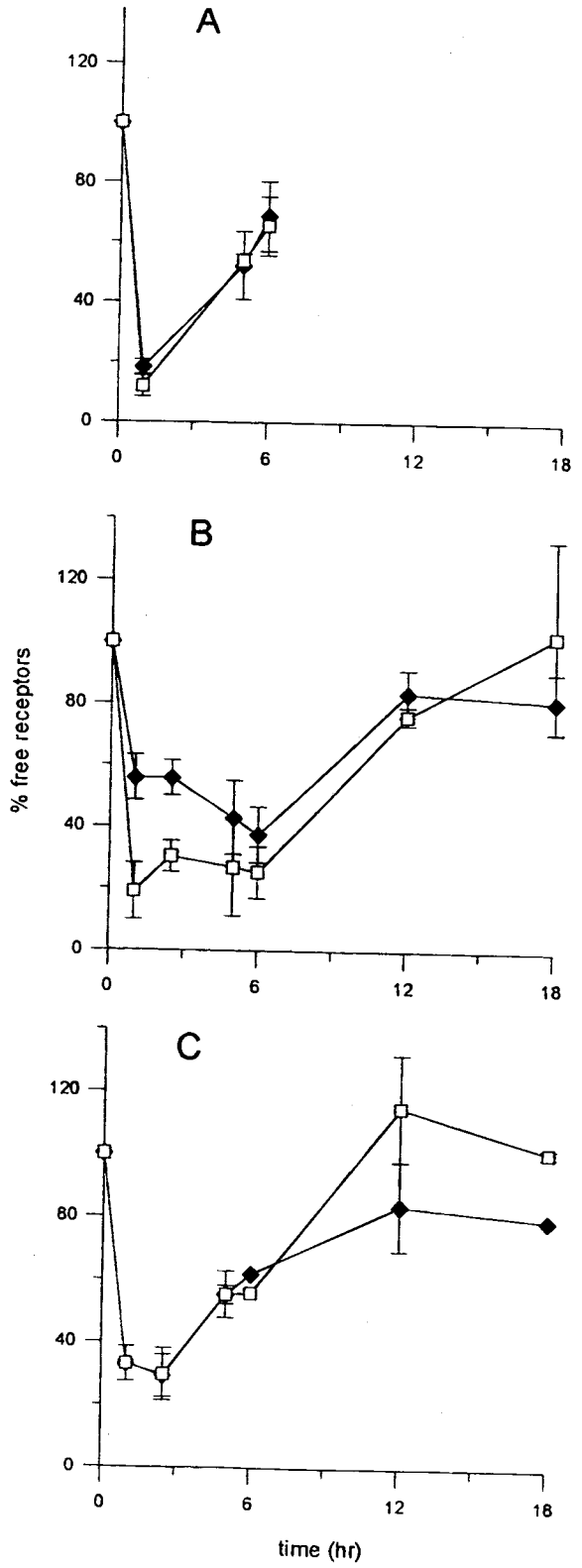

Fig. 2. Lung (□) and liver (◆) glucocorticoid receptor occupancy profiles after administration of 100 μg/kg of TAP: A) intratracheal instillation of TAP-sol; B) intratracheal instillation of TAP-lip; C) intravenously administered TAP-lip. Error bars represent mean ± S.D.

similar to what we have previously shown for TA-sol when administered in the same fashion (Fig. 3), suggesting a fast

Table I. Cumulative Receptor Occupancy (AUC), Lung Targeting Factor, and Mean Pulmonary Effect Times (MET) After Administration of TAP Liposomes (TAP-lip) and TAP Solution (TAP-sol)

	TAP-lip it adm		TAP-sol it adm	TAP-lip iv adm	
			AUC (%*h)		
	0–6h	0–18h	0–6h	0–6h	0–18h
Lung	450	770	350	490	370
Liver	280	620	340	500	600
Targeting Factor (AUC_{lung}/AUC_{liver})	1.6	1.2	1.0	1.0	0.6
Mean Pulmonary Effect Time (h)	5.7		3.0	3.8	

activation of TAP. In addition, the distinct pulmonary targeting of liposomal TAP suggests that this activation has to occur in the lung.

Incorporation of drugs into liposomes changes their pharmacokinetic behavior after pulmonary instillation (5), resulting in longer mean residence time in the lung and the potential for sustained pulmonary effects. Based on our experiments, intratracheal administration of TAP-lip resulted in a pronounced and sustained occupancy of pulmonary receptors when compared to pulmonary delivery of TAP-sol or intravenously administered TAP-lip (Fig. 3, Table I). This suggests that the sustained receptor occupancy is linked to the pulmonary administration of the liposomal preparation, while the use of TAP-sol or the intravenous administration of TAP-lip do not result in the sus-

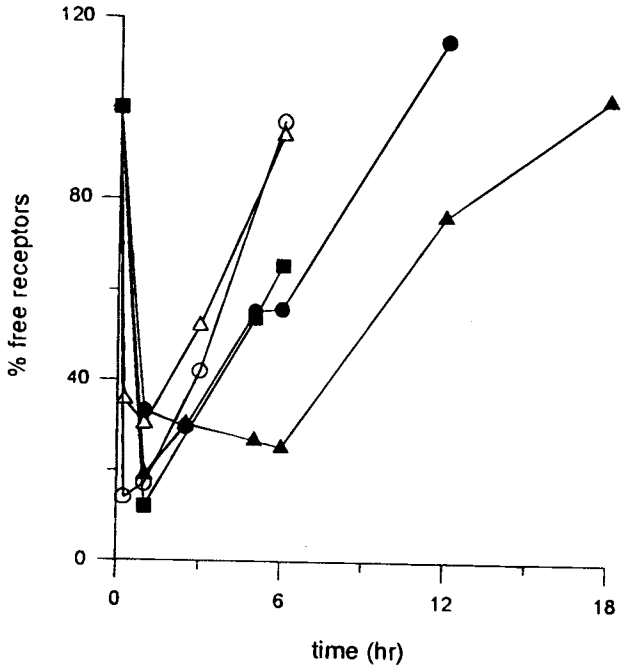

Fig. 3. Lung glucocorticoid receptor occupancy profiles after administration of 100 μg/kg of TAP: Intratracheal TAP-sol (■), intratracheal TAP-lip (▲), intravenous TAP-lip (●). Comparison is made to intratracheal TA-sol (△) and intravenous TA-sol (○) profiles from Ref. (2).

tained occupation of pulmonary receptors (Fig. 3). These results are consistent with the *in vitro* stability of the TAP liposomes in biological fluids (Fig. 1) and prolonged pulmonary release of TAP from the liposomes. In pharmacokinetic-pharmacodynamic (PK-PD) simulations, we have shown that the MET depends on the release rate of the drug delivery system in such a way that the slower the release, the larger the MET (unpublished observations). On the basis of this relationship, the longer MET value found for TAP-lip argues also for a prolonged release of this formulation.

Besides providing a sustained receptor occupancy, the main goal of this study was to test whether liposomes can improve pulmonary targeting after topical delivery. Previous studies (2) using TA suspensions and solutions suggested that pulmonary targeting, as indicated by a ratio of the cumulative pulmonary and hepatic receptor occupancy, is achieved only if the drug is absorbed slowly from the lung; e.g. due to a slow dissolution rate of the instilled particles. This study shows that TAP-lip, with presumably prolonged release characteristics, but not TAP-sol, confers pulmonary targeting. The resulting targeting factor of 1.6 was significantly larger than estimates for TAP-sol (1.0) or IV liposomes. Although achieving a pronounced targeting for tissues with high organ blood flow, such as the airways is theoretically difficult (13), our results show that liposomes increase the therapeutic availability of drugs in lung tissue (presumably due to prolonged release) therefore increasing tissue targeting.

Our study shows that triamcinolone acetonide phosphate can be incorporated in a stable liposomal formulation which appears suitable for pulmonary delivery. With intratracheal delivery, TAP-lip provides sustained receptor occupancy properties and improved pulmonary targeting superior to TAP-sol alone. Whether this formulation will ultimately result in superior clinical activity in pathologic states in humans cannot be ascertained, but deserves further study.

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