resistance for this drug. While the alkylating properties of these adducts have not yet been investigated, it would be anticipated that these conjugates are less reactive than melphalan itself. If so, conjugation with glutathione could provide a mechanistic basis for the development of cellular resistance to the drug, already known to be associated with elevated glutathione and transferase levels. In addition, development of the analytical methodology to characterize these adducts may be useful in further study of the resistance phenomenon *in vivo*. Currently, studies are under way to determine whether glutathione conjugates of melphalan are formed in melphalan-resistant cells.

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Glutathione turnover in perfused rabbit lung. Effect of external glutathione

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In the past few years there has been an interest in studying the utilization of external glutathione (GSH), GSH esters, or its precursors for augmenting the intracellular reserve of GSH [1–9]. In the body, the kidney is the primary organ which utilizes circulating plasma GSH by virtue of the high transpeptidase activity [10, 11]. Utilization of external GSH by perfused rat lung for conjugation reaction and as cellular GSH reserve has also been reported [8, 9]. These authors suggest the involvement of transpeptidase activity in the catabolism of the circulating GSH leading to the utilization of precursors thus formed, by the lungs. Since there are considerable species differences between rat and rabbit lung drug-metabolizing enzymes [12, 13], we wished to study the pulmonary utilization of externally provided GSH in rabbit lungs.

Materials and methods

Male New Zealand white rabbits (2 to 2.5 kg), obtained from a local source and maintained at our Central Animal Facilities, were used for the present study. Rabbits were treated with diethylmaleate (DEM) (6 mmoles/kg in 3 ml corn oil/kg, i.p.), and the controls received the corn oil vehicle only, 1 hr prior to surgery. Rabbits were anesthetized (Nembutal, 50 mg/kg) and heparinized (sodium heparin, 1000 units/rabbit) by i.v. injection through the marginal ear vein. Lungs were surgically removed from normal and DEM-pretreated rabbits. The oxygenated Krebs–Ringer bicarbonate buffer, supplemented with 4.5% bovine serum albumin (BSA) and 5 mM glucose (pH 7.4), was used for perfusion. For some perfusions, BSA was replaced by dextran (5%). The perfusion apparatus and the entire procedure for the recirculating system have been described previously [14, 15]. Lung perfusions were allowed to equilibrate for 10 min before the addition of GSH. Perfusate samples were collected over 1 ml perchloric acid (PCA) (final concentration 1.75%) at different time intervals. Upon termination of the perfusion after 1 hr, lungs were quickly washed with 75 ml saline in order to wash out any perfusate from the vasculature. Tissue samples were minced in PCA (at a final concentration of 1.75%), homogenized, and centrifuged. GSH and oxidized glutathione (GSSG) in the tissue and perfusate were analyzed by the HPLC method of Reed *et al.* [16].

DEM-pretreated lungs were found to deteriorate to a greater extent and more rapidly during perfusion. This could have occurred due to a rapid and severe loss of cellular GSH. Histological changes due to O_2 sensitivity in DEM-treated rat lungs have been reported recently [17]. Since depletion of GSH was a necessary experimental manipulation for some of our experiments, the criterion of 10% increase in lung weight was relaxed to 20%. Lower doses of DEM (1 and 3 mmoles/kg) failed to deplete GSH

to a considerable extent, therefore we used DEM at 6 mmoles/kg. Approximately 50% of the lungs from DEM-pretreated rabbits included in the results showed an increase in lung weight of between 10 and 20%. Signs of nausea were observed in some DEM-pretreated rabbits.

Blank experiments were conducted to determine the stability of GSH in the perfusate containing BSA or dextran while circulating in the perfusion apparatus, without the lung. GSH (1 mM) was added to the recirculating perfusate under perfusion conditions identical to the lung perfusion. Perfusate samples were collected at various time points for analysis of GSH, and GSSG, by HPLC. Recoveries of added GSH, GSSG, in BSA- and dextran-perfusates were calculated by comparison with standards. Recoveries for GSH and GSSG were 92 and 100%, respectively, in BSA-perfusate, while in the dextran-perfusate recoveries were 85 and 90% respectively. All sample values for dextran-perfusate are corrected for 100% recovery.

Comparisons between tissue levels of GSH and GSSG were subjected to Student's *t*-test. Criterion for statistical significance was set at $P < 0.05$.

Results and discussion

GSH values reported for the perfused rabbit lung [18, 19] are comparable to the values obtained in the present investigation (Table 1). The species differences in normal GSH levels with lung tissue are apparent between rats and rabbits [9]. To obtain a considerable degree of GSH depletion, we used DEM (6 mmoles/kg) because under conditions of severe GSH (cellular thiol) deficiency the mechanism for the utilization of external GSH, if it existed, would be maximized. Pulmonary GSH depletion was ascertained by using liver values for comparison. Pulmonary and hepatic GSH levels were decreased by DEM pretreatment (Table 1). For the perfusion, we used two kinds of colloidal components: BSA and dextran. In the blank experiment it was observed that added GSH rapidly disappeared from the perfusate containing BSA in the absence of lung, indicating the interaction of GSH with BSA (data not shown). Therefore, it became necessary to find a suitable replacement for BSA in these studies. As an alternative we replaced BSA with heavy molecular weight dextran (mol. wt ~81,500). GSH was ten times more stable in dextran-perfusate (data not shown). GSH and GSSG were released from control and DEM-pretreated lungs perfused in the absence of external GSH. The values of GSH and GSSG released from control lung (2.9 to 3.5 and 1.0 to 1.9 nmoles/ml respectively) in the dextran-perfusate were greater than in the BSA-perfusate (1.5 to 1.9 and 0.5 to 0.9 nmoles/ml respectively). This difference could have been due to instability of GSH and GSSG in the BSA-perfusate. The marked decrease in the release of GSH and GSSG by DEM pretreatment (0.6 to 1.1 and 0.4 to 0.5 nmoles/ml, respectively, with BSA-perfusate and 0.8 to 1.2 and 0.5 to 0.9 nmoles/ml, respectively, with dextran-perfusate) may have been due to decreased levels of GSH and GSSG in the lung (Table 2). The increase in pulmonary GSSG after perfusion in the presence of external GSH (Table 2) may have been due to a small quantity of perfusate containing GSSG (formed by oxidation of GSH, Fig. 1) left in the lung tissue after the saline wash.

Externally added GSH did not replenish pulmonary GSH in the control lungs (Table 2). Perfusion with dextran-perfusate caused an apparent greater depletion of GSH in the control lung; however, it was statistically insignificant. GSH inclusion in the BSA-perfusate resulted in a small but significant increase of GSH in perfused DEM-pretreated lungs (Table 2); this represented replenishment of only a small fraction of the GSH deficiency. Qualitatively these findings do agree with the findings of Berggren *et al.* [9].

Table 1. Hepatic and pulmonary GSH and GSSG levels in control and DEM-pretreated, unperfused rabbit lungs

	GSH (μmoles/g)		GSSG (μmoles/g)	
	Lung	Liver	Lung	Liver
Control	2.92 ± 0.13	8.00 ± 0.58	0.05 ± 0.01	0.09 ± 0.02
DEM-pretreated	0.46 ± 0.05*	1.40 ± 0.23*	0.02 ± 0.01	0.04 ± 0.01

Lungs from corn oil vehicle-pretreated (control) and DEM-pretreated rabbits were perfuse-washed with saline (75–100 ml); livers were not washed. Tissues were immediately processed for HPLC analysis of GSH and GSSG. Values represent mean ± S.E. of four to seven rabbits/group.

* Indicates significant difference from control value, $P < 0.05$.

Table 2. Effect of including GSH in the perfusate on GSH and GSSG levels in perfused lungs from control and DEM-pretreated rabbits

	Lung tissue GSH (μmoles/g)	GSSG	Lung tissue GSH (μmoles/g)	GSSG
	Perfusate with BSA		Perfusate with dextran	
Control				
−GSH	2.58 ± 0.40	0.03 ± 0.01	2.11 ± 0.32	0.03 ± 0.004
+GSH	2.26 ± 0.11	0.13 ± 0.05*	2.14 ± 0.12	0.06 ± 0.01
DEM-pretreated				
−GSH	0.40 ± 0.02†	0.01 ± 0.003	0.37 ± 0.06†	0.01 ± 0.004
+GSH	0.87 ± 0.15*†	0.09 ± 0.02*	0.65 ± 0.10†	0.05 ± 0.01*

Lungs from control and DEM-pretreated rabbits were perfused for 1 hr in the presence or absence of 1 mM GSH in the perfusate. Perfusate contained either BSA (4.5%) or dextran (5%). Values represent mean ± SE of three to four lung perfusions.

* Indicates significant difference ($P < 0.05$) from respective -GSH groups.

† Indicates significant difference ($P < 0.05$) from respective control lung-perfusions.

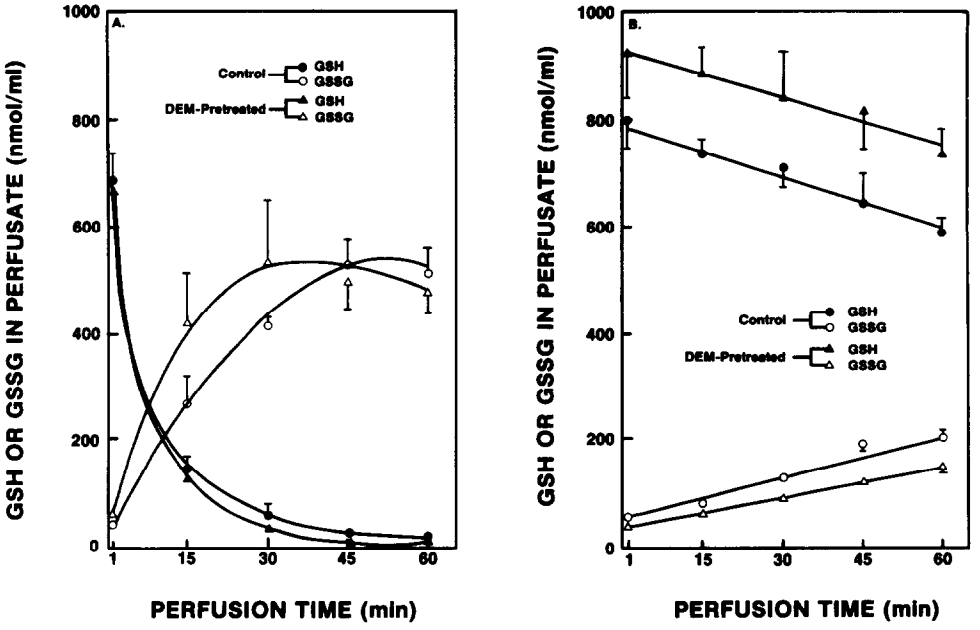

Fig. 1. Fate of externally added GSH in the perfusate during lung perfusion. Perfusate was prepared using 4.5% BSA (A) or 5% dextran (B). Lungs from control and DEM-pretreated rabbits were initially perfused for 10 min without adding GSH. GSH was then added to achieve a 1 mM concentration. Perfusate samples were collected for analysis of GSH and GSSG. Values represent mean ± S.E. of three to four individual perfusions. The $T_{1/2}$ values for GSH were calculated by semilogarithmic plots of the data points of 45-min perfusion because 98–99% of added GSH was lost from the perfusate after that time. The calculated $T_{1/2}$ values in the BSA-perfusate were 6.9 ± 1.0 min for control lung and 6.2 ± 1.0 min for DEM-pretreated lungs. The $T_{1/2}$ values of GSH in the dextran-perfusate were 123 ± 36 min for control and 165 ± 34 min for DEM-pretreated lungs (calculated by extrapolation). GSSG values are expressed as GSH equivalents.

However, quantitatively the replenishment of GSH in the present investigation is only one-third of the values reported for perfused DEM-pretreated rat lung [9]. The difference in the replenishment could be due to species differences. This inability to effectively utilize external GSH was unrelated to the rapid disappearance of GSH in the presence of BSA since lungs perfused with dextran-perfusate utilized GSH even less efficiently (Table 2). It is unlikely that even the small replenishment of GSH in the perfused lung was due to utilization of GSH as such because a considerably greater proportion of pulmonary GSH deficiency would have been ameliorated in the presence of dextran-perfusate, which allowed extended preservation of external GSH. It is also evident from the present investigation that the lung tissue did not play a significant role in the catabolism of GSH because the $T_{1/2}$ of GSH in the presence of lung did not differ significantly from the blank perfusions with dextran-perfusate (Fig. 1B). The kidney plays a major role in the catabolism of circulating GSH in the body [10, 11], by virtue of the high transpeptidase activity. Using rat lung perfusions the authors [8, 9], assigned a greater role of transpeptidases in the catabolism of external GSH. In both studies, BSA was used for the perfusate preparation. Our findings with the rabbit lung indicate that recycling of added GSH by transpeptidases may not be significant in meeting tissue demand of thiols. Once again, the differences may be simply a reflection of the species differences.

In conclusion, GSH was not effectively utilized or metabolized by rabbit lungs to meet the cellular demand of GSH. Partial replenishment of GSH observed in the DEM-pretreated perfused rabbit lung may have been due to the uptake and utilization of precursor components formed by reaction of GSH with BSA. To a small degree, similar processes may occur in the circulating plasma since albumin or other proteins are available for interaction with GSH in the blood circulation. Even under conditions in which such mechanisms might be expected to operate maximally, it appears unlikely that these processes would be able to provide sufficient GSH to meet the cellular demand.

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Effects of serum-fractions from patients with Eaton–Lambert syndrome on rat cortical synaptosomal [^{3}H]acetylcholine release

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Eaton–Lambert syndrome is a rare disorder characterized by deficits in neuromuscular transmission that result from a presynaptic inhibition of acetylcholine (ACh) release [1, 2]. The electrophysiological characteristics of the disease include normal miniature end-plate potential (MEPP) amplitudes, reduced number of quanta at low levels of nerve stimulation, and marked increases in the amplitude of the compound muscle action potential evoked by repetitive, supramaximal nerve stimulation [1]. Biochemically, it is known that the number of nicotinic ACh receptors [3], the ACh levels [4], and choline acetyltransferase [4] are unaltered in biopsied muscles from Eaton–Lambert patients. The identity and mechanism of action of the circulating inhibitor are unknown. Some neoplastic forms of the syndrome may involve the synthesis and release of neuroactive tumor-peptides, since tumor-extracts from patients with the disease can inhibit neuromuscular transmission *in vitro* [5, 6]. Alternatively, some cases of Eaton–Lambert syndrome may involve an autoimmune disorder since IgG isolated from Eaton–Lambert patients and injected into mice for several weeks can attenuate neuromuscular transmission [7] and reduce the spontaneous and electrically stimulated release of ACh from skeletal tissue [8].

In this study we used rat cortical synaptosomes to ascertain whether a neurochemically heterogeneous, non-neuromuscular tissue was also sensitive to the cholinolytic actions of the circulating inhibitor in Eaton–Lambert syndrome. We also compared the effects of the circulating inhibitor on high affinity uptake and release for the first time. Our results indicate that this preparation will be useful for characterizing the neurochemical selectivity of the circulating release-inhibitory factor.

Methods

[*Methyl*-^{3}H]Choline (80 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). Choline kinase was purchased from the Sigma Chemical Co. (St. Louis, MO). All solutions were made in Krebs–Ringer bicarbonate buffer (KR) as described previously [9].

Synaptosomal preparation. Synaptosomes were prepared from the cerebral cortices of adult male Sprague–Dawley rats (175–225 g) as described previously [10]. The synaptosomes were washed twice and resuspended in 2.5 ml of oxygenated KR buffer. To measure high affinity [^{3}H]choline uptake and [^{3}H]ACh synthesis, synaptosomes (0.5 to 1 mg protein/ml KR) were incubated for 3 min at 37° with 1 μM [^{3}H]choline plus or minus 5 μM hemicholinium-3, and then washed twice by centrifugation with cold KR. Some samples were treated with serum-fractions by substituting KR with equal volumes of serum-fractions. The synaptosomal pellet was then lysed in 0.5 ml of 5 mM sodium phosphate buffer (pH 7.4) containing 50 μM eserine, and the total hemicholinium-3 sensitive [^{3}H]choline uptake and [^{3}H]ACh synthesis were assayed as described previously [11].

Synaptosomes were incubated with 1 μM [^{3}H]choline for 10 min at 37° to load them with [^{3}H]ACh. They were then washed and resuspended in 20 ml of KR containing 50 μM eserine. Aliquots (0.3 ml, approximately 0.4 mg protein) of the synaptosomal suspension were added to 0.7 ml of the same buffer or equal volume substitution of serum-fraction. They were incubated for 3 min in the presence or absence of specified serum-fractions and depolarizing K^+ concentrations. Release-incubations were terminated by cooling the tissues in an ice bath for 5 min, followed by centrifugation at 10,000 *g* for 10 min. The inhibition of spontaneous and depolarization-induced release was normalized for daily variations in release by expressing values as a percentage of controls (untreated, 5.5 mM KCl). The average of duplicate values for each preparation of synaptosomes was obtained, multiplied by 100, and then averaged with other normalized values from other preparations. Labeled ACh in the synaptosomal supernatant fractions was separated from choline by liquid cation exchange as described previously [11]. Comparisons among several means were determined with one-way analysis of variance (F-test).

Protein determinations. Synaptosomal protein levels were determined using the Bio-rad procedure which involves Coomassie blue staining of the proteins.

Patient summaries. L. B. was a 61-year-old white woman who presented to the Medicine service with a 3-month history of weakness. On neurological examination, she was found to have normal cranial nerves. She had mild proximal muscle weakness in both upper and lower extremities without change with repetitive movements. Distal strength was normal. Sensory examination was normal, and deep tendon reflexes were equal bilaterally. Chest X-ray revealed a solitary pulmonary nodule in the right lung which on biopsy