Biochemical Pharmacology, Vol. 32, No. 18, pp. 2683–2688, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

CHLORPHENTERMINE-INDUCED ALTERATIONS IN PULMONARY PHOSPHOLIPID CONTENT IN RATS

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(*Received* 3 *September* 1982; *accepted* 21 *February* 1983)

Abstract—Daily, intraperitoneal administration of the anorectic drug chlorphentermine (30 mg/kg) for 5 days to rats significantly increased phosphatidylcholine and total phospholipid content after 1 week and reached a maximal level 4 weeks after treatment in whole lung tissue (unlavaged lungs) and in sessile tissue in which alveolar lipids and macrophages were removed by pulmonary lavage (lavaged lungs). In lavaged lung, a significant rise in the content of sphingomyelin, phosphatidylserine plus phosphatidylinositol component, and phosphatidylethanolamine plus phosphatidylglycerol fraction occurred after 2 weeks, remained at this increased level for 4 weeks, and was followed by a return to control amounts after 5 weeks. In unlavaged lung, the chlorphentermine-induced elevation in sphingomyelin content seen after 1 week persisted at this same significant level even 5 weeks after treatment. Regardless of experimental duration, pulmonary glycogen levels were not altered markedly by chlorphentermine in unlavaged or lavaged tissue. Phenobarbital (30 mg/kg) did not markedly alter pulmonary glycogen and phospholipid component levels. Simultaneous phenobarbital and anorectic drug administration prevented the chlorphentermine-induced rise in total phospholipid, sphingomyelin, and phosphatidylcholine in unlavaged lung without a change in glycogen. A 7-day withdrawal from chlorphentermine treatment in rats previously injected with drug for 2 weeks resulted in a return to control in the levels of sphingomyelin, phosphatidylcholine, and total phospholipid in unlavaged lung. Extension of withdrawal from treatment for 2 weeks produced a significant decrease in all phospholipid components below control values, suggesting that a possible imbalance in synthetic and catabolic activity may persist after drug removal. The concentration of lung glycogen was not altered significantly by chlorphentermine treatment or withdrawal from drug administration. Our results indicate that the chlorphentermine-induced rise in phospholipid components was time-dependent in lavaged and unlavaged lungs, and the increase in phosphatidylcholine occurred independently of a change in glycogen. In addition, the present study shows that the chlorphentermine-induced changes in phospholipid levels are reversible and almost completely prevented by phenobarbital.

Morphologic examination of pulmonary tissue reveals massive accumulations of hypertrophic alveolar macrophages in rats chronically administered chlorphentermine [1]. The observed chlorphentermine-induced ultrastructural alterations are accompanied by a dramatic increase in phospholipid content in alveolar macrophages [2] and whole, unlavaged lung [3]. It is of interest that the elevation in alveolar macrophage phospholipid content extracted from chronic chlorphentermine-treated lungs is 10- to 19-fold higher than is obtained from controls [2, 4, 5]. The finding by Schmien *et al.* [4] that chlorphentermine increases phospholipid content in unlavaged lung by a factor of 3 suggested that most of the changes in phospholipid are associated with alveolar macrophages and alveolar lipids rather than with the other fixed populations of pulmonary cells. Two aims of this study were: (a) to determine whether the chlorphentermine-induced elevation in whole lung phospholipid content is time dependent in rats, and (b) to determine effects of chlorphentermine on phospholipid levels in lavaged tissue.

It has been established that the chlorphentermine-induced accumulation of phospholipid-laden alveolar macrophages can be modified by pharmacological manipulation. Kacew and Narbaitz [6] demonstrated that hyperoxygenation of rats facilitates the production of pulmonary histiocytosis by a dose of chlorphentermine which would not otherwise produce this morphologic change. In contrast, simultaneous treatment with chlorphentermine and phenobarbital either completely prevents or reduces the number of hypertrophic alveolar macrophages in rat lung [7, 8]. Similarly, withdrawal from chlorphentermine treatment is associated with a disappearance of these macrophages from rat pulmonary alveoli [9, 10]. Experiments were also undertaken to examine the influence of concurrent chlorphentermine and phenobarbital administration as well as the effects of withdrawal from chlorphentermine on lung phospholipids.

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MATERIALS AND METHODS

Animals. Male Long–Evans hooded rats, weighing between 250 and 300 g, were purchased from Charles

River Laboratories, Wilmington, MA. All animals were maintained on Wayne Laboratory Chow and had free access to water throughout the course of the experiments.

Experimental procedures. In time–course studies, chlorphentermine hydrochloride was administered intraperitoneally at a dose of 30 mg/kg for 5 days/week for either 1, 2, 3, 4 or 5 weeks. Corresponding controls were pair-fed and received an equal volume (1 ml/kg) of physiological saline. Since no significant differences were found in the pulmonary glycogen and phospholipid levels between controls at 1, 2, 3, 4 and 5 weeks, the controls were combined into a single value, which was then used for comparison with drug-treated groups. Twenty-four hours after the last chlorphentermine or saline injection, rats were anesthetized with sodium pentobarbital (100 mg/kg) and exsanguinated by severing the abdominal aorta. For unlavaged samples, lungs were removed and immediately frozen in liquid nitrogen. In the case of lavaged tissue, alveolar macrophages were removed from lung according to the method described by Reasor and Koshut [5]. After removal of lavage fluid, the remaining tissue was excised and immediately frozen in liquid nitrogen for subsequent biochemical assays.

In certain experiments to evaluate the effects of concurrent drug treatment, chlorphentermine (30 mg/kg) was administered daily i.p. in the morning for 1 week (5 days/week) while phenobarbital (30 mg/kg/day) was given p.o. an hour after the anorectic on each day. Separate groups of rats were also injected with either saline or chlorphentermine (30 mg/kg) i.p. as well as being given distilled water or phenobarbital (30 mg/kg) p.o. alone for 1 week (5 days/week). Twenty-four hours after the last injection, animals were killed as described previously, and lungs were excised and frozen in liquid nitrogen.

In the withdrawal studies, rats were administered either chlorphentermine (30 mg/kg, i.p.) or saline for 2 weeks (5 days/week), and these groups of animals were then maintained without further treatment for a period of either 1 or 2 weeks. After drug removal for 1 or 2 weeks, animals were killed with pentobarbital, and lungs were excised and frozen in liquid nitrogen.

Biochemical assays. The total phospholipid content of lavaged or unlavaged pulmonary tissue was extracted according to the method described by Folch *et al.* [11]. The method of Rouser *et al.* [12] was used for the separation of individual phospholipid classes by thin-layer chromatography. Both total phospholipid and individual phospholipid class phosphorous were determined spectrophotometrically using the method of Chen *et al.* [13]. In addition, a portion of lung was immersed in 1 ml of boiling 30% potassium hydroxide for assaying glycogen by the anthrone method of Seifter *et al.* [14].

Drug and chemicals. All reagents were of the purest grade available and dissolved in double glass-distilled water. Chlorphentermine (Warner–Chilcott Inc., Morris Plains, NJ) was dissolved in physiological saline. Precoated chromatoplates with silica gel 80Å Whatman type K5 were purchased from Chromatographic Specialities Ltd., Brockville, Ontario. Lipid standards were obtained from Serdary Research Laboratories, London, Ontario. All other biochemicals used for various assays were obtained commercially from the Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. In the time–course study using lavaged or unlavaged lungs, data were analyzed statistically employing Student's *t*-test; significant differences between the mean values are indicated when the P value was <0.05. In the withdrawal and concurrent drug administration studies, data were analyzed statistically by a simple one-way analysis of variance. When significant differences ($P < 0.05$) were indicated, the data were subjected to Duncan's multiple range test to pinpoint the groups that were significantly different.

RESULTS

Time–course studies. Since chlorphentermine is known to restrict food and water consumption [15], the possibility exists that the observed changes in total phospholipid (TPL) were due to starvation. To determine the role of starvation, the effect of chlorphentermine on glycogen, a major source of energy in lung [16], was examined. Data in Table 1 show that, regardless of duration of chlorphentermine administration, pulmonary glycogen levels remained insignificantly different from controls. Our results support the view that the chlorphentermine-induced alterations presented in this study are not due to starvation and that the use of pair-fed controls was proper. The administration of chlorphentermine to rats resulted in a significant increase in TPL after 1 week of treatment (Table 1). The elevation remained at the subsequent treatment times with the maximal elevation (approximately 2.7-fold) occurring after 4 weeks. Following removal of alveolar macrophages and alveolar lipids by pulmonary lavage, the TPL was still elevated in lavaged lung at a relative level corresponding to that seen with unlavaged lung.

Data in Table 2 illustrate the effects of chlorphentermine on the levels of the individual phospholipid (PL) classes of unlavaged lungs. A significant increase in the sphingomyelin (S) content was observed after 1 week of treatment, and this did not change throughout the remaining treatment period. The level of phosphatidylcholine (PC) was also increased after 1 week of chlorphentermine but, in contrast to S, the content of PC continued to increase in a time-dependent manner with a maximum elevation of 3.6-fold seen after 4 weeks of drug administration. Although no marked change was found in phosphatidylethanolamine (PE) plus phosphatidylglycerol (PG), a significant rise in the level of phosphatidylserine (PS) plus phosphatidylinositol (PI) occurred after 4 and 5 weeks of chlorphentermine.

The levels of the individual PL classes in lavaged lungs are shown in Table 3. As with unlavaged lungs, the content of S was elevated significantly after 2 weeks and remained at this level throughout the remainder of the treatment. Similarly, PC was increased at all times studied. However, in contrast to the unlavaged lungs, the content of PS plus PI and PE plus PG was elevated during weeks 2 through 4 of drug treatment.

Table 1. Effect of chlorphentermine on total phospholipid and glycogen levels in unlavaged and lavaged rat lung*

Experimental duration (weeks)	Unlavaged		Lavaged	
	Total phospholipid (mg/g)	Glycogen (mg/g)	Total phospholipid (mg/g)	Glycogen (mg/g)
Pair-fed control	31.9 ± 2.2	0.68 ± 0.12	14.6 ± 0.4	0.43 ± 0.09
1	51.2 ± 1.9†	0.81 ± 0.08	22.5 ± 2.5‡	0.49 ± 0.02
2	60.1 ± 2.3†	0.60 ± 0.07	26.0 ± 1.4‡	0.43 ± 0.05
3	55.2 ± 4.7†	0.75 ± 0.10	26.5 ± 1.2‡	0.61 ± 0.04
4	85.1 ± 7.6†	0.62 ± 0.03	36.3 ± 2.2‡	0.42 ± 0.04
5	82.8 ± 6.1†	0.65 ± 0.01	29.4 ± 1.7‡	0.45 ± 0.23

* Each value is the mean ± S.E.M. of five animals per group. Adult rats were injected with chlorphentermine (30 mg/kg, i.p., for 5 days/week) for either 1, 2, 3, 4 or 5 weeks, while corresponding controls received physiological saline. Since no significant differences were noted between controls after 1, 2, 3, 4 or 5 weeks, values from 2-week saline-injected rats were used as representative values. The values for 5-week pair-fed control levels of phospholipid and glycogen were: unlavaged total phospholipid, 33.8 ± 3.1 mg/g; unlavaged glycogen, 0.66 ± 0.06 mg/g; lavaged total phospholipid, 16.5 ± 0.9 mg/g; and lavaged glycogen, 0.44 ± 0.8 mg/g.

† Statistically significant difference when compared with the unlavaged control values ($P < 0.05$).

‡ Statistically significant difference when compared with the lavaged control values ($P < 0.05$).

Table 2. Time–course of chlorphentermine-induced alterations in unlavaged rat lung phospholipid composition*

Experimental duration (weeks)	Phospholipid class (mg/g)			
	S	PS + PI	PE + PG	PC
Pair-fed control	7.41 ± 0.55	7.99 ± 0.32	6.61 ± 0.52	12.88 ± 1.01
1	10.09 ± 0.40†	7.96 ± 0.73	7.91 ± 0.38	25.07 ± 1.37†
2	10.28 ± 0.79†	9.05 ± 0.77	6.97 ± 0.38	33.79 ± 1.83†
3	9.64 ± 0.52†	8.60 ± 1.53	8.86 ± 1.13	28.06 ± 1.83†
4	8.92 ± 0.26†	12.66 ± 1.63†	8.24 ± 1.10	46.92 ± 1.86†
5	10.01 ± 0.56†	13.03 ± 1.29†	6.69 ± 0.46	45.27 ± 0.89†

* Each value is the mean ± S.E.M. of five animals in each group. For experimental details, see the legend to Table 1. The values for 5-week pair-fed control levels of individual phospholipids measured were: S, 7.23 ± 0.49 mg/g; PS + PI, 7.85 ± 0.35 mg/g; PE + PG, 8.29 ± 0.76 mg/g; and PC, 14.06 ± 1.35 mg/g.

† Statistically significant difference when compared wih the control values ($P < 0.05$).

Table 3. Time–course of chlorphentermine-induced changes in lavaged lung phospholipid composition*

Experimental duration (weeks)	Phospholipid class (mg/g)			
	S	PS + PI	PE + PG	PC
Pair-fed control	3.41 ± 0.29	4.39 ± 0.19	3.81 ± 0.25	3.54 ± 0.25
1	4.12 ± 0.20	5.24 ± 0.82	5.04 ± 0.68	8.04 ± 1.23†
2	5.37 ± 0.42†	5.65 ± 0.33†	5.31 ± 0.39†	9.36 ± 0.75†
3	5.91 ± 0.51†	6.20 ± 0.96†	5.24 ± 0.63†	9.09 ± 0.56†
4	4.28 ± 0.25†	5.61 ± 0.51†	7.35 ± 0.91†	14.62 ± 1.14†
5	5.73 ± 0.44†	4.46 ± 0.25	4.51 ± 0.32	10.38 ± 1.48†

* Each value is the mean ± S.E.M. of five animals in each group. For experimental details, see the legend to Table 1. The values for 5-week pair-fed control levels of individual phospholipids measured were: S, 3.76 ± 0.25 mg/g; PS + PI, 4.45 ± 0.23 mg/g; PE + PG, 4.01 ± 0.14 mg/g; and PC, 4.43 ± 0.37 mg/g.

† Statistically significant difference when compared with the control values ($P < 0.05$).

Table 4. Effect of chlorphentermine and/or phenobarbital on total phospholipid and pulmonary glycogen levels*

Treatment	Phospholipid (mg/g)	Glycogen (mg/g)
Pair-fed control	33.9 ± 1.7	0.68 ± 0.12
Phenobarbital	33.4 ± 0.5†	0.83 ± 0.07
Chlorphentermine	51.5 ± 2.0‡	0.81 ± 0.08
Phenobarbital and chlorphentermine	38.7 ± 2.6†	0.67 ± 0.05

* Each value is the mean ± S.E.M. of five animals in each group. For experimental detail, see the legend to Fig. 1.

† Statistically significant difference when compared with chlorphentermine alone values ($P < 0.05$).

‡ Statistically significant difference when compared with the control values ($P < 0.05$).

Concurrent chlorphentermine and phenobarbital. Treatment with chlorphentermine for 1 week significantly increased pulmonary TPL levels (Table 4). While phenobarbital by itself did not markedly alter lung TPL content, simultaneous barbiturate and chlorphentermine resulted in a significant decrease in PL levels when compared to anorectic alone. Regardless of treatment regimen, results in Table 4 show that lung glycogen levels were not altered significantly. Data in Fig. 1 illustrate that in unlavaged lungs of 1-week chlorphentermine-treated rats a significant rise in S and PC levels was noted. In contrast, phenobarbital administration alone did not appear to alter any pulmonary PL components. It is of interest that concurrent phenobarbital and chlorphentermine treatment significantly decreased all PL component levels compared to chlorphentermine alone, and in the case of S as well as PS plus PI this fall was below those amounts found in control lungs. Although concurrent barbiturate and chlorphentermine administration lowered PC levels compared to chlorphentermine alone, this value was still significantly higher than control.

Table 5. Effect of chlorphentermine treatment and subsequent withdrawal on pulmonary total phospholipid and glycogen levels*

Treatment	Phospholipid (mg/g)	Glycogen (mg/g)
Pair-fed control	33.9 ± 1.7	0.68 ± 0.12
Chlorphentermine	60.2 ± 2.3†	0.60 ± 0.07
Chlorphentermine; 1-week withdrawal	37.0 ± 1.6‡	0.75 ± 0.05
Chlorphentermine; 2-week withdrawal	25.5 ± 0.8†‡	0.75 ± 0.05

* Each value is the mean ± S.E.M. of five animals in each group. For experimental details, see the legend to Fig. 2.

† Statistically significant difference when compared with the control values ($P < 0.05$).

‡ Statistically significant difference when compared with chlorphentermine only values ($P < 0.05$).

Chlorphentermine treatment followed by withdrawal. Two weeks of chlorphentermine treatment significantly elevated pulmonary TPL levels (Table 5). In rats initially treated with anorectic for 2 weeks and then withdrawn from drug treatment for 1 week, TPL levels returned to approximate control values. However, extension of withdrawal from 1 to 2 weeks resulted in a significant fall in absolute TPL amounts compared to controls. Data in Table 5 also show that neither chlorphentermine administration nor withdrawal from drug treatment significantly affected lung glycogen levels. As shown previously, treatment with chlorphentermine for 2 weeks significantly elevated the concentration of pulmonary S and PC (Fig. 2). In rats administered drug for 2

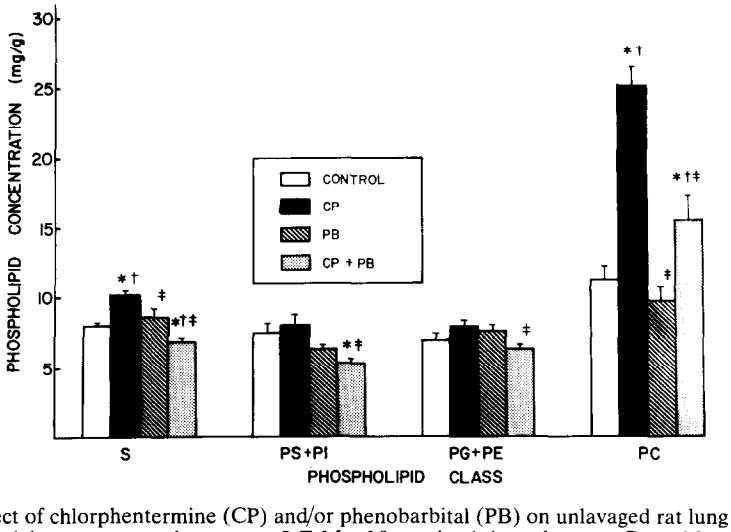

Fig. 1. Effect of chlorphentermine (CP) and/or phenobarbital (PB) on unlavaged rat lung phospholipid content. Each bar represents the mean ± S.E.M. of five animals in each group. Rats either were injected i.p. with CP (30 mg/kg) or given PB (30 mg/kg) p.o. daily for 5 days. Corresponding controls received the vehicle, physiological saline. In addition, a group of animals was injected for 5 days with 30 mg/kg chlorphentermine i.p. in the morning and given 30 mg/kg phenobarbital p.o. an hour later (CP + PB). Key: (*) statistically significant when compared with the control values ($P < 0.05$); (†) statistically significant difference when compared with phenobarbital alone values ($P < 0.05$); and (‡) statistically significant difference when compared with chlorphentermine alone values ($P < 0.05$).

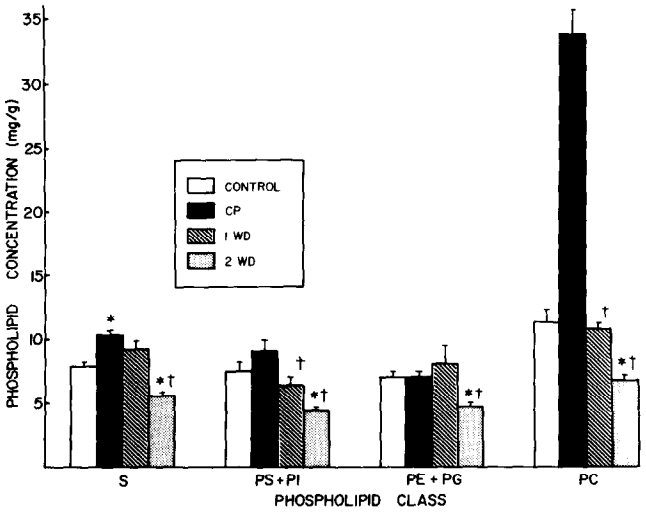

Fig. 2. Influence of chlorphentermine treatment and subsequent withdrawal on phospholipid content of unlavaged rat lung. Each bar denotes the mean ± S.E.M. of five animals in each group. Rats were injected i.p. with either saline (control) or chlorphentermine (CP, 30 mg/kg for 5 days), daily for 2 weeks. In addition, groups of rats were injected with chlorphentermine (30 mg/kg, i.p.) for 2 weeks and then maintained without further treatment for an additional period of 1 or 2 weeks and are designated as 1 WD and 2 WD respectively. Key: (*) statistically significant difference when compared with the control values ($P < 0.05$); and (†) statistically significant difference when compared with chlorphentermine only values ($P < 0.05$).

weeks followed by withdrawal for 1 week, a resultant fall in PC as well as PS and PI compared to chlorphentermine alone was noted. However, in lungs of animals withdrawn for 2 weeks from chlorphentermine treatment, all phospholipid components were decreased significantly compared to drug alone. It is of interest that the values observed in the 2-week-withdrawal rats fell to a level even lower than that seen in controls.

DISCUSSION

It was established previously that the chronic, daily administration of approximately 50 mg/kg chlorphentermine for 4 to 8 weeks elevates the concentrations of TPL, PC, S, PE, PS, PI and PG in unlavaged rat lung [3, 4, 17]. Karabelnik and Zbinden [18] demonstrated that, with an increase in chlorphentermine dose to 80 mg/kg, a rise in whole rat lung PI, PC, PG and TPL is noted after 2 weeks. Data in the present study show that, with a dose as low as 30 mg/kg chlorphentermine, significant increases in TPL, S and PC levels were found in unlavaged lung as early as 1 week after initiation of treatment. The drug-induced rise in the TPL and PC content was time-related in unlavaged lung reaching maximal quantitative values after 4 weeks under our experimental conditions. The increase in PS plus PI levels was also time-dependent in whole lung attaining significant levels after 4 weeks. The finding that not all phospholipid components were elevated by chlorphentermine in whole lung is in agreement with those of Karabelnik and Zbinden [18] who reported no marked change in S and PE content.

Within pulmonary tissue, chlorphentermine is known to induce an accumulation of phospholipid principally in alveolar macrophages [19]. Although ten times more lipid is extracted from chlorphentermine-treated alveolar macrophages compared to control cells, Schmien *et al.* [4] reported that chronic drug administration for 8 weeks also increases lipid content in pulmonary tissue other than alveolar macrophages. To evaluate the effect of chlorphentermine on sessile lung tissue, we measured the TPL content of lung following removal of alveolar macrophage and alveolar lipids. Unfortunately, these latter two fractions were not available for biochemical analysis. However, the ability of chlorphentermine to markedly increase alveolar macrophage TPL levels is well documented [2, 4, 20]. In agreement with Schmien *et al.* [4], our results show that chlorphentermine produced a time-related elevation in PC and TPL in sessile tissue of the lung reaching maximal quantitative levels after 4 weeks. Further, we also found that, in addition to PC and TPL, chlorphentermine increased S, PS plus PI, and PE plus PG after 2 weeks, and this level was maintained after 4 weeks. Why the quantitative amounts of all phospholipid components in 5-week-treated unlavaged and lavaged lungs were lower in comparison to 4-week values is, at present, not known. It is possible that processes were beginning to occur in pulmonary tissue in an attempt to correct the metabolic imbalance which gave rise to this disorder.

An increase in synthesis was probably not the basis for the elevation in the different PLs, as Karabelnik and Zbinden [21] found that treatment with chlorphentermine results in a depression in rat pulmonary PL synthesis. It is proposed that the observed accumulation of PLs in this disorder was due to an impairment in their metabolism resulting from the formation of a drug–lipid complex which was less susceptible to the action of catabolic phospholipases [1]. Accordingly, Hostetler and Matsuzawa [22] found that chlorphentermine inhibits the activity of

phospholipases A and C. It was apparent that PC increased quantitatively to a greater extent compared to other PL classes. Therefore, it is possible that there was a preferential inhibition of PC catabolism relative to the other classes, resulting in the marked buildup of this component.

The chlorphentermine-induced accumulation of masses of foam cells in pulmonary alveoli of rats was subject to modification by phenobarbital. Svendsen [7] demonstrated that in rats simultaneously administered chlorphentermine and phenobarbital there is a resultant lack of development of foam cells in lung. It was suggested that this phenomenon might be due to enhanced metabolic degradation of anorectic by barbiturate-induced drug-metabolizing enzymes. Indeed, Kacew *et al.* [8, 23] reported that an increase in lung microsomal aminopyrine *N*-demethylase activity is associated with a decrease in phospholipidosis in rats concurrently given barbiturate and chlorphentermine. However, since no procedure was available to measure chlorphentermine metabolism, this theory at present is speculative. In the present study, phenobarbital also diminished the chlorphentermine-induced rise in individual and total PL components in rat lung. The finding that PC levels were reduced yet still higher than control values in lungs of simultaneously-injected rats is in agreement with previous observations that phenobarbital does not completely abolish the phospholipidotic action of this anorectic [8, 23].

The pulmonary morphologic alterations induced by chlorphentermine are reversed upon removal of drug and maintenance of animals without further treatment [9, 24]. Reasor and coworkers [5, 25] reported that in alveolar macrophages isolated from chlorphentermine-treated lung the drug-induced rise in PL content returns to control levels upon withdrawal of compound. Data in our study also show that chlorphentermine treatment, followed by withdrawal for 1 week, resulted in a return of individual and total lung PL to control levels. Why a fall in TPL and individual PL components below control amounts in 2-week-withdrawn lungs was observed is, at present, not known. The possibility exists that certain consequences of chlorphentermine-induced phospholipidosis persisted after withdrawal for 2 weeks as reported previously by Reasor and Castranova [25]. It is also conceivable that the activities of the phospholipases initially inhibited by chlorphentermine recovered more rapidly than lipid synthesizing enzymes during withdrawal, producing a resultant decrease in PL levels.

In mammalian lung, it is believed that glycogen is a major source of carbohydrate substrate utilized in the synthesis of PC and that glycogen depletion is sequentially followed by increased PC synthesis [16, 26]. Since chlorphentermine administration is accompanied by decreased food and water consumption [15], the observed anorectic drug-induced increase in lung TPL might have been due to emaciation as reflected by glycogen depletion. The findings in the present study, that the chlorphentermine-induced increase in PC was not associated with a marked change in pulmonary glycogen, suggest that hunger may not have played a major role in the observed phospholipidosis. In a recent study, Kacew [27] found that treatment with chlorcyclizine also produces an accumulation of hypertrophic macrophages in lung accompanied by a decrease in body weight as well as a rise in PC levels. However, in contrast to chlorphentermine, the chlorcyclizine-induced increase in pulmonary PC is associated with a fall in glycogen levels. These findings support the view that chlorphentermine-induced changes in lung TPL and PC appear to be unrelated to decreased food and water consumption.

Acknowledgements—This research was supported jointly by a grant from the Medical Research Council of Canada and a NIH Research Grant, HL-24305. Warner–Chilcott Inc., Morris Plains, NJ generously supplied the chlorphentermine for these studies. The authors are indebted to Miss D. Hogan, Mr. M. R. Parulekar, Ms. C. Heyneman and Mr. M. Sullivan for their excellent technical assistance.

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