CHAPTER-6

INFLUENCE OF ESTROUS CYCLICITY AND OVARIECTOMY ON PLASMA AND LIVER PHOSPHOLIPID CONCENTRATIONS

Universal occurrence of phospholipids in cells is a well established fact. A variety of evidences have clearly demonstrated close integration of phospholipids in varying degrees with different lipoproteins (Levy *et al.*, 1971). Therefore, the phospholipid components of lipoprotein complexes clearly play important structural as well as functional roles in cell economy. It is a well known fact that membrane biogenesis, inclusive of phospholipid biosynthesis, is on essential feature of cellular development and growth (Dawidowicz, 1987; Grove and Korach, 1987). Ample available data suggest a dynamic function for phospholipids in the regulation of flux of molecules across the membrane.

Normal rate of lipid turnover and degradation are vital for cellular physiology (Dawidowicz, 1987). Phospholipid turnover occurs rapidly in most animal cells, for every one or two cell divisions wherein almost half of the total phospholipid is degraded (Vanden *et al.*, 1980; Esko and Raetz, 1983), an important example is the turnover of phosphoinositides (Downes and Michell, 1985). It was already suggested by Dawson (1973) that such lipid turnover may be connected with maintenance of cellular viability and may somehow be involved with integrity of membranes in living cells. Impairment of turnover and degradation of cellular lipids get mostly manifested in lipid storage diseases (Glew *et al.*, 1985).

Hepatic cells have the ability to synthesize fatty acids (Bloch and Kramer, 1948) and cholesterol (Little and Bloch, 1950; Srene *et al.*, 1950; Zabin and Bloch, 1950). Liver is also considered to be one of the important sites for synthesis of phospholipids as well as the concerned enzymes (Esko and Raetz, 1983; Vance, 1985; Kennedy, 1986). Another investigation has demonstrated that such enzymes are located in both endoplasmic reticulum and golgi apparatus of rat liver (Jelsema and Moore, 1978).

It is known that both endogenous and exogenous sex steroids are capable of altering serum lipid profiles (Bradley *et al.*, 1978; Knopp *et al.*,1982; Powell *et al.*, 1984). Additionally, it has been shown that the effects of sex steroids on plasma lipid profiles partly reflect corresponding alteration in the rate of biosyn-

thesis of triglyceride-rich very low density lipoprotein in liver (Ökner *et al.*, 1978). Goswami *et al.* (1963) have demonstrated that significant changes occur in uterine lipid component during normal estrous cycle of mice and due to ovariectomy as well as replacement therapy. Liver lipid profiles have also been reported to vary due to estrous cycle (Biswas and Mukherjea, 1973).

In the light of above information a quantitative evaluation of total lipid and cholesterol was carried out (Chapter-2) during 4 phases of estrous cycle, after spaying and subsequent hormone replacement (Chapter 4). From the results reported in Chapter-2 and 4, it was noticed that total lipid levels vary during different phases of estrous cycle. The total lipid level was low at the proestrous and estrous phases and started rising during metestrous, reaching the highest level during diestrous phase. Ovariectomy was found to lead to accumulation of total lipids in liver and E_2 replacement was observed to reduce it below normal level. On the other hand, the combination dose of E_2 + P was capable of restoring it to normal state. Moreover, Biswas and Mukherjea (1973) reported that total lipid and cholesterol concentration in liver, kidney and uterus decreased during estrous phase, but phospholipid concentration increased. It is therefore, logical to envisage that such variations could be attributable to some 'particular lipid components of total lipids moiety. Hence, the present investigation was undertaken to investigate the influence of estrous cyclicity as well as ovariectomy on phospholipid fractions of liver lipids and plasma lipid profiles in rats.

MATERIAL AND METHODS

Adult female rat weighing between 140 $\pm$ 20 g served as experimental animals.

I) **NORMAL ESTROUS CYCLE:-** For the study of normal cyclic variations, only those females which had normal 4-day estrous cycle were sacrificed at 9.00 am during each phase. A group of ten animals at each of the four phases was utilized.

II) **OVARIECTOMY (OVX):-** Only diestrous females were bilaterally ovariectomized or sham-operated under mild ether anaesthesia. The effect of OVX on liver was studied at the end of following three post-operative intervals : 24, 48 and 72 h.

Blood samples were collected by puncturing the nictitating membrane with slightly jagged end of anticoagulant coated capillary tubes. The plasma sample were obtained from the collected blood for estimating plasma lipids, cholesterol

and total phospholipids. Later, the animals were sacrificed under mild ether anaesthesia. The spigelian and right lobe of liver were quickly removed, trimmed free of adherent connective tissue. Blood was blotted and the tissue were weighed.

Separation and quantification of different fractions of hepatic phospholipids was performed by employing TLC technique. The full details of methods are described in chapter-1.

RESULTS

Table-6.1 shows values obtained for lipids, cholesterol and phospholipids in blood plasma during 4 phases of normal estrous cycle and at post-OVX intervals of 24, 48 and 72 h.(Fig.42 & 43).

Lowest concentrations of total lipid and cholesterol were observed during diestrous. Thereafter, these parameters exhibited a gradual increasing trend through proestrous to estrous phase, but these registered a decreasing trend during metestrous to finally reach lowest at diestrous level.

Plasma phospholipid level was found at its lowest level during estrous phase. This was seen to start increasing gradually during metestrous and diestrous phases, but during the proestrous phase the phospholipid level was noticed to begin decreasing to ultimately dip down to estrous level.

Ovariectomy was noted to lead to decrease in plasma total lipids upto 48 h, but it tended to reach to normal by 72 h post-operatively. Though there was a slight increment in cholesterol concentration by 24 h of OVX, it was found to be lowered, again to level similar to diestrous level, by 48 h post OVX interval and remained so through 72 h. If one takes into consideration cholesterol percentage values of total lipids, it can be seen that cholesterol was almost restored to normal level by 48 h of spaying. Just after 24 h of operation significant increase was observed in plasma phospholipids, which was found to be lowered by 48 h. However, once again the level shot up by 72 h of OVX.

Variations in liver lipids, phospholipids and subfractions thereof during normal estrous cycle are depicted in Table-6.2. Concentration of total phospholipids, in both liver lobes, were found to exhibit lowest levels during diestrous phase. These were observed to show a rising trend from proestrous to estrous where the highest levels were recorded. During metestrous phase the same remained more or less close to estrous level. However, taking into

Table-6.1 Influence of estrous cyclicity and ovariectomy on total lipid, total cholesterol and phospholipid in blood plasma.

PARAMETERS	PROESTROUS	ESTROUS	METESTROUS	DIESTROUS	POST-OPERATIVE INTERVALS 24 h	48 h	72 h
Total lipids mg/100 ml	316.896 ± 4.034	368.686 ± 3.629	276.086 ± 4.769	254.960 ± 3.629	249.383 ± 4.225	238.230* ± 5.406	250.097 ± 3.384
Cholesterol mg/100 ml	64.249 ± 3.078	87.766 ± 2.602	68.666 ± 2.488	56.332 ± 2.877	58.728 ± 3.246	53.638 ± 3.485	53.883 ± 3.178
Phospholipid mg/100 ml	105.173 ±3.235	87.731 ± 2.707	126.365 ± 2.297	130.953 ± 2.810	157.320**** ± 4.127	146.190**** ± 3.576	179.776**** ± 6.243
Cholesterol % of total lipid	20.194 ± 0.816	22.387 ± 0.621	24.704 ± 0.740	21.936 ± 0.801	23.495 ± 0.897	21.978 ± 1.105	21.419 ± 1.124
Phospholipid % of total lipid	33.159 ± 0.600	23.784 ± 0.545	45.816 ± 0.851	51.472 ± 1.478	63.276**** ± 2.283	61.660**** ± 2.433	71.730**** ± 2.519

Each value is Mean ± SE of six different samples pooled from 10 animals.

* $P < 0.05$ ** $P < 0.025$ *** $P < 0.01$ **** $P < 0.005$.

Table-6.2 Influence of estrous cyclicity on hepatic lipid fractions and subfractions of phospholipids (TLC).

PARAMETERS	PROESTROUS		ESTROUS		METESTROUS		DIESTROUS	
	SP	R	SP	R	SP	R	SP	R
Total lipids mg/100 mg of fresh tissue	5.074 ± 0.203	5.523 ± 0.468	5.324 ± 0.409	5.532 ± 0.326	7.841 ± 0.312	8.518 ± 0.356	9.161 ± 0.847	9.022 ± 0.694
Total phospholipids mg/100 mg of fresh tissue	2.405 ± 0.056	2.887 ± 0.028	3.443 ± 0.143	3.289 ± 0.069	2.977 ± 0.104	3.159 ± 0.057	1.992 ± 0.071	1.788 ± 0.103
Phospholipid expressed as % of total lipids	42.404 ± 0.742	52.281 ± 0.518	64.673 ± 2.885	59.452 ± 1.248	37.973 ± 1.337	37.092 ± 0.671	21.744 ± 0.782	19.817 ± 1.148
Sphingomyelin expressed as % of total phospholipids	4.157 ± 0.124	6.18[illegible] ± 0.487	2.469 ± 0.080	4.211 ± 0.334	7.121 ± 0.461	5.832 ± 0.283	8.370 ± 0.477	7.506 ± 0.582
Phosphatidyl choline % of total phospholipid	35.013 ± 0.732	29.876 ± 0.511	43.581 ± 0.580	40.713 ± 0.964	38.055 ± 1.195	35.343 ± 0.980	29.070 ± 0.988	28.031 ± 0.967
Phosphatidyl serine % of total phospholipid	11.000 ± 0.361	12.830 ± 0.930	6.909 ± 0.367	6.541 ± 0.649	11.941 ± 0.223	10.922 ± 0.617	12.894 ± 0.281	18.031 ± 1.194
Phosphatidyl inositol % of total phospholipid	17.581 ± 0.548	20.655 ± 0.387	22.406 ± 0.961	19.209 ± 0.920	13.023 ± 0.426	15.184 ± 0.119	16.921 ± 0.113	19.426 ± 0.406
Phosphatidyl ethanolamine % of total phospholipid	24.445 ± 0.468	21.260 ± 0.531	23.464 ± 0.550	20.173 ± 0.632	26.318 ± 0.711	25.570 ± 0.329	24.555 ± 0.183	24.348 ± 0.983

Each value is Mean ± SE of six different samples pooled from 10 animals, SP-Spigelian lobe R-Right lobe

Table-6.3 Effect of ovariectomy on hepatic lipid fractions and subfractions of phospholipids (TLC).

PARAMETERS	NORMAL INTACT FEMALES DURING DIESTROUS PHASE		POST-OPERATIVE INTERVALS 24 h		48 h		72 h	
	SP	R	SP	R	SP	R	SP	R
Total lipids mg/100 mg of fresh tissue	9.161 ± 0.847	9.022 ± 0.069	8.035 ± 0.299	9.134 ± 0.198	11.944*** ± 0.567	8.113** ± 0.400	13.627**** ± 1.078	11.704**** ± 0.336
Total phospholipids mg/100 mg of fresh tissue	1.992 ± 0.071	1.788 ± 0.103	2.617**** ± 0.060	2.646**** ± 0.017	2.542**** ± 0.017	1.134**** ± 0.020	2.796**** ± 0.068	3.089**** ± 0.159
Phospholipid expressed as % of total lipids	21.744 ± 0.782	19.817 ± 1.148	32.575**** ± 0.748	28.974**** ± 0.196	21.283 ± 0.149	16.551*** ± 0.258	20.524 ± 0.449	24.826**** ± 0.569
Sphingomyelin expressed as % of total phospholipids	8.370 ± 0.477	7.506 ± 0.582	8.164 ± 0.227	7.702**** ± 0.726	8.877**** ± 0.259	8.864**** ± 0.618	5.301**** ± 0.155	6.491 ± 0.328
Phosphatidyl choline % of total phospholipid	29.070 ± 0.988	28.031 ± 0.967	31.521** ± 0.483	25.895* ± 0.499	25.395*** ± 0.531	24.391*** ± 0.516	23.155**** ± 0.608	20.903*** ± 0.546
Phosphatidyl serine % of total phospholipid	12.894 ± 0.281	18.031 ± 1.194	11.840*** ± 0.650	15.715* ± 0.361	9.688**** ± 0.139	9.534**** ± 0.392	12.625 ± 0.493	8.934**** ± 0.369
Phosphatidyl inositol % of total phospholipid	16.921 ± 0.113	19.426 ± 0.406	21.932**** ± 0.373	24.237**** ± 0.346	16.672 ± 0.265	15.890**** ± 0.342	17.557 ± 0.638	24.154**** ± 0.777
Phosphatidyl ethanolamine % of total phospholipid	24.555 ± 0.183	24.348 ± 0.983	21.057**** ± 0.216	21.816** ± 0.636	32.337**** ± 0.624	36.784**** ± 0.911	32.193** ± 0.724	37.953** ± 0.687

Each value is Mean ± SE of six different samples pooled from 10 animals. SP-Spegelian lobe R-Right lobe
* $p < 0.05$ ** $p < 0.025$ *** $p < 0.01$ **** $p < 0.005$.

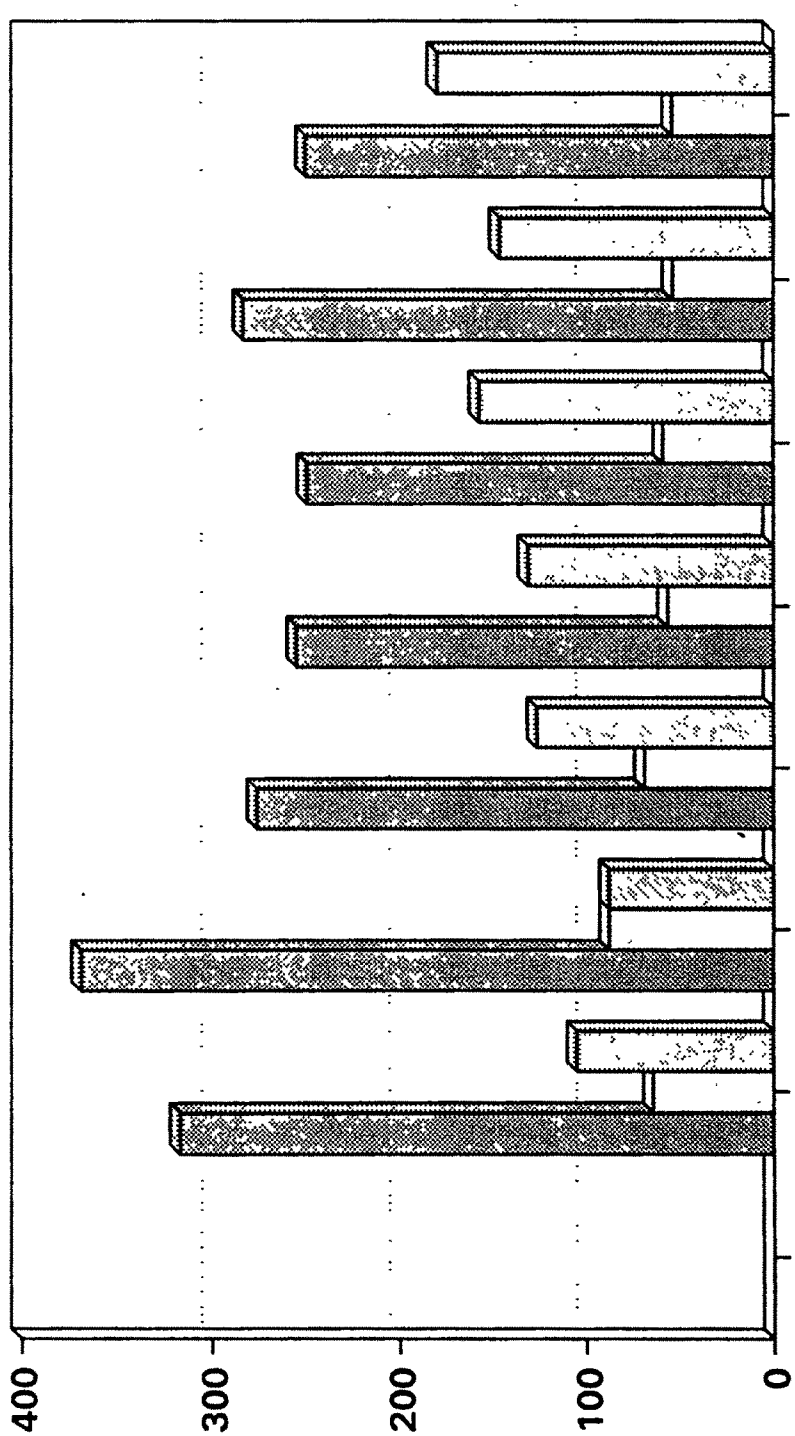
400
300
200
100
0
P
E
M
D
24 h
48 h
72 h
POST-OVX INTERVALS
TOTAL LIPID
CHOLESTEROL
PHOSPHOLIPID
PLASMA LIPID, CHOLESTEROL AND PHOSPHOLIPID
mg/ 100ml plasma

FIG. 42.

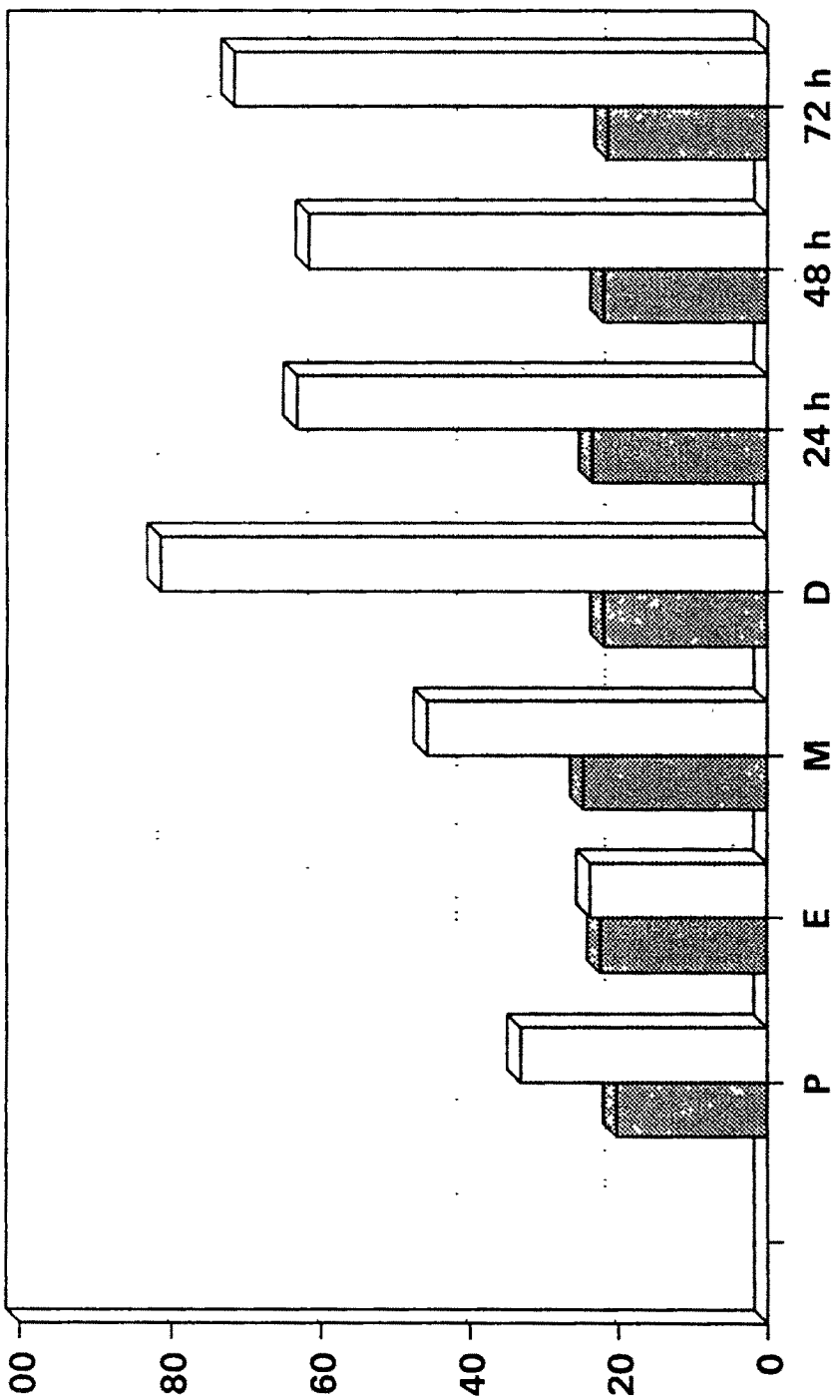
100
80
60
40
20
0
P
E
M
D
24 h
48 h
72 h
POST-OVX INTERVALS
CHOLESTEROL
PHOSPHOLIPID
PLASMA CHOLESTEROL AND PHOSPHOLIPID
% OF TOTAL PLASMA LIPID

FIG. 43.

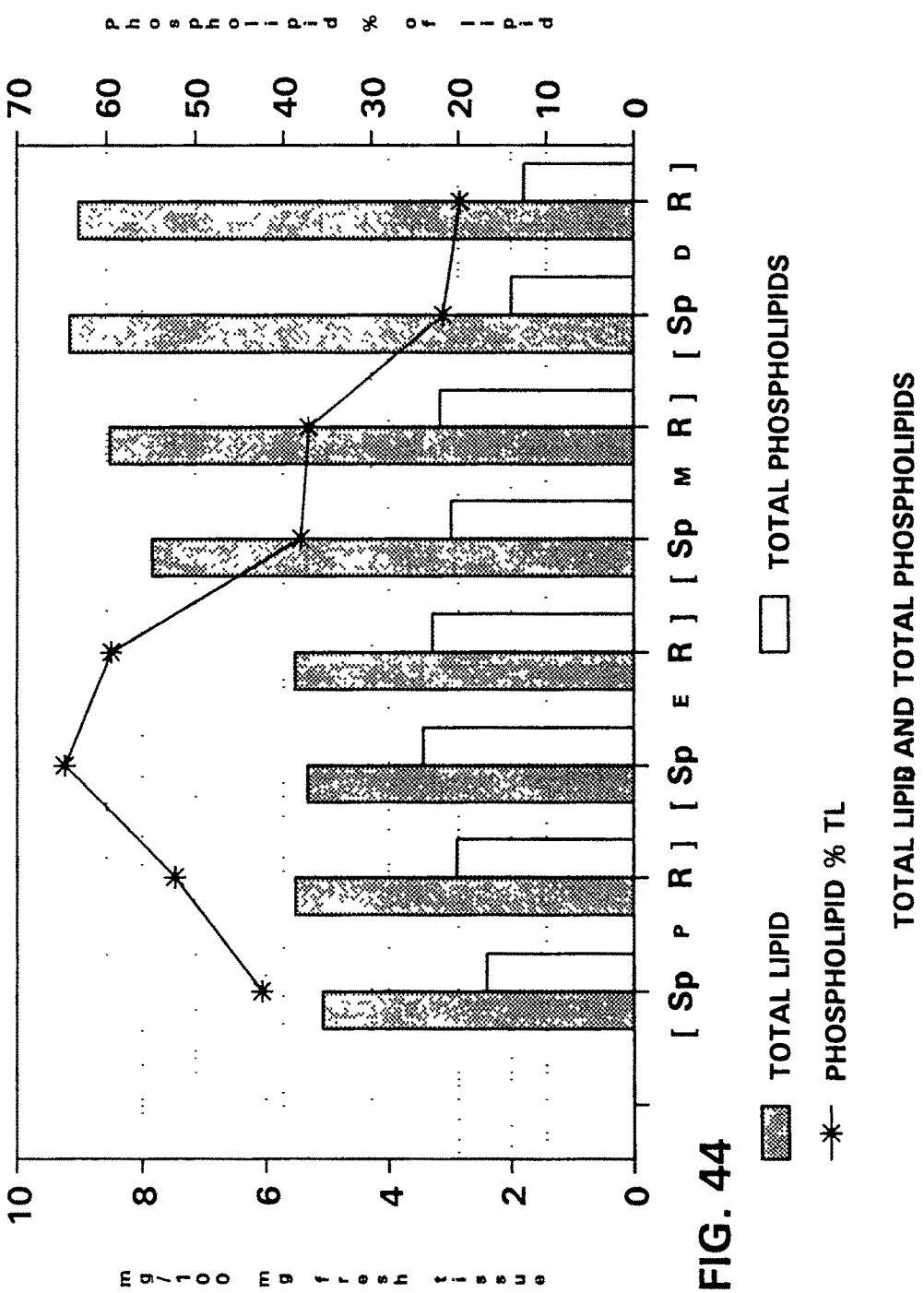
Phospho-lipid % of lipid
70
60
50
40
30
20
10
0
10
8
6
4
2
0
mg/100 mg fresh tissue
[Sp P R]
[Sp E R]
[Sp M R]
[Sp D R]
TOTAL LIPID
TOTAL PHOSPHOLIPIDS
PHOSPHOLIPID % TL
TOTAL LIPID AND TOTAL PHOSPHOLIPIDS

FIG. 44

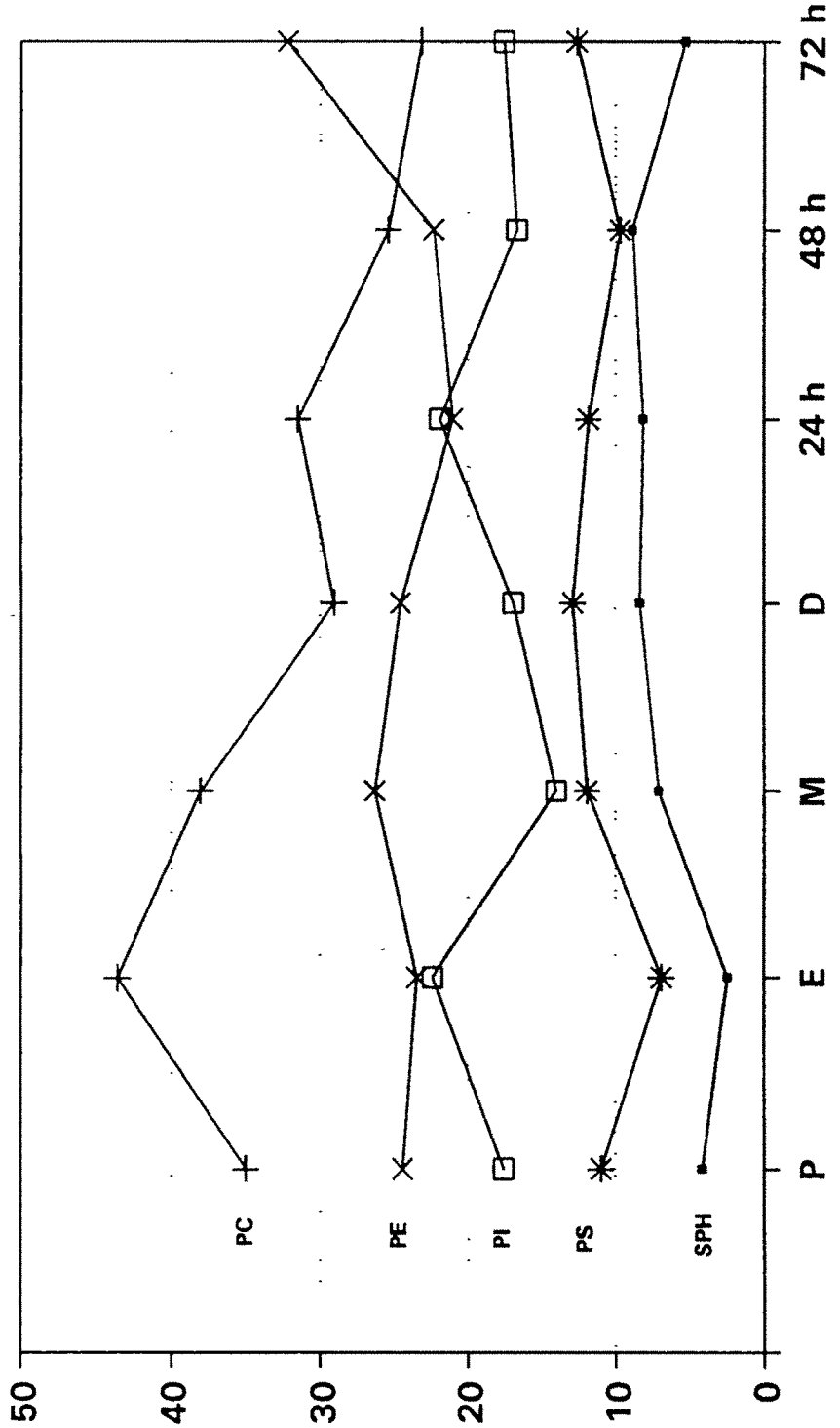
50
40
30
20
10
0
PC
PE
PI
PS
SPH
P
E
M
D
24 h
48 h
72 h
POST - OVX INTERVALS
SPH
PC
PS
PI
PE
PHOSPHOLIPID FRACTIONS IN SPIGELIAN LOBE
% of total phospholipid

FIG. 45.

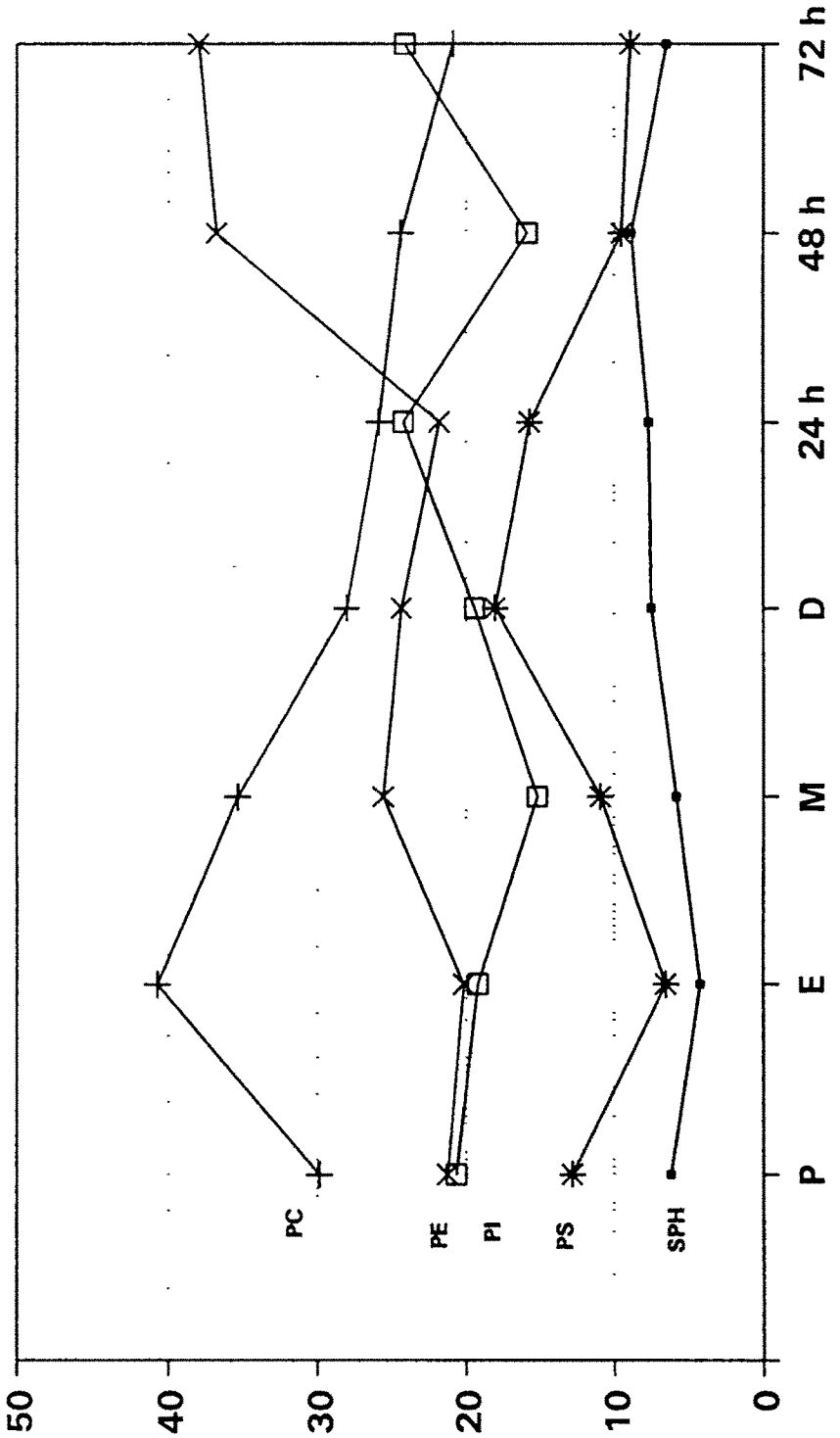
50
40
30
20
10
0
P
E
M
D
24 h
48 h
72 h
POST - OVX INTERVALS
PC
PE
PI
PS
SPH
SPH
PC
PS
PI
PE
PHOSPHOLIPID FRACTIONS IN RIGHT LOBE
% of total phospholipid

FIG. 46.

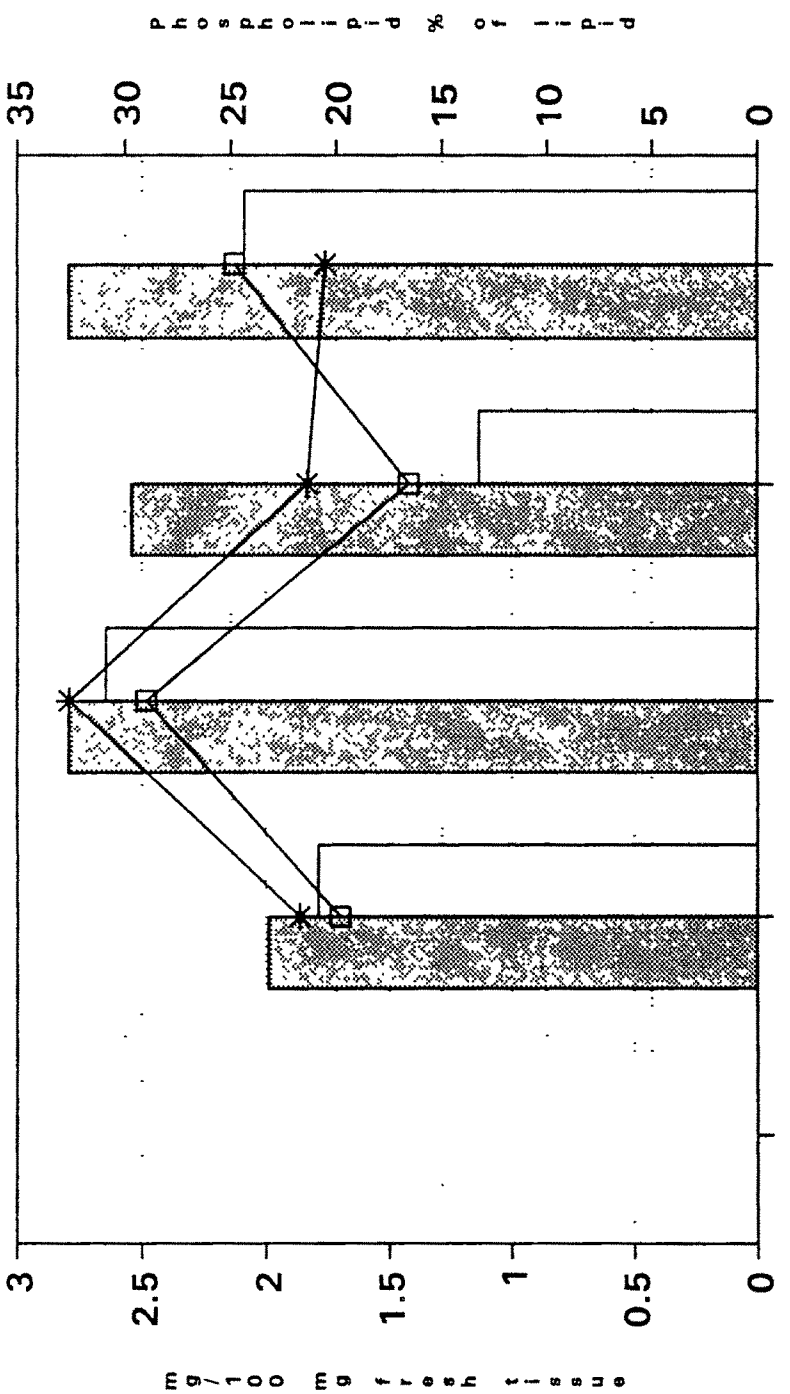
Phospholipid % of total lipid
35
30
25
20
15
10
5
0
3
2.5
2
1.5
1
0.5
0
mg/100 mg fresh tissue
NORMAL
24 h
48 h
72 h
POST - OVX INTERVALS
Sp
R
Sp
R
PHOSPHOLIPIDS

FIG. 47.

consideration the levels of phospholipid as percentage values of total lipids, it could be seen that there was a clearcut decrease from estrous to diestrous phase (Fig.44).

From the data given in Table-6.2 (Fig.45 & 46) it was evident that among the phospholipid subfractions, at any phase of the estrous cycle, the phosphatidyl choline (PC) comprised of major bulk of total phospholipid content, whereas sphingomyelin (SPH) represented the least of the fractions. Sphingomyelin was found at its lowest level during estrous phase of cycle. It exhibited an increasing trend from metestrous to diestrous but, thereafter, it started decreasing during proestrous to finally reach the minimum level noted during estrous phase. As opposed to this, the phosphatidyl choline subfraction was noted to be higher during estrous phase but the same started declining from metestrous to diestrous. Hereafter a slight dichotomy of lobe-wise response was noted. The spigelian lobe recorded heightening of PC by proestrous phase only but the right lobe showed delayed increase as it occurred only during estrous phase of the cycle.

Phosphatidylserine (PS) concentration in both the liver lobes was lowest during estrous phase which increased during the metestrous phase and remained more or less constant during rest of the phases. There was a marked increase in the PS that was observable in the right lobe only during diestrous phase, but that was lowered to metestrous level during proestrous phase. Thereafter, in both of lobes the PS content was found to be decreased to minimum level as noted during estrous phase.

Concentrations of phosphatidylinositol (PI) were noted to be at the lowest levels during metestrous phase in case of both the liver lobes. A clearcut lobewise differential response was noted in this case. The spigelian lobe registered a gradual increase from diestrous to estrous. On the other hand, the right lobe showed enhanced percentage of PI during diestrous phase but thereafter it did not exhibit variation worth noticing at proestrous and estrous phases of the cycle.

As far as phosphatidylethanolamine (PE) concentrations were concerned; it was observed that both liver lobes showed higher levels during metestrous. Here too, lobe-wise differences were apparent. A gradual decrease was noted in the case of right lobe upto estrous phase. On the other hand, the PI level of spigelian lobe exhibited a certain decrease and thereafter, not much of variation was evident upto estrous. The magnitude of decrease in PI levels of spigelian lobe was negligible as compared to that in the right lobe.

EFFECT OF OVARIECTOMY (Table-6.3):- Ovariectomy was seen to lead to increase in total phospholipid content of both the liver lobes at 24 h interval. It

remained higher in spigelian lobe upto 72 h, but it was lowered in the right lobe at 48 h interval only to be increased again by 72 h post-operatively (Fig.47). A noteworthy fact that needs to be mentioned here concerns the lobe-wise difference on different basis. Eventhough the values of total phospholipids were seen to be increased after OVX, when considered in terms of percentage values of total tissue lipid content 48 h of OVX, it was not found to differ much from those of diestrous level in the case of spigelian lobe. Along the same line, however the right lobe showed a fluctuating response. In other words, the spigelian lobe was found to normalize phospholipid percentage by 48 h OVX but the right lobe was not capable of the same even at 72 h interval. Sham-operation did not affect the normal estrous cyclicity hence the values were not taken into consideration.

Sphingomyelin, expressed as percentage of total phospholipid, was slightly increased in both liver lobes after 48 h of spaying. This was lowered significantly after 72 h only in the spigelian lobe after 72 h. showed an initial increase [PC of spigelian lobe] after 24 h. PC levels, in both liver lobes, were lowered by 48 h with further suppression upto 72 h. Gradual lowering was observed in the PS of both the lobes at 24 and 48 h but it was restored to normal level in spigelian lobe. In contrast, it was found to be lowered further in the right lobe at 72 h interval. The PI concentration in both of the liver lobes was increased just after 24 h of OVX. This was seen to be restored to normal level by 48 h in the spigelian lobe and remained so even at 72 h of OVX. On the other hand, the PI level of the right lobe exhibited a fluctuating response at 48 h and 72 h post OVX interval. It was lowered significantly by 48 h but once again went up by 72 h interval which was significantly higher than normal level. In other words the right lobe was found to be incapable of restoring normal level of its PI. Phosphatidylethanolamine (PE) levels were observed to show decrease in both lobes after 24 h of spaying. Remarkable increase in the same in both of the liver lobes was noted at 48 h interval. The values remained higher in both the lobes even after 72 h of OVX (Fig.45 & 46).

DISCUSSION

The results of the present study clearly bring forth the fact that changes associated with estrous cycle occurred in the liver as well as plasma and were characterized by significant alterations in total lipid concentration and components of phospholipid. The most conspicuous change was increase in phospholipid concentration during estrous and decrease during subsequent phases of estrous cycle. This was accompanied by an exactly reverse pattern of variation in case of plasma phospholipids. Further, it was noted that most of these changes

were seen to be abolished by ovariectomy. These observations suggest that variations in liver phospholipids during different phases of estrous cycle phases are in all probability manifestations of influence of cyclic variations in ovarian hormones.

The present observations also brought up another noteworthy fact concerning the phospholipid concentration. More meaningful inferences could be drawn if phospholipids were considered as percentage of total lipid content rather than when the values of phospholipids *per se* were considered. A very clear evidence in favour of this contention can be seen during the metestrous phase *vis a vis* other phases of the estrous cycle. Additionally, the reduction of phospholipid percentage at metestrous could be said to be due to antagonistic action of progesterone. It has been well documented that rise in phospholipids is associated with protein biosynthesis (Thompson and Ballou, 1956; Hendler, 1959; Goswami *et al.*, 1963). Here it may be recalled that hepatic protein levels were observed (Chapter-2) to rise during metestrous. In the present context, therefore, it can be said that rising levels of estrogen from proestrous to estrous resulted in increase in hepatic phospholipids. During metestrous phase the estrogenic influence was followed by progesterone and that supposedly facilitated the utilization of phospholipids as source of energy for protein synthesis, hence the observed lowering of phospholipid percentage.

Another aspect of hepatic lipids and estrous cyclicity became clear from the present observations. Decrease in hepatic total lipids and cholesterol during proestrous and estrous was accompanied invariably by increase of these two parameters in blood plasma. This inverse relationship may safely be said to indicate influence of estrogen on liver. Marked increase in plasma lipids and cholesterol levels evidently point to lipid mobilization and phospholipid accumulation in liver under the influence of estrogen. The patterns of variation reported here concerning the hepatic total lipid and phospholipid levels are apparently similar to those reported for mouse uterus (Goswami *et al.*, 1963) and for rat uterus (Biswas and Mukherjea, 1973). Biswas and Mukherjea, (1973) further added that possibly modulating action of estrogens responsible for determining the lipid patterns is not restricted to target tissue (uterus) alone, but it could be a generalized effect, since such changes in lipid patterns have also been observed in extragonadal tissues like liver and kidney.

Nishigori and Aizawa (1968a) reported an estrogen stimulated increase in incorporation of 1-2-^{14}C-choline into lipids of uterus, but not in liver, of mature ovariectomized rats. However, they also reported that 2-^{14}C-ethanolamine was

incorporated into uterine phosphatidylcholine. Further Young (1971) has shown that changes in specific activity of enzymes governing phosphatidylcholine biosynthesis may account for increased *in vivo* incorporation of methyl groups of L-methionine into hepatic phosphatidylcholine in female and estradiol treated male rats. According to him, estradiol stimulates the hepatic PC synthesis by the stepwise methylation of phosphatidylethanolamine. In this context remarkable rise in phosphatidylcholine during estrous phase and low level of PE indirectly indicates conversion of PE to PC. Further, it could be said that this influence might be due to high level of circulating E_2 during estrous phase and this probably act through the methylation pathway suggested by Nishigori and Aizawa (1968b) and Young (1971).

Of the different components of phospholipids, sphinomyelin, serine and ethanolamine together followed a certain common pattern of variation whereas choline and inositol followed a different trend of variation through various phases of the estrous cycle. Nevertheless, differential responses by the two lobes of the liver were apparent.

Ovariectomy during diestrous phase lead to accumulation of lipids and phospholipids in liver. However, differential response by two lobes of liver became obvious as the time lapsed after OVX leading to increase in lipid concentration and decrease in phospholipid percentage in the spigelian lobe, which only showed normalizing response but not the right lobe.

From the results obtained for different fractions of phospholipid after 24, 48 and 72 h of OVX it can be seen that maximum alterations in all fractions occurred by 48 h post OVX. This corroborates other observations reported here elsewhere and those reported previously in case of male rats (Ambadkar and Gangaramani, 1980). Greater reduction in choline at and simultaneous marked increase in ethanolamine 48 h interval confirms the cessation of methylation of ethanolamine to choline due to absence of estrogen after spaying.

It should be admitted here that on the basis of data on hand about reduced levels of SPH, PC and PS and, increased levels of PI and PE after OVX no definite inferences could be drawn. Only further work on some of the concerned enzyme activities and other metabolic aspects may throw some light.

By way of summary, the data presented here indicate-

1) Normal hormonal alterations of estrous cycle have obvious influence on hepatic lipid metabolism particularly, the hepatic phospholipid components.

2) There appears to be an inverse relationship between hepatic lipid components and blood plasma.

3) Spigelian lobe exhibits distinctly different responses to hormonal variations during normal estrous cycle as compared to those of the right lobe.

4) Maximal alterations are observable by 48 h post OVX interval.