CHAPTER-IV

EFFECTS OF CORTICOSTERONE TREATMENTS ON LIPID/PHOSPHOLIPID CONTENT/COMPOSITION AND ^{3}H ACETATE INCORPORATION INTO BRAIN MITOCHONDRIAL PHOSPHOLIPIDS AND MEMBRANE FLUIDITY DURING DEVELOPMENT.

Glucocorticoids are known to alter synthesis of triacylglycerol, cholesterol and phospholipids in variety of tissues including brain (Chapter I). The observed changes in mitochondrial oxidative energy metabolism and in kinetic properties of mitochondrial ATPase in brain upon glucocorticoid administration (Chapter II and III) could be attributed to possible alterations in lipids of mitochondria; many of the mitochondrial membrane-bound enzymes including ATPase are known to have requirement for lipid environment for their activity (1,2).

Glycerol 3 phosphate dehydrogenase (GPDH), a key enzyme in synthesis of the phospholipids gets stimulated by glucocorticoid treatment in rat brain in age-dependent manner (3). Warringa et al. (4) have shown that hydrocortisone stimulates the development of oligodendrocytes in primary glial cell cultures and stimulates GPDH activity. Incorporation of ^{35}S sulfate in sulfolipids was greatly enhanced by hydrocortisone and incorporation of ^{14}C acetate into cholesterol and fatty acids was also stimulated but to a smaller extent. It is also known that adrenalectomy increases whereas glucocorticoid treatment decreases the myelination in rat pups (5,6). However, myelin of adrenalectomized rats contained lesser amounts of galactolipids and phosphatidylcholine compared to the controls (6). Bhargava et al. (7) have reported effects of corticosterone administration

on lipid metabolism in brain regions of rat during development and shown alterations in ^{14}C glucose incorporation into phospholipids and cholesterol. The effects of corticosterone treatment were age specific. In 40- and 90-day-old rats none of the brain regions studied responded to the treatment and young animals were more susceptible to glucocorticoid overload (7).

The studies on lipid metabolism at whole tissue level can only give an overall but approximate picture. Hence studies at the level of subcellular organelles or at membrane level are desired to get better insights on influence of corticosterone treatment on lipid microenvironment of a particular enzyme and its function. Although information is available about glucocorticoid effects on lipid composition of subcellular organelles such as mitochondria (8) and microsomes (9) from liver tissue (e.g. also see Chapter VI), no information is available on brain membranes especially the mitochondrial membrane during development.

Hence the objective of the present study was to examine the effects of <u>in vivo</u> corticosterone treatments -acute and chronic- on the following parameters in brain mitochondria during postnatal development : 1) Mitochondrial content of total phospholipids, cholesterol and molar ratios of total phospholipids/cholesterol, 2) Mitochondrial phospholipid

composition (as % of total) and content (µg/mg protein) of individual phospholipids, 3) Incorporation of ^{3}H acetate into different phospholipid classes of mitochondria and 4) Mitochondrial membrane fluidity.

MATERIALS AND METHODS

Chemicals

1,6-diphenyl-1,3,5 hexatriene(DPH) and 2,5- diphenyl-oxazole (PPO) were purchased from Sigma Chemicals Co. USA.

Kieselgel H and cholesterol were purchased from E.Merck, Germany and J.T.Baker Chemical Co. N.J. USA respectively.

Sources of all other chemicals were the same as described in Chapter II.

Radiochemicals

^{3}H acetate (Specific radio activity: 1000 mCi/m mol) was purchased from BRIT, Bombay, India.

Injection of Radioactivity

^{3}H acetate was injected to animals intraperitoneally (i.p.) 1 hour before killing, at a dose of 50 µCi/100 g body weight.

Lipid extraction

Extraction of mitochondrial lipids was carried out according to the method of Folch et al.(10) as described by Bangur et al. (11). Aliquots of mitochondrial suspension (10

to 20 mg mitochondrial protein) were taken in test tubes and final volume was made upto 1.5 ml with isolation medium. To this, 4.0 ml of freshly prepared 2:1 (v/v) chloroform : methanol mixture was added and the tubes were vortexed vigorously several times and then subjected to centrifugation at 3000 rpm for 10 minutes in a table top centrifuge. The lower solvent phase was transferred to another tube using a long broad gauge needle attached to a glass syringe. Re-extraction was carried out using 2.0 ml of the above mentioned solvent mixture. Tubes were vortexed vigorously and centrifuged again at 3000 rpm for 10 minutes. The lower phase was collected and pooled with previous extract and the volume of the pooled extract was measured. For removal of traces of proteins and proteolipids, 0.2 volumes of 0.017% (w/v) $MgCl_2$ solution was added to pooled extracts and tubes were vortexed vigorously and centrifuged at 3000 rpm for 10 minutes. The lower solvent phase was transferred to another tube and the final volume of the extract was made to 4.0 ml with 2:1 (v/v) chloroform:methanol mixture. From this, measured aliquots were dispensed in different tubes for the determination of total phospholipid, cholesterol and for separation of individual phospholipids by thin layer chromatography (TLC) to determine phospholipid content and composition and to study incorporation of ^{3}H acetate into individual phospholipid classes.

Separation of phospholipids by TLC

Separation of different phospholipid components was carried out by one dimentional thin layer chromatography (12). 20 X 20 cm glass plates were used for chromatographic separtion. The plates were dipped overnight in chromic acid solution and washed first with running tap water and then twice with distilled water. Finally, the plates were cleaned with acetone to remove traces of grease and dried.

A slurry of silicagel H (6 g Kieselgel H in 14 ml of distilled water per plate) was prepared by gentle mixing and poured into an applicator. The slurry was evenly spread on the plates with the help of the applicator, the thickness of the layer being adjusted to 0.25 mm. The layer was allowed to dry by leaving the plates overnight at room temperature. Before spotting the samples, TLC plates were activated in an oven at 100°C for 30 minutes.

The aliquots of lipid extract for TLC were made to zero volume by evaporating the solvent with the help of a hair dryer and reconstituted in about 0.1 to 0.2 ml of chloroform : methanol, 2:1 (v/v) mixture. Aliquot of reconstituted sample (corresponding to 10 to 15 µg of phospholipid phosphorous) was spotted on the activated TLC plate in such a way that the diameter of the spot should be minimum to acheive better resolution. The TLC chamber was

saturated with solvent mixture at least 1 hour before the run. The solvent system used was: chloroform:methanol:glacial acetic acid:water :: 25:15:4:2 (v/v). After spotting the samples, plates were kept in oven for 2 minutes and then introduced in saturated TLC chamber. After the run, plates were taken out and kept at room temperature for 3 to 4 hours to remove the solvents. The spots of individual phospholipids were visualized and marked after brief exposure to iodine vapours by keeping the plates in iodine chamber.

From the origin, the sequence of the phospholipids was lysophosphatidic acid (LYSO), sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG). After the spots were marked, plates were kept at room temperature till iodine sublimed off, and then spots were scraped for phospholipid phosphorous estimation and determination of ^{3}H acetate incorporation in phospholipids. For incorporation studies in phospholipids, equal amount of the same sample was spotted in duplicate, from which one spot was used for phospholipid phosphorous estimation while other from next lane for scintillation counting.

Estimation of phospholipid phosphorous

After scraping the individual phospholipid spots, the samples were digested with 0.5 ml of 10 N sulfuric acid by heating in a sand bath for 2.5 hours. The tubes were allowed to cool and a drop of 70% perchloric acid was added and again the tubes were heated in sand bath for 2.5 hours till all the traces of perchloric acid are removed. The aliquots taken for total phospholipid estimation were also treated in the similar manner. The estimation of phosphorous was carried out as described by Bartlett (13).

Reagents for Bartlett's method

1. Phosphorous standard

a) Stock: Dissolve 35.1 mg KH_2PO_4 in 50 ml distilled water, then add 1.0 ml of concentrated sulfuric acid and make up the volume to 100 ml to give final concentration of 80 µg phosphorous/ml.

b) Working standard: Dilute the stock standard 1:40 times with distilled water to give 2 µg phosphorous/ml.

2. Ammonium molybdate reagent

Prepared freshly by dissolving 5 g of ammonium molybdate in distilled water and volume was made up to 100 ml.

3. ANSA reagent

Same as described previously in Fiske and Subba Row's method (Chapter III).

Procedure

Different aliquots of working standard were taken to give concentration of phosphorous in the range of 0.4 to 4.0 µg. Then 0.5 ml of 10 N sulfuric acid was added. Reagent blanks contained 0.5 ml of 10 N sulfuric acid. The volume was made up to 3.7 ml with distilled water in all the tubes. Then 0.2 ml of 5% (w/v) aqueous ammonium molybdate was added followed by 0.1 ml of ANSA reagent. The tubes were vortexed immediately and kept in a boiling water bath for 10 minutes for the development of blue color. Tubes were removed, cooled and read at 830 nm against reagent blanks in a spectrophotometer. Sample tubes were centrifuged at 3000 rpm for 10 minutes to sediment silica gel and clear supernatants were used to measure O.D. at 830 nm. The slope was calculated from the standard graph and used to find out the phosphorous concentrations in samples. This procedure gives slope of 0.18 (1 ug phosphorous gives 0.18 O.D).

Estimation of cholesterol

Cholesterol was estimated according to the method of Zlatkis et al. (14) with some modifications.

Reagents

1. cholesterol standard

1 mg cholesterol/ml (w/v) was prepared in isopropanol.

2. Ferric chloride reagent

a) 10% (w/v) ferric chloride was prepared in glacial acetic acid.

b) 100 ml of concentrated sulfuric acid was taken and to this 1.0 ml of 10% ferric chloride solution was added and mixed properly. This reagent was prepared fresh before use.

Procedure

Sample aliquots were made to zero volume and to this 0.5 ml of isopropanol was added. For standard curve, aliquots were dispenced to get the concentration of cholesterol in the range of 20 to 200 µg. The reagent blanks contained 0.5 ml of isopropanol in standard tubes.Then all the tubes: blanks, standards and samples were treated in a similar manner. To all the tubes 1.5 ml of glacial acetic acid was added and then 1.5 ml of ferric chloride reagent was added slowly to give two clear layers. The tubes were then vortexed vigorously and kept at room temperature for 20 minutes. The brown color developed was read against reagent blank in colorimeter using a filter of 530 nm. Slope was obtained from the standard graph and used to findout cholesterol concentrations in samples. This method gives slope of 0.0038.

Counting of radioactivity

For radioactivity counting, spots of individual phospholipids were scraped (after iodine had sublimed off) and transferred to screw cap scintillation vials. To this 3.0 ml

of scintillation cocktail (0.3% (w/v) PPO in toluene) was added followed by one drop of concentrated triton X-100. After closing the cap, the contents were mixed by inverting the vials several times. The vials were left overnight and counted on the next morning in Rack Beta Liquid Scintillation Counter with 50% efficiency of counting using a programme for ^{3}H window. Each sample was counted for 300 seconds followed by external standard counting for 15 seconds. The counts obtained were converted to DPM (disintegrations per minute) using external standard ratios. Finally, the results were expressed as DPM/μmol of phospholipid phosphorous.

Measurement of mitochondrial membrane fluidity

Fluidity measurements were carried out in freshly isolated mitochondria using 0.32 M sucrose containing 10 mM tris.HCl, pH 7,4. The probe used was 1,6,diphenyl-1,3,5-hexatriene (DPH). Stock DPH solution (2 mM) was prepared in tetrahydrofuran and stored refrigerated in amber bottle. For measurements of fluorescence polarization, mitochondrial samples were taken in 3.0 ml of buffered sucrose medium at a final protein concentration of 0.2 mg/ml, and the stock DPH solustion was added so that the molar ratio of probe to lipid was between 1:200 to 1:300 (11,15). The mixture was vortexed vigorously and left in dark for 30 minutes to permit equilibration of probe into the membranes.

Fluorescence polarization was measured in a Shimadzu RF - 5000 spectrophotofluorimeter with a polarizer attachment. The instrument has a program for calculating and printing out fluorescence polarization (P) values. Excitation and emission wavelengths were 360 and 430 nm respectively; bandwidths were 5 and 10 nm respectively. Data were accumuated for 5 seconds for each polarization setting : vertical (parallel) and horizontal (perpendicular) (11,15).

From the fluorescence polarization (P) values the calculations of fluorescence anisotropy (r), limited hindered anisotropy (r_∞) and order parmeter (S) were by using following formulas:

$$r = 2P/(3-P)$$

$$r_\infty = (4r/3) - 0.1 \quad \text{and}$$

$$S = \sqrt{r_\infty/r}$$

Fluorescence anisotropy (r) and limited hindered anisotropy (r_∞) are used to assess the static component of membrane fluidity (16).

RESULTS

The results on effects of corticosterone treatment on mitochondrial lipid composition, content and synthesis and membrane fluidity are summarized in this chapter.

Table 1 shows effects of corticosterone treatments on total phospholipids (TPL) and cholesterol (CHL) and TPL/CHL molar ratios of brain mitochondria. Acute treatment with corticosterone caused 25 to 55% increase in mitochondrial TPL content in 14- and 21-day-old animals without having any significant effect on 35-day and adult groups. On the other hand, chronic treatment had no effect on TPL content in 14- and 21-day-old groups but it increased TPL content by 13% in 35-day-old animals whereas in case of adults it decreased the TPL content by about 45%.

Acute treatmaent had no effect on mitochondrial CHL content of all the age groups studied and the effects of chronic treatment were age-dependent. In 14-day-old animals chronic treatment doubled the CHL content but in 21- and 35-day-old rats the CHL content decreased by about 25%, in adults it was unaffected (Table 1).

As a result of significant changes in mitochondrial TPL and CHL contents, corticosterone treatments caused significant alterations in TPL/CHL molar ratios in animals of all the age groups. Acute treatment did not have any significant effect on TPL/CHL molar ratios except a 26% increase in 21-day-old animals. Effects of chronic treatment were age-dependent; in 14-day-old pups and adults it caused significant (45 to 50%) decrease but in other two age groups it increased TPL/CHL molar ratio from 24 to 50%.

Table 1

Effect of corticosterone treatment on total phospholipid (TPL) and cholesterol (CHL) content and TPL / CHL molar ratios of brain mitochondria during development.

Age	Treatment	Total phospholipid content (µg/mg protein)	Cholesterol content (µg/mg protein)	Total phospholipid/ cholesterol (mol:mol)
	Control	409.2 ± 23.0(10)	71.2 ± 3.21(8)	2.8 ± 0.23 (8)
14 Day	Acute	508.8 ± 22.5[b](10)	84.0 ± 9.01(8)	3.0 ± 0.30 (8)
	Chronic	403.1 ± 15.9(10)	143.8 ± 17.8[c](8)	1.4 ± 0.08[d](8)
	Control	511.7 ± 29.5(10)	76.3 ± 4.32(8)	3.4 ± 0.14 (8)
21 Day	Acute	794.5 ± 50.7[d](7)	92.3 ± 9.63(8)	4.3 ± 0.19[c](7)
	Chronic	501.9 ± 23.8(12)	58.5 ± 6.14[a](8)	4.2 ± 0.21[b](8)
	Control	511.5 ± 18.7(11)	90.6 ± 7.66(12)	2.8 ± 0.16(11)
35 Day	Acute	499.1 ± 15.8(14)	89.7 ± 3.44(12)	2.8 ± 0.15(12)
	Chronic	580.5 ± 15.4[b](12)	68.7 ± 3.91[a](8)	4.2 ± 0.17[d](8)
	Control	681.6 ± 52.3(12)	78.0 ± 7.94(8)	4.4 ± 0.20(8)
Adults	Acute	602.2 ± 22.8(7)	76.4 ± 4.57(8)	3.9 ± 0.18(7)
	Chronic	382.3 ± 22.7[d](12)	76.5 ± 3.07(8)	2.5 ± 0.30[d](8)

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.002$ and [d]$P < 0.001$ compared to the corresponding controls.

In an age-dependent and treatment-specific manner, both the corticosterone treatments affected the phospholipid composition (as % of total phospholipids) of brain mitochondria. These results are summarized in Tables 2 to 5.

The major effects of corticosterone treatments on mitochondrial phospholipid composition were observed only in the 14-day-old rats; other age groups were least affected by corticosterone. In case of 35-day-old animals, both the treatments failed to produce any significant effect on phospholipid composition of brain mitochondria (Table 4).

In 14-day-old animals, acute treatment caused 2 to 3 fold increase in SPM, PI and PS with about 40% reduction in DPG component. Chronic treatment also caused 30% reduction in DPG, but the increase in SPM was about 80% as against 200% increase observed in acute treatment group (Table 2).

Acute treatment to 21-day-old animals caused 160% increase in PS content, an effect similar to that observed in 14-day-age group. Besides, there was slight but significant increase in PE which is noteworthy (Table 3). Chronic treatment had no effect on phospholipid composition of brain mitochondria from 21-day group.

Table 2

Effect of corticosterone treatment on phospholipid composition of brain mitochondria from 14-day-old rats.

Phospholipid class	Phospholipid composition (% of total)		
	Control(12)	Acute(12)	Chronic(6)
LYSO	2.02 ± 0.49	2.12 ± 0.37	1.36 ± 0.47
SPM	2.15 ± 0.45	6.70 ± 0.78[d]	3.87 ± 0.26[c]
PC	44.70 ± 1.63	41.65 ± 1.46	43.17 ± 1.74
PI	2.38 ± 0.39	4.60 ± 0.71[b]	3.24 ± 0.57
PS	0.98 ± 0.28	2.44 ± 0.59[a]	2.20 ± 0.54
PE	35.14 ± 1.02	34.79 ± 0.99	37.81 ± 1.73
DPG	12.02 ± 1.19	7.15 ± 0.45[d]	8.35 ± 0.25[c]

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$; [b]$P < 0.02$; [c]$P < 0.01$ and [d]$P < 0.001$ compared to the corresponding controls.

Table 3

Effect of corticosterone treatment on phospholipid composition of brain mitochondria from 21-day-old rats.

Phospholipid class	Phospholipid composition (% of total)		
	Control(9)	Acute(10)	Chronic(6)
LYSO	3.92 ± 1.00	2.87 ± 0.35	1.77 ± 0.59
SPM	5.72 ± 0.49	6.62 ± 0.67	8.38 ± 1.22
PC	40.53 ± 0.56	40.78 ± 1.58	43.54 ± 1.46
PI	4.47 ± 0.55	5.49 ± 0.51	3.99 ± 1.23
PS	1.65 ± 0.47	4.32 ± 0.40[b]	1.57 ± 0.30
PE	37.36 ± 1.05	34.09 ± 1.04[a]	35.83 ± 2.66
DPG	6.35 ± 0.32	5.83 ± 0.51	4.96 ± 0.79

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$ and [b]$P < 0.001$ compared to the corresponding controls.

Table 4

Effect of corticosterone treatment on phospholipid composition of brain mitochondria from 35-day-old rats.

Phospholipid class	Phospholipid composition (% of total)		
	Control(7)	Acute(8)	Chronic(8)
LYSO	4.11 ± 1.12	2.33 ± 0.61	4.14 ± 0.62
SPM	7.67 ± 0.99	6.90 ± 1.13	6.79 ± 1.35
PC	36.52 ± 1.71	37.99 ± 1.33	34.15 ± 0.93
PI	4.21 ± 0.87	4.85 ± 1.22	4.39 ± 0.94
PS	4.06 ± 1.33	2.96 ± 1.11	5.18 ± 1.40
PE	35.60 ± 1.70	37.62 ± 1.37	35.83 ± 1.26
DPG	7.82 ± 0.96	7.34 ± 0.46	8.78 ± 0.53

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

Table 5

Effect of corticosterone treatment on phospholipid composition of brain mitochondria from adult rats.

Phospholipid class	Phospholipid composition (% of total)		
	Control(11)	Acute(10)	Chronic(8)
Lyso	2.67 ± 0.70	3.64 ± 0.57	2.73 ± 0.66
SPM	5.29 ± 0.43	6.24 ± 0.28	4.98 ± 0.69
PC	39.69 ± 1.02	35.86 ± 0.88[b]	37.24 ± 1.25
PI	3.61 ± 0.18	4.13 ± 0.49	3.63 ± 0.36
PS	2.74 ± 0.24	2.92 ± 0.33	2.79 ± 0.34
PE	39.59 ± 0.46	39.66 ± 1.23	43.52 ± 1.25[a]
DPG	6.43 ± 0.35	7.25 ± 0.89	5.79 ± 0.71

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$ and [b]$P < 0.02$ compared to the corresponding controls.

In case of adults, acute treatment caused 10% decrease in PC whereas the chronic treatment caused 10% decrease in PE. Composition of other phospholipids was not affected by either of the two corticosterone treatments (Table 5).

Tables 6 to 9 summarize the effects of corticosterone treatments on actual contents of individual phospholipids (μg/mg protein) of brain mitochondria from animals of different age groups.

In 14-day-old animals, acute treatment caused significant increase in content of almost all the individual phospholipid except the lysophospholipids which remained unaltered and DPG had decreased by 25%. The contents of SPM, PC, PI, PS and PE increased, with the extent of increase being much higher in SPM and PS compared to PI and PC. Chronic treatment caused about 80 to 120% increase in SPM and PS; DPG content had decreased by 30%. The effects of chronic treatment were more or less similar to those observed in acute treatment group, but the magnitude of change in individual phospholipid contents were different in the two treatment groups (Table 6).

Similarly, in 21-day-old rats also the acute treatment led to 40 to 300% increase in content of all the phospholipids except lysophospholipids. The increase in PS content was 4 fold upon acute treatment. On the other hand, chronic treatment decreased significantly the contents of LYSO and DPG but increased SPM content of brain mitochondria (Table 7).

Table 6

Effect of corticosterone treatment on phospholipid content of brain mitochondria from 14-day-old rats.

Phospholipid class	Phospholipid content (µg/mg protein)		
	Control(12)	Acute(12)	Chronic(6)
Lyso	8.27 ± 1.23	10.78 ± 1.17	5.48 ± 1.04
SPM	8.80 ± 1.16	34.10 ± 2.75[d]	15.60 ± 0.83[d]
PC	182.97 ± 8.45	211.96 ± 8.48[a]	174.00 ± 6.93
PI	11.44 ± 1.27	23.37 ± 2.33[d]	13.05 ± 1.41
PS	4.00 ± 0.67	12.41 ± 1.77[d]	8.85 ± 1.26[b]
PE	142.89 ± 6.10	177.00 ± 6.50[d]	152.40 ± 6.50
DPG	49.17 ± 3.82	36.36 ± 1.96[b]	13.66 ± 1.17[c]

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.002$ and [d]$P < 0.001$ compared to the corresponding controls.

Table 7

Effect of corticosterone treatment on phospholipid content of brain mitochondria from 21-day-old rats.

Phospholipid class	Phospholipid content (µg/mg protein)		
	Control(9)	Acute(10)	Chronic(6)
Lyso	20.00 ± 3.13	22.80 ± 2.11	8.88 ± 1.65[c]
SPM	29.23 ± 2.10	52.60 ± 4.34[d]	42.15 ± 4.06[b]
PC	207.20 ± 7.40	325.00 ± 16.66[d]	218.53 ± 8.84
PI	22.86 ± 2.06	43.67 ± 3.44[d]	20.02 ± 3.56
PS	8.44 ± 1.44	34.37 ± 3.68[d]	7.90 ± 0.93
PE	190.98 ± 8.20	270.87 ± 12.77[d]	179.83 ± 10.93
DPG	32.46 ± 1.75	46.37 ± 3.51[c]	24.89 ± 2.55[a]

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$; [b]$P < 0.02$; [c]$P < 0.01$ and [d]$P < 0.001$ compared to the corresponding controls.

Table 8

Effect of corticosterone treatment on phospholipid content of brain mitochondria from 35-day-old rats.

Phospholipid class	Phospholipid content (µg/mg protein)		
	Control(7)	Acute(8)	Chronic(8)
Lyso	21.01 ± 3.25	11.62 ± 1.70[a]	24.00 ± 2.11
SPM	39.23 ± 3.25	34.43 ± 3.36	39.42 ± 4.44
PC	186.81 ± 7.80	189.60 ± 6.32	198.23 ± 5.32
PI	21.52 ± 2.61	24.21 ± 3.43	25.48 ± 3.06
PS	20.76 ± 3.78	14.75 ± 3.00	30.05 ± 4.45
PE	182.10 ± 7.68	187.76 ± 6.37	208.00 ± 6.41[a]
DPG	40.00 ± 3.19	36.63 ± 1.72	59.99 ± 2.59[b]

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$ and [b]$P < 0.001$ compared to the corresponding controls.

Table 9

Effect of corticosterone treatment on phospholipid composition of brain mitochondria from adult rats.

Phospholipid class	Phospholipid content (µg/mg protein)		
	Control(11)	Acute(10)	Chronic(8)
Lyso	18.20 ± 3.07	21.78 ± 2.10	10.43 ± 1.55[a]
SPM	36.03 ± 2.78	37.58 ± 1.47	19.02 ± 1.87[c]
PC	269.78 ± 13.78	215.97 ± 6.77[b]	143.86 ± 6.78[c]
PI	24.61 ± 1.59	24.76 ± 1.87	13.67 ± 1.47[c]
PS	18.68 ± 1.53	17.63 ± 1.33	10.73 ± 0.95[c]
PE	269.77 ± 11.90	238.08 ± 8.18[a]	166.39 ± 7.25[c]
DPG	43.75 ± 2.78	43.67 ± 3.48	22.17 ± 2.00[c]

Results are given as mean ± SEM of number of independent observations for each group as indicated in parentheses.

[a]$P < 0.05$; [b]$P < 0.01$ and [c]$P < 0.001$ compared to the corresponding controls.

The mitochondrial content of the individual phospholipids in 35-day-old animals was least affected by corticosterone treatments. Acute treatment reduced LYSO by 45% and chronic treatment led to increase in PE and DPG by 15 to 50%.

In case of adults, acute treatment caused significant decrease in PC and PE content. Chronic treatment decreased the contents of all the mitochondrial phospholipids by 40 to 50% (Table 9).

Since corticosterone treatments induced host of changes in phospholipid composition and content of brain mitochondria, it was of interest to study the effects of corticosterone treatments on phospholipid synthesis during development. Table 10 shows effects of corticosterone treatments on ^{3}H acetate incorporation into individual phospholipids in brain mitochondria from animals of different age groups.

In control animals, the brain mitochondria from 14-day-age group showed highest rate of ^{3}H acetate incorporation in all the phospholipid classes, and the incorporation of label decreased with advancement of age. In case of adults, compared to 14-day-old animals, the rates of radioactivity incorporation had decreased by 87 to 97% in all the phospholipid classes except PI.

Effects of corticosterone treatment on ^{3}H acetate incorporation into different phospholipids were also age-

Table 10

Effect of corticosterone treatments on ^{3}H acetate incorporation in phospholipids in brain mitochondria during development.

Age/ Treatment	Label incorporation (DPM/μmol) in different phospholipids						
	LYSO	SPM	PC	PI	PS	PE	DPG
14 Day							
Control	531	1959	566	689	1520	274	18022
Acute	174	286	356	313	1018	83	9539
Chronic	532	257	236	1156	1156	91	6201
21 Day							
Control	222	465	140	377	480	139	3651
Acute	148	98	210	228	397	67	4350
Chronic	501	177	133	212	123	213	3997
35 Day							
Control	234	177	38	50	341	59	1203
Acute	101	5	33	158	79	24	1179
Chronic	107	216	35	153	405	10	646
Adult							
Control	85	98	52	775	109	23	492
Acute	322	498	93	773	439	62	705
Chronic	—	—	27	179	316	43	737

Results for each datapoint are average of two observations in duplicate

dependent and treatment-specific. In mitochondria from 14-day age group, acute treatment caused about 30 to 85% reduction in all the phospholipids and chronic treatment also had similar effects with an exception that it increased label incorporation into PI by 70%. The extent of decrease was different for different phospholipids and also treatment specific; maximum decrease was observed in SPM (85 to 87% decrease) in both the treatment groups.

The effects of corticosterone in 21-day-old group were more or less similar to those mentioned earlier for 14-day-group. However, there were some differences: in 21-day-group both the treatments showed slight (10 to 20%) increase in label incorporation in DPG and acute treatment caused 50% increase in PC whereas chronic treatment led to 50% increase in rate of label incorporation into PE.

In 35-day-old animals, acute treatment caused about 60 to 97% decrease in incorporation of ^{3}H acetate in LYSO, SPM, PS and PE; maximum (97%) decrease was observed in LYSO. The incorporation into PC and DPG was not affected but in case of PI it was increased by about 200%. Chronic treatment had similar effects on LYSO, PC, PI and PE components; in case of SPM and PS it increased by about 20% and DPG showed 45% decrease in label indorporation.

Table 11

Effect of corticosterone treatment on membrane fluidity of brain mitochondria from 14-day-old rats.

Parameter	Treatment		
	Control	Acute	Chronic
Fluorescence Polarization (P)	0.205 ± 0.002	0.193 ± 0.001[a]	0.172 ± 0.003[a]
Fluorescence anisotropy (r)	0.146 ± 0.001	0.137 ± 0.001[a]	0.122 ± 0.002[a]
Limited hindered anistropy (r∞)	0.095 ± 0.002	0.083 ± 0.001[a]	0.062 ± 0.003[a]
Order parameter (S)	0.805 ± 0.004	0.777 ± 0.004[a]	0.714 ± 0.010[a]

Results are give as mean ± SEM of 8 independent observations in each group.

[a] $P < 0.001$ compared to the corresponding controls.

Table 12

Effect of corticosterone treatment on membrane fluidity of brain mitochondria from 21-day-old rats.

Parameter	Treatment		
	Control	Acute	Chronic
Fluorescence Polarization (P)	0.159 ± 0.003	0.163 ± 0.003	0.175 ± 0.002[a]
Fluorescence anisotropy (r)	0.112 ± 0.002	0.115 ± 0.002	0.124 ± 0.002[a]
Limited hindered anistropy (r_∞)	0.049 ± 0.003	0.053 ± 0.003	0.065 ± 0.002[a]
Order parameter (S)	0.660 ± 0.013	0.676 ± 0.015	0.725 ± 0.008[a]

Results are give as mean ± SEM of 8 independent observations in each group.

[a] $P < 0.001$ compared to the corresponding controls.

Table 13

Effect of corticosterone treatment on membrane fluidity of brain mitochondria from 35-day-old rats.

Parameter	Treatment		
	Control	Acute	Chronic
Fluorescence Polarization (P)	0.147 ± 0.003	0.138 ± 0.003	0.152 ± 0.003
Fluorescence anisotropy (r)	0.103 ± 0.002	0.096 ± 0.002[a]	0.107 ± 0.002
Limited hindered anistropy (r∞)	0.037 ± 0.003	0.028 ± 0.003	0.043 ± 0.002
Order parameter (S)	0.570 ± 0.027	0.531 ± 0.027	0.629 ± 0.014

Results are give as mean ± SEM of 8 independent observations in each group.

[a] $P < 0.05$ compared to the corresponding controls.

Table 14

Effect of corticosterone treatment on membrane fluidity of brain mitochondria from adult rats.

Parameter	Treatment		
	Control	Acute	Chronic
Fluorescence Polarization (P)	0.171 ± 0.006	0.188 ± 0.006	0.196 ± 0.006[a]
Fluorescence anisotropy (r)	0.121 ± 0.005	0.134 ± 0.005	0.140 ± 0.005[b]
Limited hindered anistropy (r∞)	0.061 ± 0.006	0.078 ± 0.006	0.087 ± 0.006[c]
Order parameter (S)	0.698 ± 0.021	0.758 ± 0.017[a]	0.783 ± 0.014[c]

Results are give as mean ± SEM of 8 independent observations in each group.

[a] $P < 0.05$; [b] $P < 0.02$ and [c] $P < 0.01$ compared to the corresponding controls.

Adult group upon acute treatment showed 78 to 400% increase in ^{3}H acetate incorporation in all phospholipid classes except PI, which remained unaltered. The maximum (400%) increase was observed in SPM. Chronic treatment led to about 50 and 75% decrease in incorporation in PC and PI respectively. The incorporation of label into LYSO and SPM was undetectable. PS, PE and DPG showed 50 to 190 % increase in label incorporation.

Tables 11 to 14 summarize the effects of corticosterone treatments on various membrane fluidity parameters of brain mitochondria from animals of different age groups. In 14-day-old animals, both the treatments caused significant increase in membrane fluidity as evident from decrease in fluorescence polarization (P), fluorescence anisotropy (r), limited hindered anisotropy (r_∞) and order parmeter (S). In case of 21-day-old and adult rats, the acute treatment had no effect but chronic treatment caused significant decrease in membrane fluidity. Both the treatments did not have much effect on mitochondrial membrane fluidity in 35-day-old animals.

DISCUSSION

The results of the present studies have shown that corticosterone treatments significantly affects mitochondrial TPL, CHL contents and TPL/CHL molar ratios. The effects were more pronounced in young animals and adults were least

affected. The relative proportion of the phospholipid and cholesterol is one of the major factors governing membrane fluidity (17) which in turn could affect the kinetic properties of many of the membrane associated enzymes. From the Arrhenius kinetics studies, it was found that in 14-days group chrnonic treatment had abolished the phase transition temperature. In 35-day-old and adult animals belonging to control as well as corticosterone treatment groups also no break could be detected in Arrhenius plots of ATPase (Chapter III). The results of the present studies revealed that the animals from the above mentioned groups contained higher amounts of cholesterol in brain mitochondria (Table 1); this could be the possible reason for disappearance of breaks in Arrhenius plots. The Arrhenius plots of mitochondrial ATPase in yeast are known to be affected by sterols; at higher concentrations sterols could abolish the break in Arrhenius plots of ATPase (18).

In young animals, corticosterone treatment significantly increased the TPL content of brain mitochondria. This could be due to the increased phospholipid synthesis. Glucocorticoids are known to induce the enzyme GPDH in brain which is one of the key enzyme in phospholipid biosynthesis (19,20). Secondly, if corticosterone damaged the mitochondrial membranes the proteins of mitochondrial matrix will leak out and the values of TPL content/mg protein would look higher. This does not

seem to be the case, as glucocorticoids alter the permeability of mitochondrial membranes but proteins do not leak out from these subcellular organelles (21).

The effects of corticosterone treatment on phopholipid composition/content of brain mitochondria were age-dependent and treatment-specific. Compositionwise maximum alterations were observed in 14-day group. Paradoxically, although the maximum deleterious effects of corticosterone on oxidative energy metabolism were observed in 35-day group (Chapter II), the phospholipid composition of mitochondria from this age group did not change upon corticosterone treatments. The content of practically all the phospholipids increased significantly upon acute treatment in 14- and 21-day-old animals. This could be attributed to significant increase in TPL content mentioned earlier in these age groups. Increased SPM content in brain mitochondria from corticosterone treated animals is supported by the reported findings. In vivo treatments with glucocorticoids have been known to increase SPM component in leucocytes (22) and fat cells (23). In fibroblast cell line dexamethasone caused increase in SPM content by inducing the enzyme in the biosynthetic pathway (24).

Incorporation studies revealed that the rates of synthesis of individual phospholipids of brain mitochondria

were highest in 14-day group and declined gradually with advancement of age; the biosynthetic rates decreased by 90% in adults compared to the 14-day-old rats. It is known that by the age of 35-days the brain development nearly becomes complete, and as the animal reaches the adult stage there is no synthesis of new cells and even the neuronal connectivities are also established in the brain (25). Hence in adult animals the observed low rate of phospholipid synthesis may be adequate for the maintainance of cerebral function.

Although corticosterone treatments in general caused increase in total and individual phospholipid contents in 14- and 21-day-old animals, the rate of ^{3}H acetate incorporation into different phospholipids of brain mitochondria was decreased. Since the phospholipids content increased in spite of the decreased synthesis, it may be due to increased transport of the phospholipids from microsomes or decreased turnover. Most of the mitochondrial phospholipids except DPG and SPM are synthesized by microsomes (2). Chronic corticosterone treatment to 17-day-old rats has been known to decrease $U^{14}C$ glucose incorporation into phospholipids in different brain regions (7).

In 35-day group also the corticosterone treatment decreased ^{3}H acetate incorporation into phospholipids. In contrast, acute treatment to adult animals caused significant increase in label incorporation into all the phospholipids

except PI; chronic treatment also had similar effect on PS, PE and DPG. In adult animals the effects of corticosterone were treatment specific. Acute treatment besides increasing the rate of phospholipid synthesis, also increased the breakdown as evidenced from 3.5 fold increase in counts in lysophospholipids. The chronic treatment, on the other hand, seems to inhibit phospholipid breakdown as no detectable counts were observed in lysophospholipids. In chronically treated animals this could be a mechanism to correct the decreased TPL content of brain mitochondria.

The data on membrane fluidity indicate that in 35-day-old rats, corticosterone treatments had no effect on fluidity. As mentioned earlier, in brain mitochondria from this age group corticosterone treatment did not alter significantly the phospholipid composition. In 14-day-old rats where both the corticosterone treatments induced host of changes in mitochondrial phospholipids, this also led to significant increase in membrane fluidity. In 21-day-old and adult rats chronic treatment significantly decreased membrane fluidity of brain mitochondria.

The alterations in phospholipids fatty acid composition can also affect the membrane fluidity. Glucocorticoid induced changes in fatty acid composition of phospholipids of liver microsomes has been reported (9). As mentioned earlier (2)

most of the mitochondrial phospholipids are synthesized by microsomes and then transported to mitochondria and other membranes. Hence, corticosterone treatments could possibly also affect the fatty acid composition of phospholipids during their synthesis. This possibility, however, needs to be varified experimentally.

REFERENCES

1. Brown,R.E. and Cunningham,C.C. (1982) Negatively charged phospholipid requirement of the oligomycin-sensitive mitochondrial ATPase. Biochim.Biophys.Acta 684, 141-145.

2. Daum,G. (1985) Lipids of mitochondria. Biochim.Biophys. acta 822, 1-42.

3. DeVellis,J. and Inglish,D. (1973) Age-dependent changes in the regulation of glycerolphosphate dehydrogenase in the rat brain and in a glial cell line. In: Neurobiological Aspects of Maturation and Aging(Ford, D.H.ed.) Elsevier, Amsterdam. pp.321-330.

4. Warringa,R.A.J., Hoeben,R.C., Koper,J.W., Sykes,J.E.C., VanGolde,L.M.G. and Lopez-Cardozo,M. (1987) Hydrocortisone stimulates the development of oligodendrocytes in primary glial cultures and affects glucose metabolism and lipid synthesis in these cultures. Dev.Brain Res. 34, 79-86.

5. Noguchi,T., Sugisaka,T., Watanabe,M., Kohsaka,S. and Tsukada,Y. (1982) Effects of porcine growth hormone on the retarded cerebral development induced by neonatal hydrocortisone intoxication. J.Neurochem. 38, 246-256.

6. Meyer,J.S. and Fairman,K.R. (1985) Early adrenalectomy increases myelin content of rat brain. Dev.Brain Res. 17, 1-9.

7. Bhargava,H.K., Tenneti,L. and Telang,S.D. (1991) Corticosterone administration and lipid metabolism in brain regions during development. Indian J. Biochem. Biophys. 28, 214-218.

8. Kaur,N., Sharma,N. and Gupta,A.K. (1989) Effects of dexamethasone on lipid metabolism in rat organs Indian Journal of Biochemistry and Biophysics 26, 371-376.

9. Melby,J.M., Wennhold,A.R. and Nelson,D.H. (1981) Corticosteroid-induced lipid changes in rat liver microsomes. Endocrinology 109, 920-923.

10. Folch,J., Lees,M. and Sloane-Stanley,G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues J.Biol.Chem. 226, 497-509.

11. Bangur,C.S., Howland,J.L. and Katyare,S.S. (1995) Thyroid hormone treatment alters phospholipid composition and membrane fluidity of rat brain mitochondria Biochem.J. 305, 29-32.

12. Mangold,H. (1969) Aliphatic lipids. In:Thin-layer chromatography (Stahl,E. ed.) Springer Verlag, Berlin. pp. 363-420.

13. Bartlett,G.R. (1954) Phosphorous assay in column chromatography J.Biol.Chem. 234, 466-468.

14. Zlatkis,A., Zak,B. and Boyle,J.A. (1953) A new method for the determination of serum cholesterol. J.Lab.Clin.Med. 41, 486-492.

15. Mehta,J,R, Braund,K.G., Hegreberg,G.A. and Thukral,V. (1991) Lipid fluidity and composition of the erythrocyte membrane from healthy dogs and Labrador retrievers with hereditary muscular dystrophy. Neurochem.Res. 16, 129-135.

16. VanBlitterswijk,W.J., VanHoeven,R.P. and Van der Meer,B.W. (1981) Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements Biochim.Biophys.Acta 644, 323-332.

17. Duval,D., Durant,S. and Homo-Delarche,F. (1983) Non-genomic effects of steroids: Interactions of steroid molecules with membrane structures and functions Biochim.Biophys.Acta 737, 409-442.

18. Demel,R.A. and Kruyff,B.D. (1976) The function of sterols in membranes. Biochim.Biophys.Acta 437, 109-132.

19. Weingarten,D.P., Kumar,S., Bressler,J. and DeVellis,J. (1984) Regulation of differentiated properties of oligodendrocytes. In: Oligodendroglia, Advances in

Neurochemistry Vol.5 (Norton,W.T. ed.) pp.299-338.

20. Meyer,J.S. (1985) Biochemical effects of corticosteroids on neural tissues. Physiol.Rev. 65, 946-1020.

21. Blecher,M. and White,A. (1960) Alterations produced by steroids in adenosine triphosphatase activity and volume of lymphosarcoma and liver mitochondria. J.Biol.Chem. 235, 3404-3412.

22. Nelson,D.H. (1980) Corticosteroid induced changes in phospholipid membranes as mediators of their action. Endocrine Rev. 1, 180-198.

23. Murray,D.K., Rhumann-Wennhold,A. and Nelson,D.H. (1982) Adrenalectomy decreases the sphingomyelin and cholesterol content of fat cell ghost. Endocrinology 111, 452-455.

24. Nelson,D.H. and Murray,D.K. (1982) Dexamethasone increases the synthesis of sphingomyelin in 3T3-L1 cell membranes. Proc.Natl.Acad.Sci.(USA) 79, 6690-6692.

25. Cotterrell,M., Balazs,R. and Johnson,A.L. (1972) Effects of corticosteroids on the biochemical maturation of rat brain: Postnatal cell formation. J.Neurochem. 19, 2151-2167.

SUMMARY

1. Both acute and chronic corticosterone treatments significantly altered the contents of TPL and CHL and TPL/CHL molar ratios in brain mitochondria. The effects were more pronounced young animals than the adults.

2. The effects of corticosterone treatments on phospholipid composition/content of brain mitochondria were age-dependent and treatment-specific. Maximum alterations in phospholipid composition were observed in 14-day group; 35-day-old rats remained unaffected.

   In 14- and 21-day-old animals, content of practically all the phospholipids increased significantly upon acute treatment, which seems to be due to significant increase in TPL content in these groups.

3. Rates of synthesis of individual phospholipids of brain mitochondria were highest in 14-day-old rats which declined gradually and by the adult age the decrease amounted to about 90% compared to the 14-day-group.

   Effects of corticosterone treatments on phospholipid synthesis were age-dependent. Young animals in general showed decreased incorporation of ^{3}H acetate into different phospholipids upon treatment with corticosterone; adults showed generalized increase in

label incorporation into phospholipids upon corticosterone treatment.

4. The membrane fluidity of brain mitochondria from corticosterone treated 35-day-old rats remained unchanged. In 14-day-old animals corticosterone treatments significantly increased the membrane fluidity. Chronic treatment to 21-day-old and adult rats led to significant decrease in membrane fluidity of brain mitochondria.