STUDIES ON THE ENDOGENOUS PHOSPHOLIPIDS OF MAMMALIAN KIDNEY AND THEIR *IN VITRO* HYDROLYSIS BY ENDOGENOUS PHOSPHOLIPASES

BY

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Acknowledgment

(Verily we created man from a drop of mingled sperm) [(Koran) chapter76 (Time), verse 2].

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Abstract*

There are several recent detailed reviews on cellular phospholipase A_2 ($cPLA_2$), yet the role of $cPLA_2$ in cell function remains unclear.

Recently, we have reported that the endogenous cardiolipin (CL) of various mammalian myocardia has been shown to be preferentially deacylated by an endogenous phospholipase (PLA) during a 60 minutes *in vitro* incubation of whole tissue homogenate of these cardiac muscles, producing monolysocardiolipin (MLCL).

In the present study, we provide additional data demonstrating that the phenomenon of CL deacylation also occurs in mammalian kidney.

In vitro incubation of whole tissue homogenate of freeze-dried kidney from various mammals (as an endogenous source of substrates and enzymes) for 60 minutes, at pH 7.4 and 38°C uniformly deacylated CL. Thin layer chromatographic analysis (TLC) and TLC densitometry provided the in vitro incubation data. Following TLC separation, MLCL as well as CL were differentially detected by the thionine reagent. Concurrent with this deacylation of CL, there was also formation of Lyso alkenyl phosphatidyl ethanolamine (LPE) and ceramide.

These data indicate a) the action of endogenous PLA_2 on sn-2 of alkenyl PE resulting in LPE and diminution of PE plasmalogen, and most probably on sn-2 fatty acid of CL producing MLCL, and b) the action of sphingomyelinase on the endogenous sphingomyelin (SM) forming ceramide.

Details pertaining to a) the *in vitro* incubation, b) the diverse chromatographic analysis used to identify the products, and c) what second messengers interpretation may be operating in vivo will be discussed.

* This abstract was submitted and accepted, in preparation for a poster presentation, at the XIX International Congress of Biochemistry and Molecular Biology, Toronto, Canada; July 20-24, 2003 (Letter of acceptance page iv)

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Introduction

The enzymology of phospholipase A_2 (PLA_2) and to a lesser extent PLA_1 have received considerable attention in recent years, largely by the application of a variety of analytical techniques limited to a single substrate (natural or concocted) in accommodation of instrumental requirements (1, 2).

Detailed examination of amino acid sequence homology data, including multiple disulfide bridge structure has facilitated the grouping of PLA_2 into groups I, II and III, small Ca^{2+}- dependent secretory phospholipase A_2 ($sPLA_2$) with monomeric molecular mass of 14 KD (2). This is in contrast with that portion of the cellular ($cPLA_2$) of various tissues with molecular mass categorically indicated as 40 KD and 85 KD, some reported to be Ca^{2+}-independent (3, 4, 5), and some have been implicated in various cellular responses such as differentiation, cytotoxicity and mutagenesis (6, 7, 8, 9). Yet the mechanisms of their activation and regulation of cell function remain unclear.

How these various PLAs accommodate to, or restrict, the different head groups of glycerophospholipids of various tissues, including diphosphatidyl glycerol [i.e. cardiolipin (CL)], remains incompletely described as does the isoforms situation. Cardiolipin is fully accessible to both PLA_1 and PLA_2, but plasmalogens (sn-1'enyl phospholipids) are only hydrolyzed by PLA_2 (Fig 1).

For some time, our laboratory has been concerned with CL and the plasmalogen components of tissues very adequately represented by the extractable phospholipids of mammalian cardiac muscle (10, 11).

In our most recent studies (12,13, 14), we have reported that the endogenous CL of mammalian myocardia is preferentially deacylated by an endogenous phospholipase (PLA_1/PLA_2) during a 45 minutes *in vitro* incubation of cardiac muscle homogenate at pH 7.4 and 38°C producing monolysocardiolipin (MLCL) and concurrent diminution of CL level. The use of thionine as the TLC spot test reagent (15) facilitated the recognition of these deacylation products as well as CL.

Our earlier report demonstrated the diminution of CL in the experimentally-induced dog myocardium infarction and the infarcted cardiac muscle of cat, rabbit and man, with subsequent appearance of lyso derivatives of CL and concurrent mitochondrial swelling (16, 17, 18). These aforementioned data strongly suggest that CL occupies a unique position in the metabolism of phospholipids, not yet well defined.

CL is an exclusive mitochondrial lipid, is associated with the enzymes of oxidative metabolism, and contains largely 18:1, 18:2 and 18:3 fatty acids (19, 20, 21, 22).

In an attempt to learn more about the actual role of CL, we raised the following question: Is the CL deacylation phenomenon, referred to above, unique to cardiac muscle or does it occur in other tissues as well? We selected the kidney as an organ known to contain numerous mitochondria in its nephrons comparable to cardiac muscle, which also has numerous mitochondria and correlates with high level of cardiolipin.

In the present study, we demonstrate that the deacylation process of CL is not only unique to mammalian myocardia, but also occurs in mammalian kidney.

We report here, TLC evidence of the *in vitro* response of mammalian kidney (from 6 species) where both the substrates (phospholipids) and enzymes (phospholipases) are endogenous. This TLC analysis demonstrates, for the first time, the deacylation of endogenous CL and the plasmalogens by endogenous phospholipases (PLA_1/PLA_2) and the hydrolysis of sphingomyelin (SM) by an endogenous sphingomyelinase producing ceramide in mammalian kidney.

Details pertaining to **a)** the *in vitro* incubation procedure, and **b)** the diverse chromatographic analysis in conjunction with densitometric measurements to identify the products as well as the phospholipid profile of these kidneys, including interpretation of the data as related to renal physiology will be described.

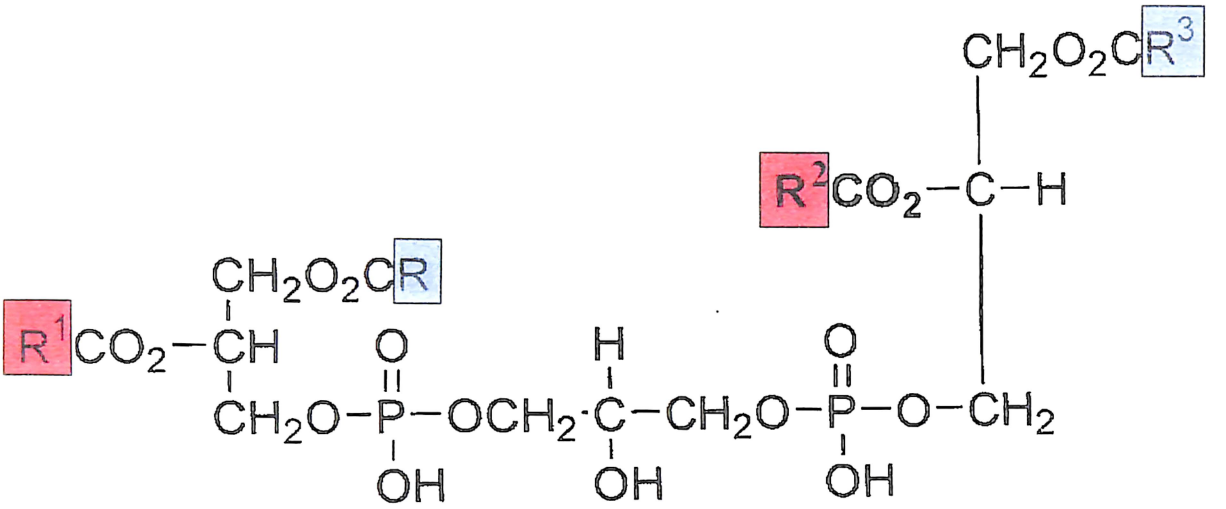


Figure 1A:
Cardiolipin can be recognized as a shared dimer of phosphatidyl glycerol. R and R^3 are sn-1 and R^1 and R^2 are sn-2, therefore, represent the respective sites of PLA_1 and PLA_2 activity

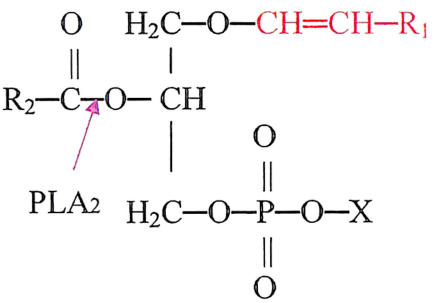


Figure 1B:
Phospholipid with an alkenyl ether, and sn-2 fatty acid (arrow), the site of PLA_2 hydrolysis.

Materials & Methods

I. Preparation of Tissues

Kidneys from six mammalian species (young adults) were used in this study as listed in the table below. Guinea pigs were obtained from Covance Research Products, Denver, PA, USA. Rats were bought from Charles River Laboratories, Wilmington, ME, USA. Mice were bought from Jackson Laboratory, Bar Harbor, ME, USA. Fresh dog and cat kidneys were obtained on ice from local abattoir.

All kidneys (cat and dog as kidney cortex and medulla) were promptly placed as thin slices, after removing the fat and connective tissue, in aluminum foil, and flash-frozen between two 1 cm-thick slabs of aluminum pre-cooled and maintained at -20°C or below. The tissue was then freeze-dried in vacuo (using Labconco freeze-dryer), and pulverized with a mortar and pestle.

Mammalian Species	Number of animals	Number of kidneys	Age of animal
Cat *Felis catus*	3	6	Young adult
Dog *Canis familiaris*	3	6	Young adult
Pig *Svs scrofa*	3	6	Young adult
Guinea pig Cavia porcelus	12	24	Young adult
Rat *Rattus norvegicus*	6	12	Young adult
Mouse *Acomys cahirinus*	6	12	Young adult

II. *In Vitro* Incubation

The activities of the endogenous enzymes (phospholipases) on the endogenous phospholipids of each of the mammalian kidney species used in the current study were determined by using a control and incubation samples that were prepared by transferring the freeze-dried powder of each kidney species to a series of 13 mmx100 mm PTFE-lined screw-capped test tubes (100 mg per test tube) under the following conditions:

1) The control test tubes, each containing 100 mg of powder, were directly extracted with chloroform: methanol mixture (C:M) 2:1, (2 ml/test tube).

2) *In Vitro* Incubation Procedure was as follow:

   The remaining series of test tubes, each containing 100 mg freeze-dried powder, 2 ml of Tris buffer (0.05 M, pH 7.4) and 20 µl of calcium chloride (0.1 M) were incubated at 38°C with frequent vortex mixing for 60 minutes. Some incubation was done with added disodium EDTA (0.1 M, 10 µl/test tube), and others without addition of Ca^{2+}. The incubation was terminated by rapid freezing of the test tubes contents (in situ) and freeze-dried. The freeze-dried tissues were scraped down into each test tube and were extracted with C: M (2:1), 2 ml/test tube.

3) Direct comparison of C: M extracts of both controls and phospholipid standards (CL, PE, PC, SM) by multiple forms of TLC technology would identify the major phospholipids of control kidney specimens. TLC spot-test analysis for amino lipids, choline lipids and phosphorus were also used for confirmation of the phospholipids of kidney species (11).

4) In addition, direct comparison of two adjacent lanes on TLC plates of the C:M extracts of both control tissues and their respective incubated samples revealed the changes that occur during incubation and accordingly, certain deductions could be made as to the possible lipolytic enzymology their respective endogenous phospholipids encountered. For example, the production of MLCL, following incubation, (shown on the incubated TLC lane when compared with its respective control figure 2 (A-F)) is a clear evidence of an endogenous phospholipase action on endogenous CL.

III Thin Layer Chromatographic Procedure (TLC)

Thin layer chromatographic analysis was performed to determine the phospholipids profile of both control and incubated samples for each animal listed above as described elsewhere (11). This was accomplished with the use of commercial 10x10 cm plastic silica gel-backed sheets, as the stationary phase (Scientific Absorbents Inc., Atlanta, GA) for phospholipids profile, and 10x10 cm plastic Aluminum Oxide IB for choline lipids identification (JT Backer Inc., Phillipsburg, Germany). Prior to use, TLC plates were washed sequentially with chloroform-methanol (2:1) and acetone for 10 minutes each then dried for 10 minutes.

The chromatographic chambers were CAMAG divided flat bottom design suitable for running two 10x10 cm TLC plates (CAMAG Scientific Inc Wilmington, NC). Mobile phase equilibrium established for a minimum of 1-hour prior the insertion of TLC plate. Tissue extracts were applied as spots using 10 µl or 5µl disposable capillaries (CAMAG Scientific Inc. Wilmington, NC).

TLC analysis consists of two modes: 1) **One-dimension**, where the mobile phase used consists of 1-propanol: chloroform: ethyl acetate: methanol: water (50:50:50:21:18) (designated as A-I-50) with running time 30-45 minutes to allow uniform distribution of the phospholipids. The primary spot test analysis employed thionine reagent (Fluka Chemical Corp, Milwaukee, WI). The thionine reaction solution consisted of 5 mg per 100 ml water, staining was done for 20-30 minutes, followed by six 5 minutes rinses in 0.05 M H_2SO_3 for background clearing (15). Aluminum Oxide TLC plates were used for detection of the choline lipids (sphingomyelin (SM), phosphatidyl choline (PC) and lyso PC), developed in chloroform: methanol: water (65:30:4), and stained with Biebrich scarlet reagent (5 mg/100 ml of water). Spot testing also used ninhydrin spray in ethanol (ACROS Organics, New Jersey, NJ) for amino phospholipid detection (PE, LPE, PS), and using phospray (Supelco, Bellefonte, PA) for phosphorus (11).

2) **Two-dimension** designated as 2D/HCl. Sample preparation was conducted by placing the lipid extract at the lower left of the chromatogram, and was developed as described in the 1st dimension. After it dried completely, the TLC plate was rotated 90° counter-clockwise for a second dimension and developed in 0.1 M HCl for 5-minutes (i.e. until it had passed the lipid lane) for alkenyl ether hydrolysis.

After air-drying (1hour), the released long chain aldhydes were separated from the phospholipids lane by developing the TLC plate in the same second dimension in hexane: ethyl ether (20:4), and sequentially spot tested by the Schiff aldehydes reagent (i.e. leucofuscin of the plasmal spot test).

IV. Standard reagents

Appropriate phospholipid standards (Cardiolipin (CL), Phosphatidyethanolamine (PE), Phosphatidyl choline (PC), Sphingomylin (SM), and Ceramide (Cer) obtained from (Avanti Polar lipids Inc, Phelham, AL, USA, Fluka Chemical Corp, Milwaukee, WI, USA), were employed.

V. Densitometric Measurements:

Densitometric measurements of the TLC plates were done at 600 nm (thionine: λ max at 600~nm) or 560 nm (Leucofuscin: λ max at 560~nm) or 520 nm (Biebrich: λ max at 520~nm) by using CAMAG Scanner II and the CATS software.

The graphs obtained were digitized by using UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The digitized (x,y) data were opened in Microsoft Excel, and then converted into graphs.

Results

We demonstrate here the first TLC evidence of the *in vitro* response of mammalian kidney from diverse species (guinea pig, pig, cat, dog, rat and mouse) where both the substrates (the phospholipids) and the enzymes (phospholipases) are endogenous.

The phosphoglyceride profile of these kidneys and their *in vitro* response to the endogenous lipolytic enzymes (for 1h, at pH 7.4, at 38 °C) were determined by TLC technology in conjunction with densitomitric measurements.

A variety of one and two dimension TLC chromatographic procedures, with selective spot testing for phosphorus, amino groups, alkenyl and cardiolipin, as described in details in materials and methods and elsewhere (11, 13), were employed to determine the phosphoglyceride profile of these tissues under investigation and the changes that occur subsequent to *in vitro* incubation and prior to phospholipid extraction. These changes are mainly related to a) the endogenous CL, b) alkenyl phophatidyl ethanolamine (PE plasmalogen), c) choline phospholipids [phosphatidyl choline (PC) and sphingomyelin (SM)], and d) their deacylations and/or hydrolysis to their respective lyso derivatives. Based on the products identified as a consequence of the *in vitro* incubation, certain deductions could be made pertaining to the phospholipases involved in the deacylation process. These principal TLC data are the following:

I. TLC analysis of chloroform: methanol (2:1) extracts of all control kidneys studied showed CL as a major phospholipid component, Fig 2, A, B, C, D, E and F where it is uniquely recognized by its lavender color due to the spot-test reagent, thionine [control lanes (C)].

In vitro incubation of tissue homogenates from these kidneys (for 60 minutes at pH 7.4 and 38 °C with added Ca^{2+} (0.1 M, 20 μl) produced monolyso cardiolipin (MLCL) and concurrent reduction in CL level as showed, in Fig 2 A, B, C, D, E and F (I lanes).

The degree of deacylation of CL in all kidney specimens is clearly illustrated in the accompanying scan (Fig 3 A, B, C, D, E, and F) made from TLC plates in Fig 2 (I lanes).

This deacylation phenomenon of CL in all kidneys studied indicates endogenous phospholipase (PLA_1 and/or PLA_2) activity. Addition of EDTA to the *in vitro* incubation medium or *in vitro* incubation without added Ca^{2+} reduced the level of MLCL formation indicating possible Ca^{2+} requirement for the activation of PLA_1 and/or PLA_2 as shown in pig kidney (Fig 2C).

II. PE alkenyl species is also a predominant phospholipid in all kidney specimens studied as revealed in Fig 4 A, B, C, D, E and F (control lanes **a**) and the accompanying scans Fig 5 A, B, C, D, E, and F (control lanes **a**).

In addition the 2D$_{HCl}$ chromatographic analysis of the incubated samples of all kidneys analyzed (Fig 4 $\mathbf{a_1}$ lanes, A, B, C, D, E, and F), and the scans made from these lanes (Fig 5 A, B, C, D, E, and F) showed the formation of lysoalkenyl phosphatidyl ethanolamine (LPE) (I lanes, $\mathbf{a_1}$) and concurrent diminution of alkenyl PE concentration when compared with their respective controls [**a** lanes (control)].

These novel observations indicate endogenous PLA_2 activity releasing sn-2 fatty acid on the endogenous alkenyl PE species. The guinea pig kidney showed a higher activity of this enzyme than the other kidneys studied, as revealed in the densitometric measurements (Fig 5 D).

These data suggest that the endogenous PLA_2, which deacylated PE plasmalogen, reacts preferentially with CL due to the high level of MLCL formation (Fig 2, 3) when compared with the very low level of LPE (Fig 4, 5) in all kidneys studied following the *in vitro* incubation.

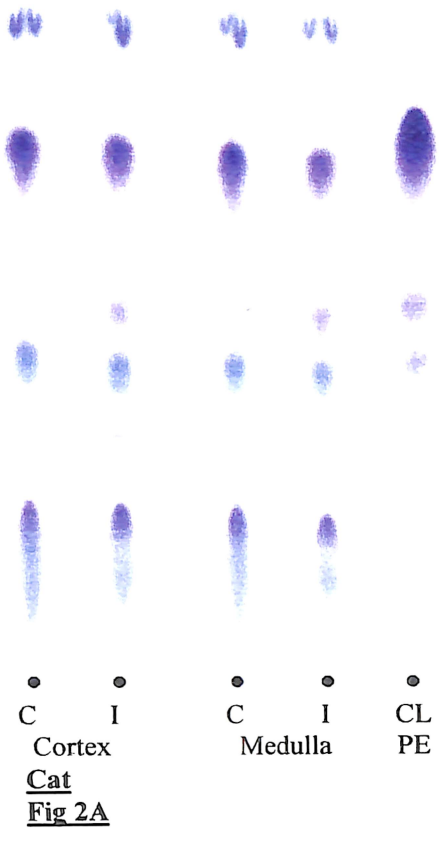
III. Phosphatidyl choline plasmalogen was also present, its level varies among the species, dog and pig kidney revealed higher concentration than the guinea pig and rat. Mouse kidney PC was barely detectable (Fig 4 A, B, C, D, E, and F).

IV. The aluminum oxide TLC preparation, (Fig 6 A, B, and C) of all kidneys studied provided clear confirmation that phophatidyl choline and sphingomyelin (SM) are among the components of the phospholipid profile of control kidneys (C lanes). Ceramide (Cer) was produced in very low concentration following *in vitro* incubation of these kidneys, as shown in (Fig 7 A, B, C, D, E, and F) (I lanes).

The formation of ceramide indicated endogenous PLC (phospholipase C) or sphingomyelinase action on SM.

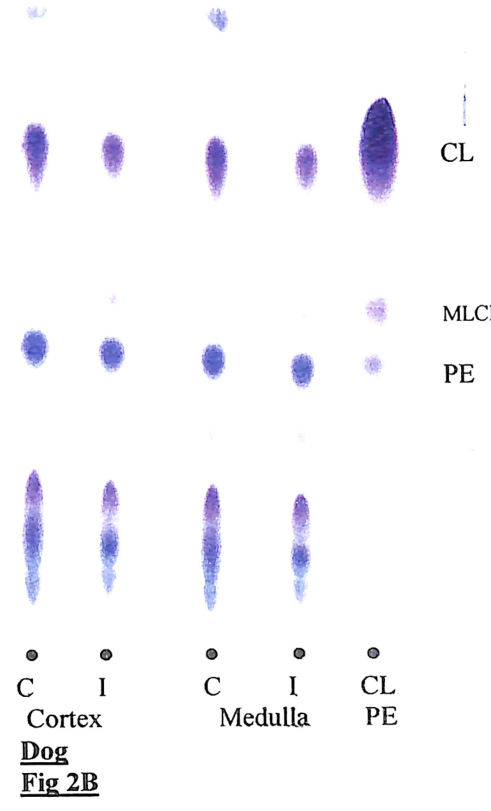
In summary: *In vitro* incubation of whole tissue homogenates of mammalian kidney from guinea pig, pig, cat, dog, rat and mouse, as a source of phospholipids and phospholipases, a) uniformly deacylated CL producing MLCL, b) slightly deacylated PE plasmalogen, producing LPE, which indicates PLA_2 action on sn-2 fatty acid of PE plasmalogen and most probably on sn-2 fatty acid of CL, and c) produced ceramide indicating the action of sphingomyelinase on SM.

Fig 2 (A, B, C & D)



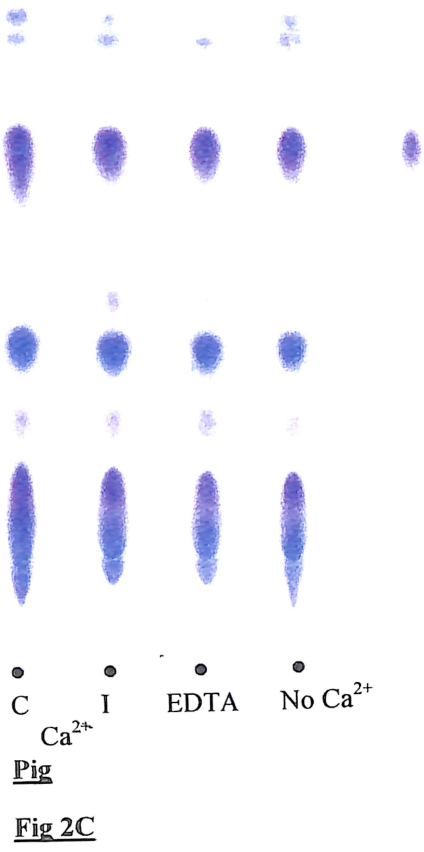
Cat

Fig 2A



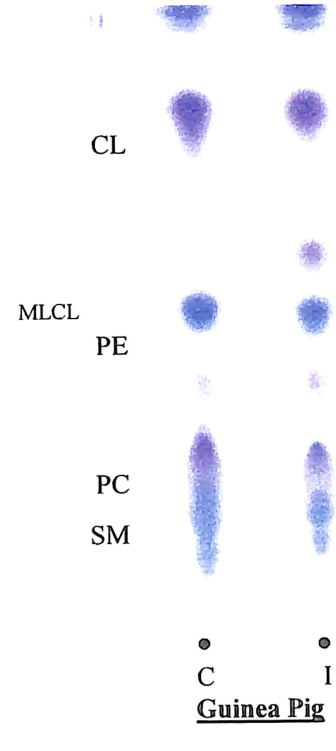
Dog

Fig 2B



Pig

Fig 2C



Guinea Pig

Fig 2D

Fig 2 (E & F)

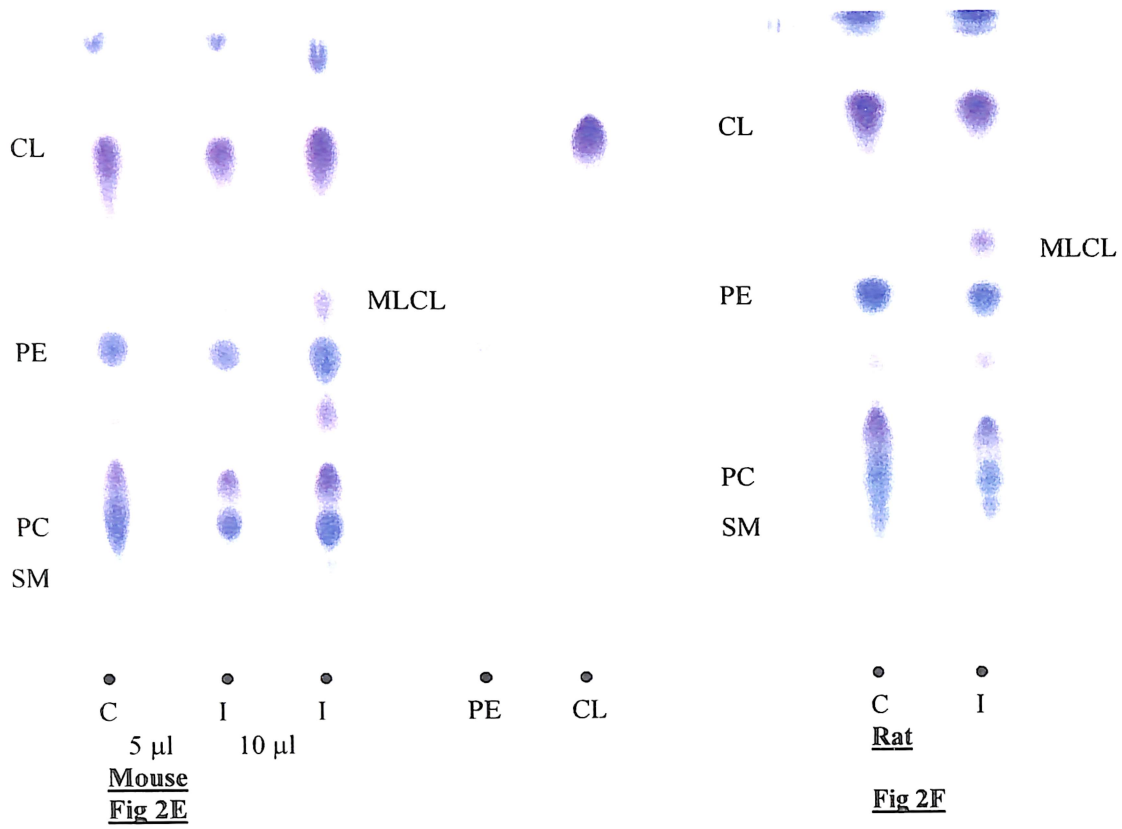


Fig 2 (A, B, C, D, E, F)

Silica Gel TLC plates developed in A-1-50 (1-propanol-chloroform-ethyl acetate-methanol, 50:50:50:21:18) and stained with the thionine reagent, revealing the unique differential staining for CL and MLCL (lavender). Comparing the control (C) lanes and incubated (I) lanes from chloroform: methanol extracts (2:1), 10µl each, from cat kidney (A), dog kidney (B), pig kidney (C), guinea pig kidney (D), mouse kidney (E), and rat kidney (F). CL and PE (C lanes) are two major phospholipids of all kidneys. Formation of MLCL with concurrent reduction of CL level (I lanes) is shown in all incubated samples.

Cardiolipin (CL), monolyso cardiolipin (MLCL), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (SM). CL, PE, and PC are standards. Incubation was done on a whole tissue homogenate, in tris. buffer pH 7.4 with added $Ca^{2}+$ (0.1M, 20µl) for 60 minutes at 38 °C with frequent vortexing. (Tissue homogenates of cat and dog represent cortex and medulla).

Fig 3 (A, B, C)

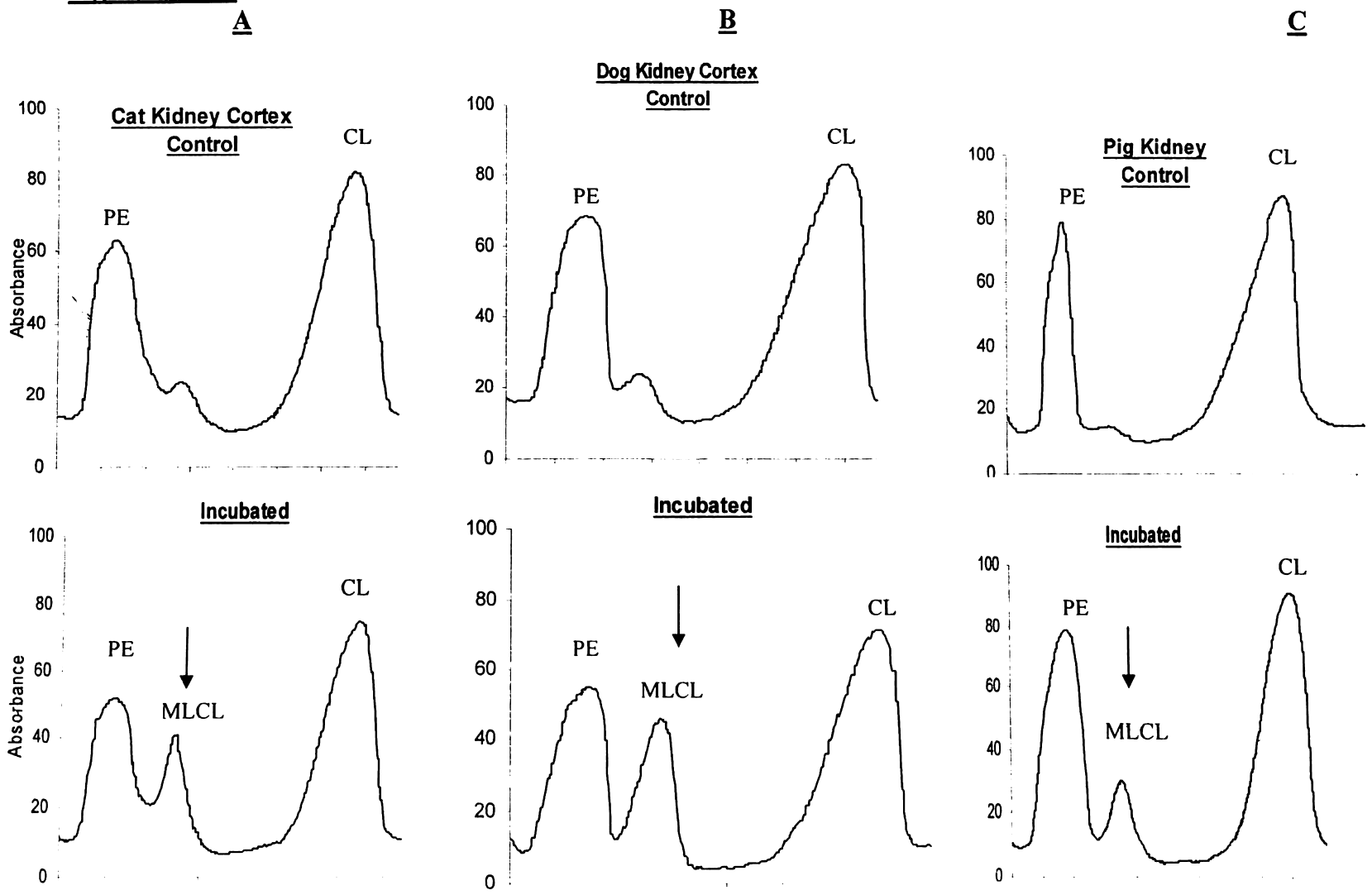

A
B
C
Cat Kidney Cortex
Control
Dog Kidney Cortex
Control
Pig Kidney
Control
PE
CL
Absorbance
100
80
60
40
20
0
Incubated
MLCL

Fig3 (D, E, F)

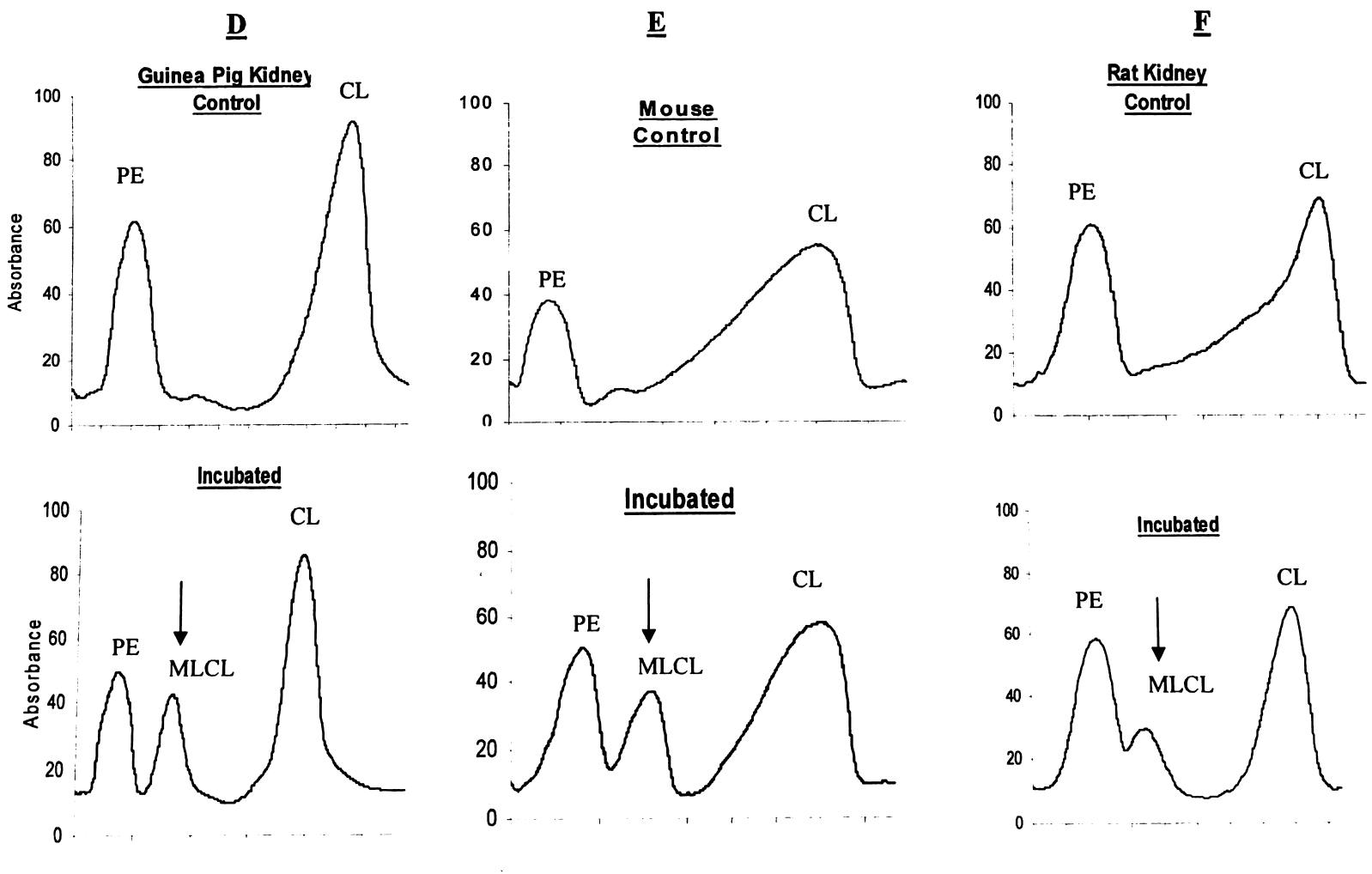
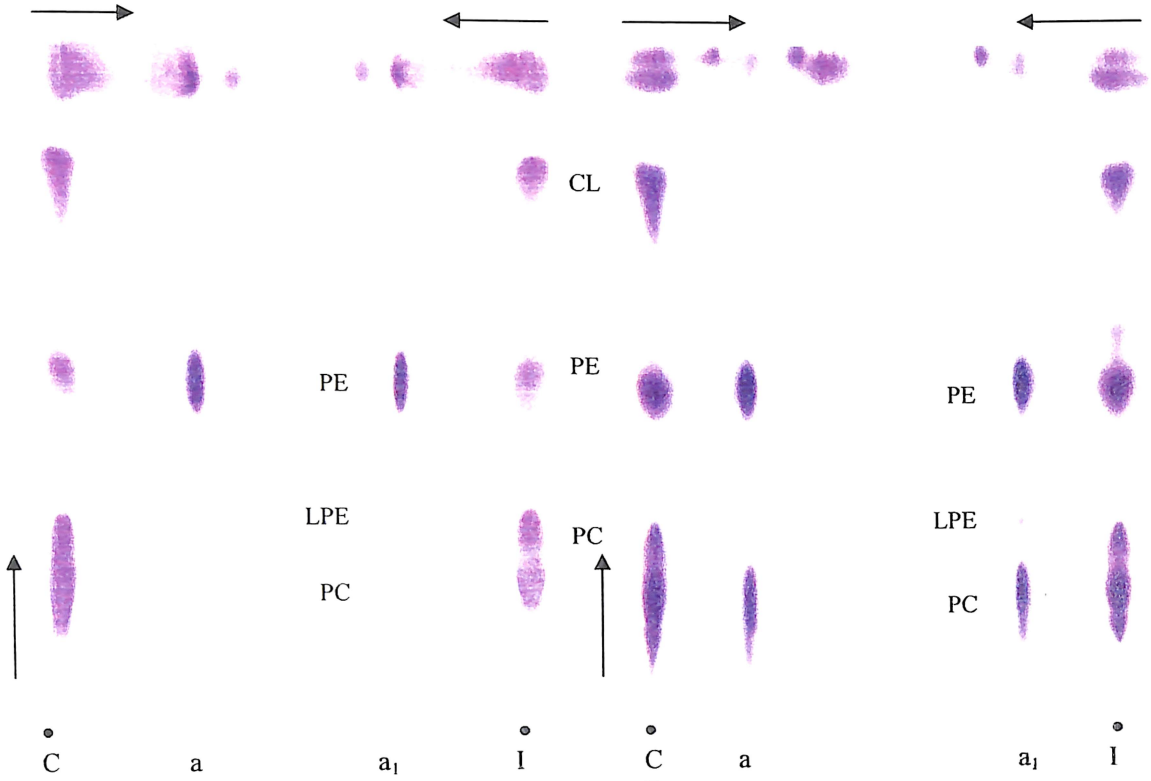


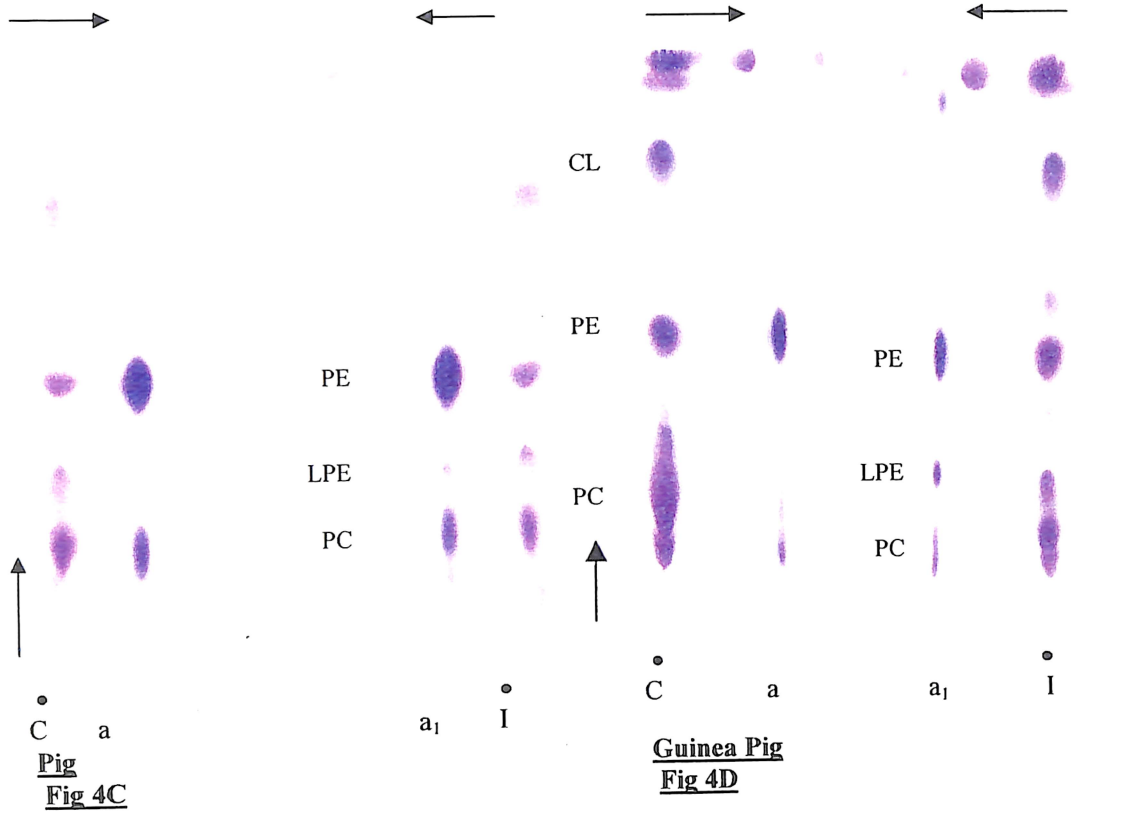
Fig 3 (A, B, C, D, E, F):
Scanning was done at 600 nm of all controls (C) and incubated lanes (I) from fig 2. The degree of deacylation of endogenous CL is clearly illustrated as shown by the level of MLCL formation subsequent to incubation in all kidneys studied.

Fig 4 (A, B, C, D)



Cat
Fig 4A

Dog
Fig 4B



Pig
Fig 4C

Guinea Pig
Fig 4D

Fig 4 (E, F)

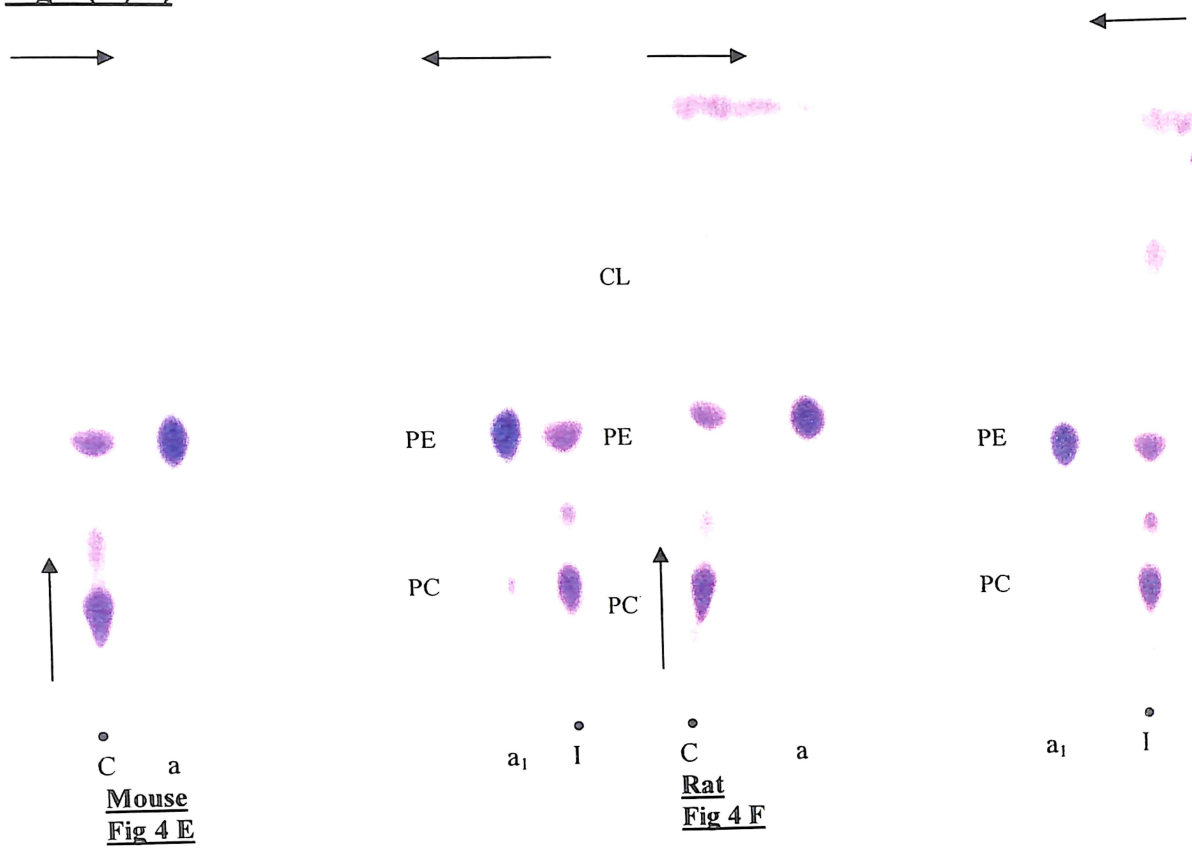


Fig 4 (A, B, C, D, E, F):

2D HCL silica gel plates of plasmalogen pattern of cat kidney cortex (A), dog kidney cortex (B), pig kidney cortex (C), guinea pig kidney (D), mouse kidney (E), and rat kidney (F). Control (C) lanes, and incubated (I) lanes, (10µl each) of chloroform: methanol extract (2:1). First dimension (lower left arrow) developed in A-1-50 (1-propanol-chloroform-ethyl acetate-methanol, 50:50:50:21:18), dried 10 minutes. Second dimension (upper left and right arrows) developed in 1% HCL, dried for one hour followed by developing (same second dimension) in Hexane: ethyl ether (4:1). Lane a, a_1 are the free aldehydes released by the action of HCL on the alkenyl phospholipids as described in materials and methods and stained in Schiff leucofuscin. Phosphatidyl ethanolamine plasmalogen (PE) is a major phospholipid component in all kidney samples analyzed (**a**) lanes. The level of phosphatidyl choline plasmalogen (PC) varies among the species studied (**a** lanes), pig and dog kidney revealed higher concentration than guinea pig and mouse kidney. Rat kidney PC is barely detectable (**a** lanes). Following incubation, lyso alkenyl phosphatidyl ethanolamine (LPE) is produced ($\mathbf{a_1}$ Lanes). The guinea pig and pig kidney incubated samples ($\mathbf{a_1}$ lanes) showed higher level of LPE when compared with the other kidneys (cat, dog, rat, & mouse.) The lyso alkenyl product demonstrates the action of endogenous PLA2 on sn-2 fatty acids of the endogenous PE plasmalogens.

Fig 5 (A, B, C)

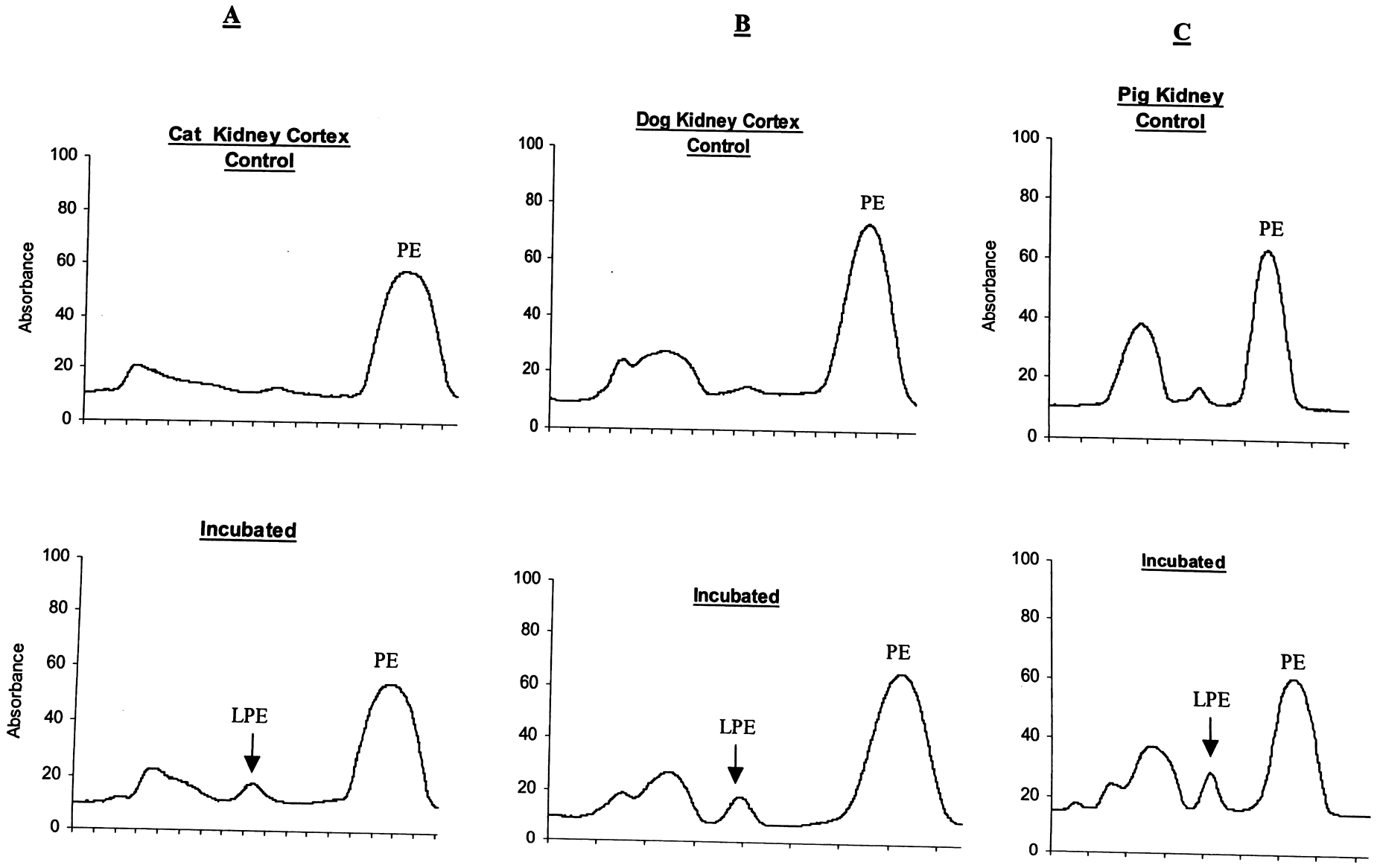

A
B
C
Cat Kidney Cortex
Control
Dog Kidney Cortex
Control
Pig Kidney
Control
Absorbance
100
80
60
40
20
0
PE
Incubated
LPE

Fig 5 (D, E, F)

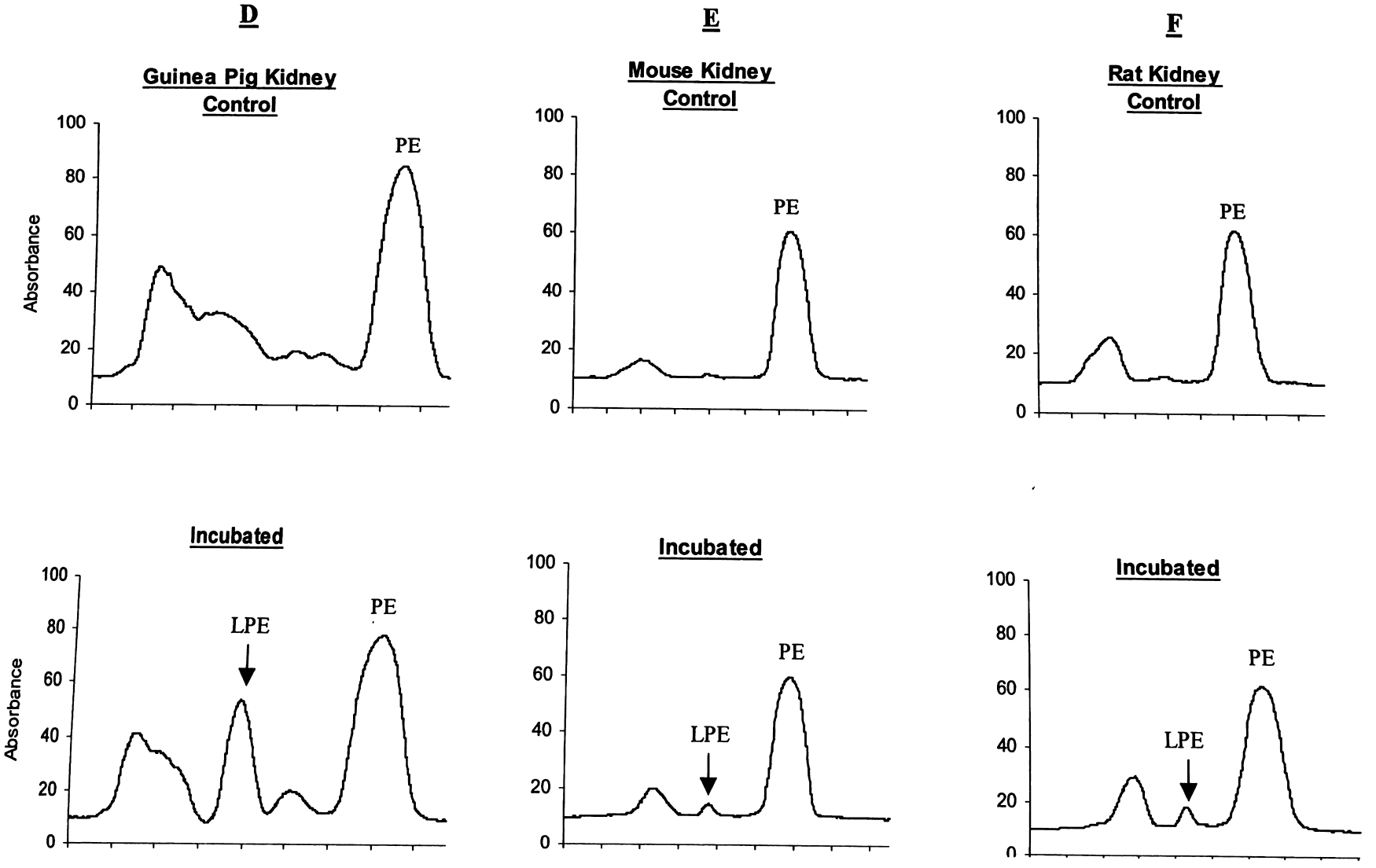


Fig 5 (A, B, C, D, E, F):

The accompanied densitometric measurements were done (wavelength 560 nm) from Fig 4 A, B, C, D, E, F, lanes **a** (control) and **a_1** (incubated) of all kidneys, and clearly confirmed the production of LPE in all incubated samples (a_1 lanes).

PLA_2 seams to be the most active in guinea pig kidney as detected by higher level of LPE formation following incubation (*lane* **a_1** *D) in* comparison to a much lower level in other kidneys (lanes **a_1** in A, B, C, E, F)

Fig 6 (A, B, C)

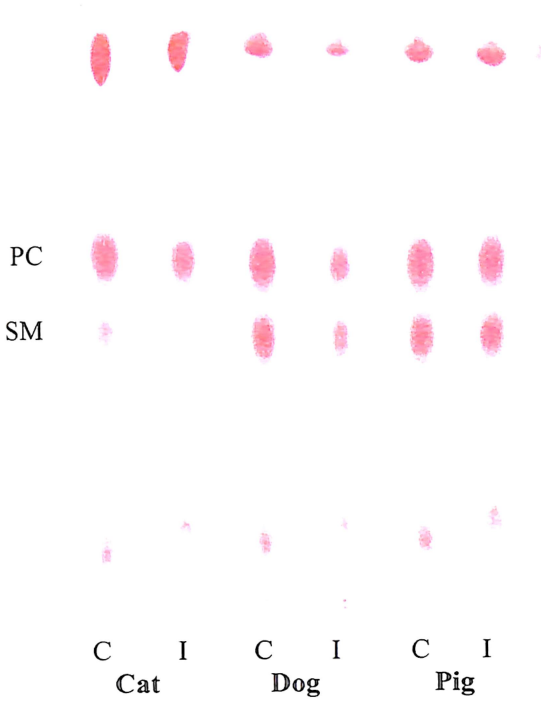


Fig 6A

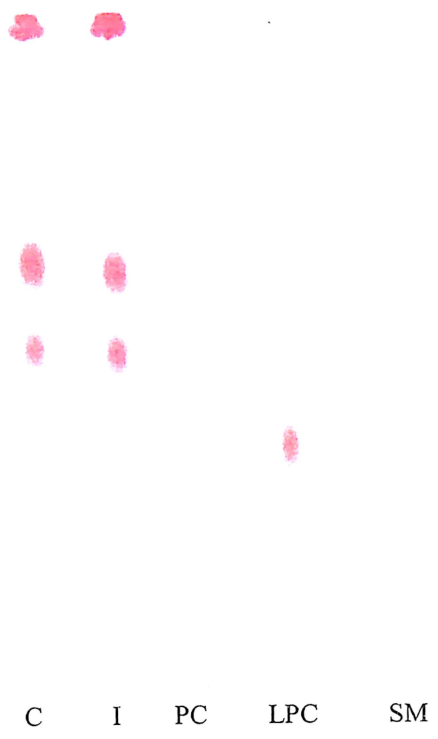


Fig 6B

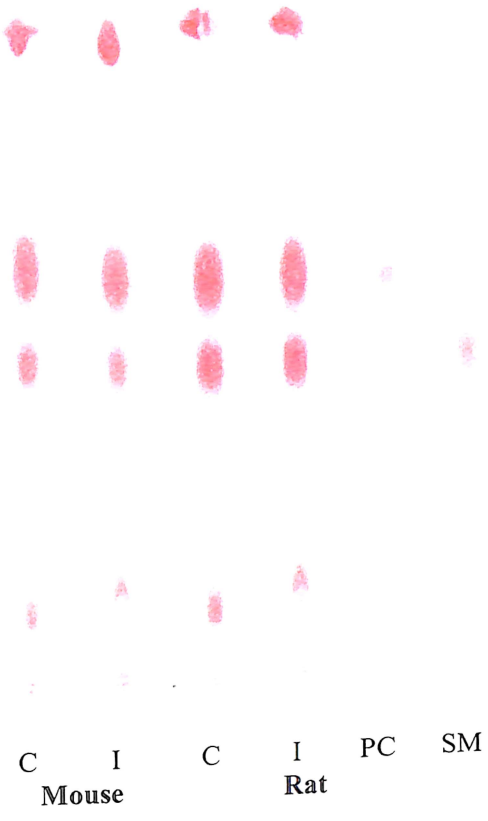


Fig 6C

Fig 6 (A, B, C):

Aluminum oxide (Alox) TLC plate, for the resolution of choline phospholipids [phosphatidyl choline (PC), and sphingomyelin (SM)], developed in (chloroform: methanol: 2-propanol: H_2O, (100:25:2:2)) and stained with Biebrich scarlet reagent. Comparing the control (C lanes) and incubated (I lanes) for chloroform: methanol (2:1) extracts (10µl each). PC and SM (C lanes) are the two choline lipids present in all kidney samples analyzed. Following incubation (I lanes) a slight reduction is observed in SM level in all kidneys analyzed when compared with their respective controls (C lanes).
PC: phosphatidyl choline, SM: sphingomyelin, LPC: lyso phosphatidyl choline.
PC, LPC and SM are standards shown in 6B.

Fig 7 (A, B, C, D)

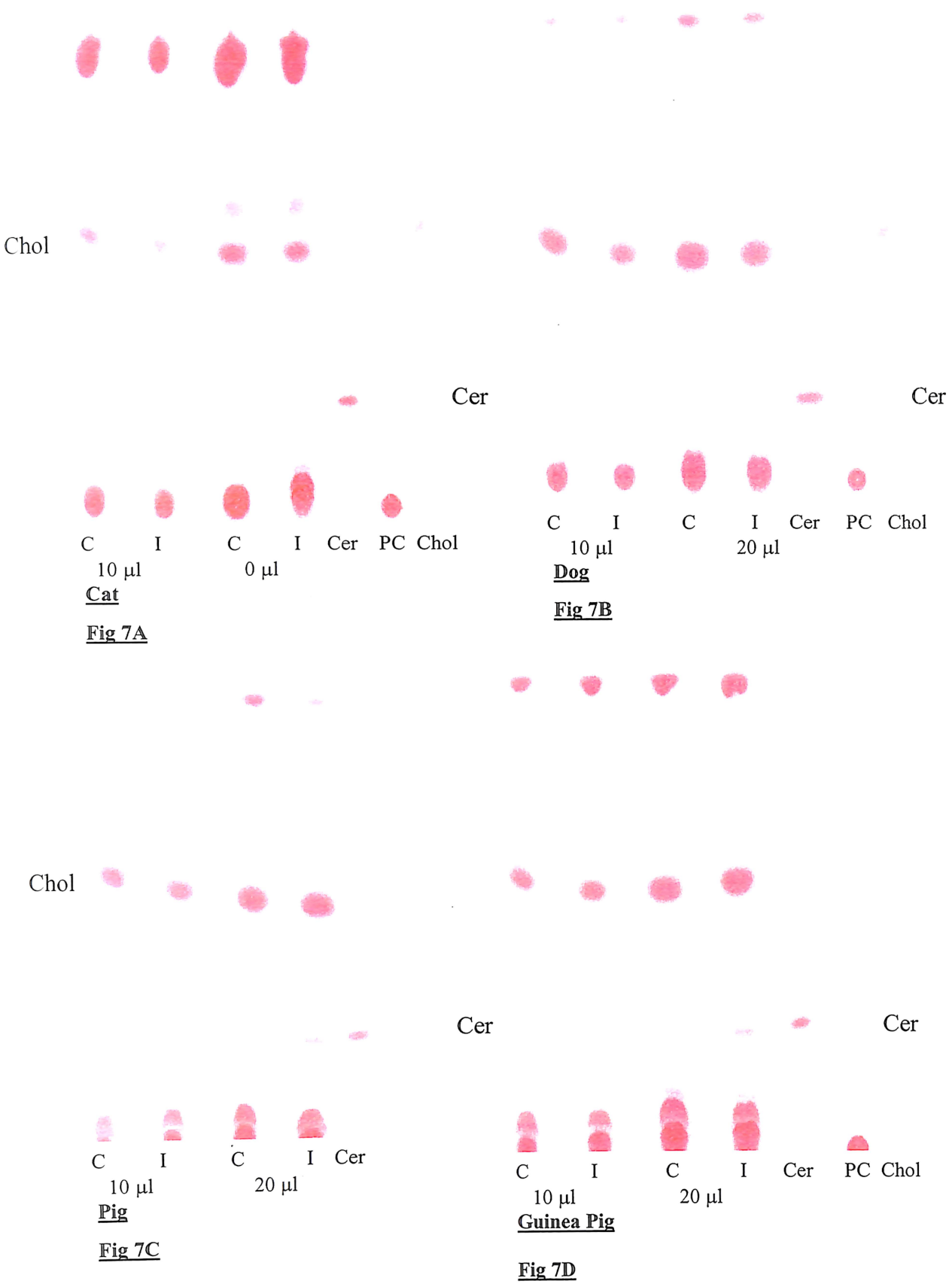

Chol
Cer
C
I
C
I
Cer
PC
Chol
10 µl
0 µl
Cat
Fig 7A
Cer
C
I
C
I
Cer
PC
Chol
10 µl
20 µl
Dog
Fig 7B
Chol
Cer
C
I
C
I
Cer
10 µl
20 µl
Pig
Fig 7C
Cer
C
I
C
I
Cer
PC
Chol
10 µl
20 µl
Guinea Pig
Fig 7D

Fig 7 (E, F)

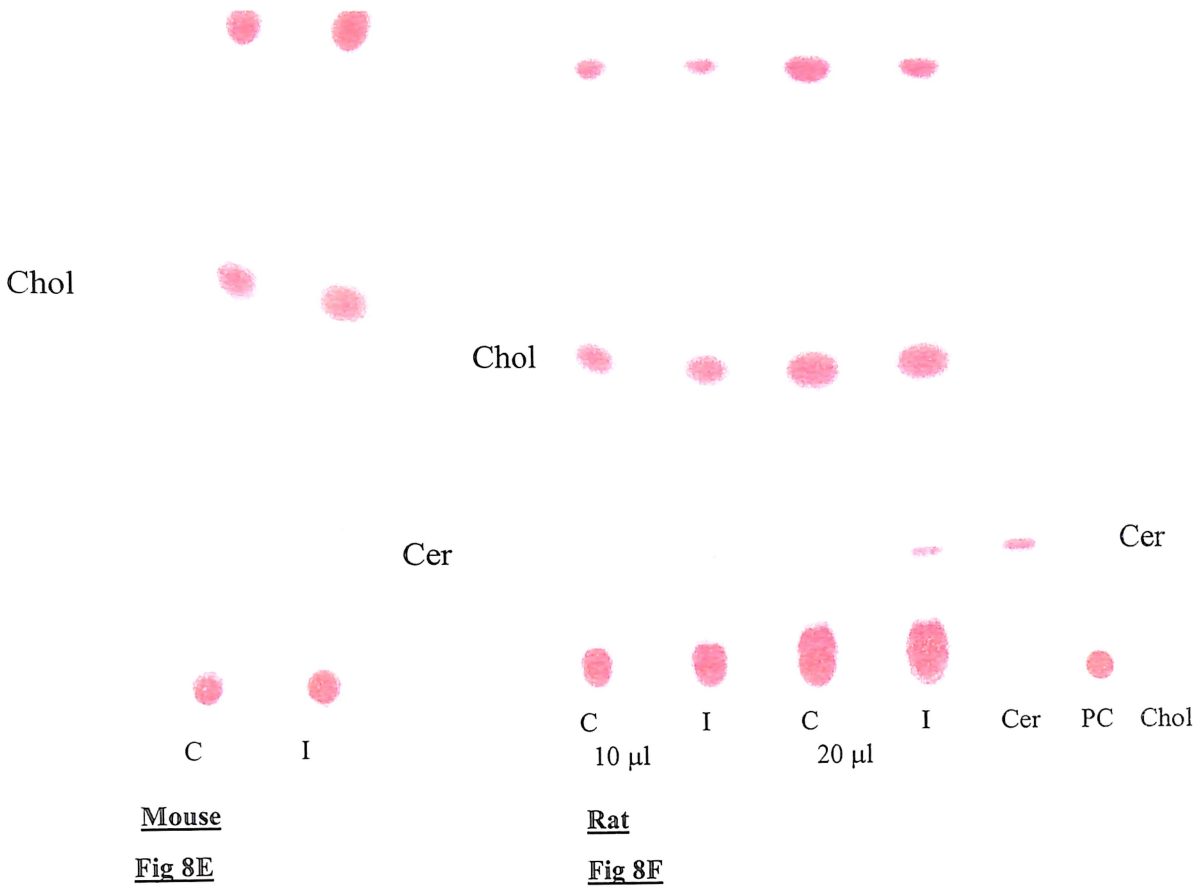


Fig 7 (A, B, C, D, E, F):

Silica Gel TLC plates developed in chloroform: benzene: methanol, 8:2:1, stained in Biebrich Scarlet reagent. When comparing control samples (C, 10µl, C, 20 µl) with their respective incubated samples (I, 10µl, I, 20µl C: M), ceramide is shown only in all incubated samples (cat, dog, pig, guinea pig, mouse and rat kidneys). Ceramide production following incubation indicates sphingomyelinase (or PLC) action on sphingomyelin (SM). Slight reduction in SM level concurrent to incubation in all kidneys studied is shown in Fig 7 (I lanes). **Cer:** Ceramide, **PC**: Phosphatidyl choline, **Chol:** Cholesterol are standard samples.

Discussion

Two techniques were developed in this laboratory. One is a TLC spot test, (the thionine reagent) which allowed the recognition of CL and its lyso derivatives, in conjunction with densitometric measurements. The unique advantage of thionine reagent is its ability to stain CL and its lyso derivatives violet (λ max 600 nm) and the plasmalogen green-blue (λ max 625 nm) (15). The second is an *in vitro* incubation procedure to test the catalytic activity of phospholipases, endogenous to the tissues under investigation, on their endogenous phospholipids and identification of the products as a consequence of phospholipase/s activation (23, 24). From the phosphoglyceride profiles revealed from C: M extracts of the controls, by multiple forms of TLC (11) and those in the incubated samples, deductions could be made as to the possible lipolytic enzymology their respective endogenous phospholipids encountered. Both techniques were used in the present study to assess the lipolytic capabilities of kidney from diverse mammals.

The results presented here represent the first report pertaining to the phosphoglyceride profiles of six species of mammalian kidney and their *in vitro* response to the endogenous lipolytic enzymes mainly in the phospholipases group. *In vitro* incubation of these kidneys (Guinea pig, pig, cat, dog, rat, and mouse), as source of phospholipases and phospholipids, produced MLCL and concurrent reduction of CL (representing the most lipolytic event). To a much lesser extent there was also formation of lyso phosphatidyl ethanolamine plasmalogen and ceramide concurrent to reduction in the level of ethanolamine plasmalogen and sphingomyelin respectively.

This deacylation of ethanolamine plasmalogen provided the most precise demonstration of endogenous PLA_2 action on sn-2 fatty acid of endogenous PE plasmalogen. The preferential deacylation of CL reported here indicates the action of endogenous PLA_1 or PLA_2. We believe that the endogenous PLA_2, which deacylated the alkenyl species, reacted preferentially with the endogenous CL due to the much higher level of MLCL produced, compared with a very low level of LPE.

It has already been reported that multiple forms of PLA_2 isoforms have been isolated from rat and rabbit kidneys (25, 26) and from *in vitro* study, the isolated PLA_2 from these kidneys selectively deacylated the exogenously added alkenyl phosphatidyl choline (the favorite exogenous substrate used in most of these studies) having arachidonic acid at sn-2 position. CL was not used as substrate in any of these studies.

The present study is the first report showing the *in vitro* deacylation of endogenous CL by an endogenous phospholipase in all kidney specimens studied. The varying level of MLCL production observed in these kidneys (higher level in guinea pig compared with lower level in pig kidney) probably relates in part to the endogenous PLA_2 isoforms unique to the tissue under investigation.

In 1990, Schlame (et al.) reported on the *in vitro* deacylation of CL by endogenous phospholipase in rat liver mitochondria and described a CL/MLCL cycle for the remodeling of CL, where CL is deacylated to MLCL, and the later is reacylated to CL (27, 19). Schlame's observation supports the deacylation phenomenon of CL to MLCL described in the present study and confirms the presence of PLA_2 in mitochondria.

Our most recent study described a similar CL deacylation phenomenon in the cardiac muscle of these same mammalian species but with a higher magnitude. This CL deacylation was age related, being very low in newborn myocardium of guinea pig and rabbit, and reach highest level in myocardium of aged animals (12, 13, 14, 28), emphasizing an important metabolic role of CL in these tissues, yet to be established.

Our earlier observations further defined the role of CL by showing diminution of CL in experimentally produced ischemic dog myocardium and infarcted cardiac muscle of cat, rabbit and man with subsequent appearance of lyso derivatives of CL and concurrent mitochondrial swelling (16, 17, 18). Smith (et al.) (1980) reported on the decrease of CL level during early phase of ischemia in mitochondria isolated from rat kidney due to mitochondrial PLA_2 activation, resulting in irreversible cellular injury (29). It has also been reported that there are marked quantitative variations in CL level of various vertebrate tissues, which seem to have no obvious direct relationship to the concentration of other phospholipids (30, 31), but are related to the number of mitochondria and the degree of folding of the inner mitochondrial membrane. Mitochondria are abundant and with numerous cristae in cardiac muscle and kidney (32, 33), and hence a higher level of CL as described here in all kidney specimens studied and in cardiac muscle of these same species as described elsewhere (13, 14).

This aforementioned information provides presumptive evidence emphasizing that CL occupies a unique position in the metabolism of phospholipids (in kidney as well as heart) associated with some requirement for oxidative metabolism to meet energy requirement due to *in vivo* ischemia and/or hypoxia,, and its high level of linoleic and linolenic fatty acids seems to be significant.

It has also been reported that the activation of PLA_2 from rat and rabbit kidney is associated with renal cell injury as a consequence of hypoxia or ischemia, which resulted in reduction of PE plasmalogen, accompanied by the release of arachidonic acid in the rabbit kidney (34, 35) and oleic acid in the rat kidney (5).

The PLA_2 purified from rat liver mitochondria, appears to be linked to the energy status of mitochondria, and become activated when ATP drops to minimal level, resulting in the degradation of PE (36, 37). It was also reported that $cPLA_2$ is activated following treating renal epithelial cells with H_2O_2 (8), which contributes to oxidant induced cytotoxicity. These observations seem to be in support of the present study.

In the present study, *in vitro* incubation of all kidney specimens also produced lyso PE plasmalogen accompanied by reduction of PE plasmalogen. Our use of closed test-tube *in vitro* incubation may be thought as a model for ***in vivo*** **ischemia**, where the endogenous PLA_2 will be activated, and deacylate PE plasmalogen releasing the fatty acid on sn-2 position and producing lyso PE plasmalogen, as well as deacylating CL. The released fatty acids from CL deacylation will, probably serve as an alternate source of energy required for renal epithelium preventing cell damage during *in vivo* hypoxia.

Lyso products of $cPLA_2$ have been shown to be an important modulator of renal Na^+/K^+ ATPase (38). In rat, rabbit and mouse kidneys, the activity of Na^+/K^+ ATPase is **a)** related to the rate of ATP hydrolysis, which is an index of the capacity of the kidney nephron to hydrolyze ATP, and **b)** dependent on mitochondrial oxidative phosphorylation that is responsible for supplying most of the energy for the kidney (39, 40).

In the absence of exogenous substrates, the proximal tubule segment of the nephron has a preference to oxidize endogenous fatty acids as a source of energy (40). The *in vitro* CL deacylation, described here, may be the provider of the endogenous free fatty acids during *in vivo* ischemia, and its high level of linolenic and linoleic acids is significant.

Ethanolamine plasmalogen was shown to be the principal plasmalogen analog of mammalian kidney as described here, as well as in other vertebrate tissues as described elsewhere (30, 31), which continue to suggest that PE plasmalogen and its *in vitro* deacylation, as described here, must play a fundamental role in the lipid metabolism of these tissues yet to be defined. This is the first report describing the concurrent deacylation of both CL and PE plasmalogen (two major endogenous phospholipids) in mammalian kidney as a consequence of *in vitro* incubation of whole tissue homogenate.

The simple and direct *in vitro* incubation procedure described here provided the lipolytic enzymes of these tissues under investigation with a natural mixture of endogenous substrates (phospholipids) for their use. This meant that we have disturbed the natural spatial relationship of their *in vivo* environment, but provided a more equal chance at the total substrates.

These studies, described here illustrate that comparative lipid-chromatographic and lipolytic procedures are capable of providing some sense of direction in terms of visualizing possible metabolic mechanisms and help us understand why there is a need for all these diverse phospholipid species in biomembranes.

A variety of cytokines and mitogens, such as tumor necrosis factor (TNF), epidermal growth factors and UV injury have been shown to induce activation of $cPLA_2$ and release arachidonic acid in various types of cells including kidney cells, macrophages, vascular smooth muscle, and keratinocytes (6, 7). The released arachidonic acid is implicated as a regulator of sphingomyelinase activation leading to ceramide production (41-45). The ceramide generated appears to modulate a number of cell regulatory activities including inhibition of cell growth, induction of cell differentiation and apoptosis (41, 44, 45).

In spite of the recent excessive information involving the breakdown of SM through the action of sphingomyelinase and formation of ceramide, the actual role of ceramide in cell function, particularly in mammalian epithelial cells, is still unclear. Our *in vitro* incubation of all mammalian kidneys also produced ceramide and concurrent reduction of sphingomyelin (SM) (SM is one of the major lipids of these kidneys) indicating the action of endogenous sphingomyelinase as well as the production of LPE and reduction of PE plasmalogen indicating $cPLA_2$ activation. Whether these factors cited above, which cause the activation of sphingomyelinase leading to ceramide production, operate *in vivo* in mammalian kidney require further investigation.

Several isoforms of cytosolic PLA_2 have been described with the preferential release of arachidonic acid (AA) from canine myocardium (46), bovine brain (47), murine macrophages, rat and rabbit kidney (48, 49). Thus far, the arachidonic acid-selective enzymes have been emphasized because of arachidonic acid is the rate limiting precursor of prostaglandins, thromboxanes, and Leukotrienes (46-49) and its association with the inflammatory response (50, 2, 9).

In mammalian kidney, eicosanoid synthesis participates in regulation of renal blood flow, glomerular filtration rate, solute and water transport, and renin secretion (33)-. However, the regulatory mechanisms of $cPLA_2$ remain unclear, controversial and require further investigation.

The proenzyme status of mammalian pancreatic PLA_2 provides *in situ* protection against premature deacylation (23, 24) while the cellular phospholipases of other tissues, including the ones described in this report, are under different control yet to be established. The discovery that the pancreatic phospholipase A_2 exists as a proenzyme activated by trypsin (23) has lead to the search of other activator and/ or inhibitors. Overall, the varying reports on Ca^{2+} requirements, pH optimum, temperature sensitivity etc (51, 52, 5) has made necessary continuing investigation into this area in search for full understanding of the *in vivo* function of all lipolytic enzymes endogenous to an organ as described here in the mammalian kidney, and for encompassing explanation of the need for such diversity of phosphoglycerides which is not yet at hand.

The present study has raised several questions pertaining to renal physiology:

1- Why these mammalian kidneys possess these endogenous lipolytic enzymes, which **a)** preferentially deacylated CL, **b)** to a much lesser extent alkenyl PE, and **c)** hydrolyzed SM producing ceramide?

2- What are the ligands, which initiate the activation of these enzymes *in vivo* as well as the factors that enhance or inhibit these hydrolytic processes?

3- Is there a relatedness *in vivo* associated with the concurrent deacylation of endogenous CL, PE plasmalogen and sphingomyelin hydrolysis?

These relationships provide us today with some exciting comparative biochemistry situations requiring both mind and machine for their explanation and require further investigation.

References

1. Heinrickson RL. Dissection and Sequence Analysis of Phospholipase A_2. In Methods in Enzymology, **197**:201-214; Academic Press, 1991.
2. Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL. A novel arachidonic acid-selective cytosolic PLA2 contains a Ca (2+)-dependent translocation domain with homology to PKC and GAP. Cell 1991; **65:** 1043-1051.
3. Mayer RJ, Marshall LA. New insights on mammalian phospholipase A2(s); comparison of arachidonoyl-selective and –nonselective enzymes. FASEB J 1993; **7:** 339-348.
4. Balsinde J, Dennis EA. Function and inhibition of intracellular calcium-independent phospholipase A2. J Bio Chem 1997; **272:**16069-16072.
5. Leslie CC. Properties and Regulation of Cytosolic Phospholipase A_2. J Bio Chem 1997; **272**:16709-16712
6. Sapirstein A, Spech RA, Witzgall R, Bonventre JV. Cytosolic Phospholipase A_2, but Not Secretory PLA_2, Potentiates Hydrogen Peroxide Cytotoxicity in Kidney Epithelial Cells. J Bio Chem 1996; **271**: 21505-21513.
7. Hayakawa M, Ishida N, Takeuchi K, Shibamoto S, Hori T, Oku N, Ito F, Tsujimoto M. Arachidonic Acid-Selective Cytosolic Phospholipase A_2 Is Crucial in the Cytotoxic action of Tumor Necrosis Factor. J Bio Chem 1993; **268**: 11290-11295.

8. Balboa MA, Balsinde J. Involvement of Calcium-Independent Phospholipase A_2 in Hydrogen Peroxide-Induced Accumulation of Free Fatty Acids in Human U937 Cells. J Bio Chem 2002; **277**: 40384-40389.

9. Asai K, Hirabayashi T, Houjou T, Uozumi N, Taguchi R, Shimizu T. Human Group IVC Phospholipase A_2 ($cPLA_2$). Roles in the Membrane Remodeling and Activation induced by Oxidative Stress. J Bio Chem 2003; **278**: 8809-8814.

10. Hack MH, Helmy FM. On The Plasmalogenation of Myocardial Choline Glycerophospholipids during Maturation of Various Vertebrates. Com. Biochem Physiol 1988; **89B**:111-118.

11. Helmy FM, Hack MH. Some Contributions to the Thin-Layer Chromatographic Analysis of Complex Natural Phospholipid and Neutral Lipid Mixtures. J Chrom 1986; **374**: 61-72.

12. Hack MH, Helmy FM, Muller TE, Juracka A. Further TLC Evidence of the Preferential Deacylation of Cardiolipin and N-Acyl phosphatidyl Ethanolamine Production in Mammalian Myocardia, Fetal and Adult. An In Vitro study. J Planar Chrom 2002; **15**: 396-403.

13. Helmy FM, Hack MH. TLC Derived Data Relating the In Vitro Deacylation of Phospholipids by Various Extracellular Phospholipase A_2 Compared with the In Vitro Deacylation of Endogenous Substrate by the Endogenous Phospholipase A_2 of Various Tissues. J Planar Chrom 1995; **8**: 369-373.

14. Hack MH, Helmy FM. On the Apparent Preferential Deacylation of Cardiolipin, in Vitro, by Various Tissues of Birds and Mammals: A Thin Layer Chromatographic Analysis. J Planar Chrom 1991; **4**: 80-84.

15. Hack MH, Helmy FM. Thin-Layer Chromatographic Analysis of Myelin Lipids, Their Differential O-Deacylation by Primary Alkylamines and Their Selective Staining by Thionine, A limited Phylogenetic Study. J Chrom 1990; **525**: 339-347.

16. Hack MH, Helmy FM. A Reappraisal of the Dog Heart Infarct Plasmalogen and Its conception as a Bis-Phosphatidic Acid and Current Recognition as an N-Acyl Phosphatidyl Ethanolamine. Comp Biochem Physiol 1982; **73B**: 873-879.

17. Hack MH, Helmy FM. Some Studies Relating to the Properties and Biochemical Significance of Cardiolipin. Comp Biochem Physiol 1967; **23**:105-112.

18. Colcolough HL. A Comparative Study of Acute Myocardial Infarction in the Rabbit, Cat and Man. Comp Biochem Physiol 1974; **49A**: 121-125.

19. Schlame M, Rua D, Greenberg ML. The Biosynthesis and Functional Role of Cardiolipin. Prog Lipid Res 2000; **39**: 257-288.

20. Eble KS, Coleman WB, Hantgan RR, Cunningham CC. Tightly Associated Cardiolipin in the Bovine Heart Mitochondrial ATP Synthase as Analyzed by ^{31}P Nuclear Magnetic Resonance Spectroscopy. J Bio Chem 1990; **265**: 19434-19440.

21. Paradies G, Petrosillo G, Ruggiero FM. Cardiolipin-Dependent Decrease of Cytochrome C Oxidase Activity in Heart Mitochondria from Hypothyroid Rats. Biochim Biophys Acta 1997; **1319**: 5-8.

22. Schlame M, Hostetler KY. Cardiolipin Synthase from Mammalian Mitochondria. Biochim Biophys Acta 1997; **1348**: 207-213.

23. Helmy FM, Hack MH. Studies on the Endogenous Phosphatides of Mammalian Pancreas and their Hydrolysis by Endogenous Phospholipases- I The Lipids of Dog Pancreas and Their In Vitro Hydrolysis Primed by Trypsin, by Phospholipase A_2. Comp Biochem Physiol 1982; **71B**:101-104

24. Helmy FM, Hack MH. A Correlative Lipid and lipolytic Study of the Pancreas and Small Intestine of the Guinea Pig. Comparative Biochem Physiol 1987; **86**: 83.

25. Nakamura H, Nemenoff RA, Gronich JH, Bonventre JV. Subcellular Characteristics of Phospholipase A2 activity in the Rat Kidney. Enhanced Cytosolic, Mitochondrial, and Microsomal Phospholipase A2 Enzymatic Activity after Renal Ischemia and reperfusion. J Clin Invest 1991; **87**: 1810-1818.

26. Portilla D, Dai G. Purification of a Novel Calcium-Independent Phospholipase A_2 from Rabbit Kidney. J Bio Chem 1996; **271**: 15451-15457.

27. Schlame M, Rostow B. Lysocardiolipin formation and Reacylation in Isolated Rat Liver Mitochondria. Biochem J 1990; **272**: 589-595.

28. Helmy FM, Hack MH. Some TLC Observations on the In Vitro Formation of N-Acyl Phosphatidyl Ethanolamine by Endogenous Components of Bovine and Porcine Retina. J Planar Chrom 1994; **7**:14-17.

29. Smith MW, Collan Y, Kahng MW, Trump BF. Changes in Mitochondrial Lipids of Rat Kidney during Ischemia. Biochem Biophys Acta 1980; **618**: 190-201.

30. Hack MH, Helmy FM. comparative Lipid Biochemistry-IV. A Correlative Study of the Lipids of Vertebrate Kidney. Comp. Biochem Physiol 1967; **20**: 65-71

31. Helmy FM, Hack MH. Some Comparative Histochemical Studies of the Vertebrate Kidney. Comp biochem Physiol 1967; **20**: 55-63.

32. Lehninger AL, Nelson DL, Cox MM. Principles of Biochemistry, pages 743. Worth Publishers, 1993.

33. Brenner BM, Rector F.C. The Kidney, 23-26. WB Saunders Company, 1991.

34. Portilla D, Creer MH. Plasmalogen Phospholipid Hydrolysis during Hypoxic Injury of Rabbit Proximal Tubules. Kidney Int 1995; **47**: 1087-94.

35. Portilla D, Shah SV, Lehman PA, Creer MH. Role of Cytosolic Calcium-Independent Plasmalogen-Selective Phospholipase A2 in Hypoxic Injury to Rabbit Proximal Tubules. J Clin Invest 1994; **93**: 1609-15

36. De Winter JM, Vianen GM, Van Den Bosch H. Purification of Rat Liver Mitochondria Phospholipase A_2. Biochim Biophys Acta 1982; **712**: 332-341.

37. Paree GW, Cunningham CC, Waite M. Mitochondria Phospholipase A_2 Activity and Mitochondrial Aging. Biochem 1978; **17**: 1634-1639.

38. Weidemann MJ, Krebs HA. The Fuel of Respiration of Rat Kidney Cortex. Biochem J 1969; **112**:149.

39. Krebs HA, Hems R, Gascoyne T. Renal Gluconeogenesis IV. Gluconeogenesis from Substrate Combinations. Acta Biol Med 1963; **11**: 607

40. Klein KL, Wang MS, Torikai S, Davidson WD, Kurokawa K. Substrate Oxidation by Isolated Single Nephron Segments of the Rat. Kidney Int 1981; **20**: 29-35.

41. Hannun YA. The Sphingomyelin Cycle and the Second Messenger Function of Ceramide. J Bio Chem 1994; **269**: 3125-3128.

42. Hannun YA, Linardic CM. Sphingolipid Breakdown Products: Anti Proliferative and Tumor-suppressor Lipids. Biochim Biophys Act Bio-Memb 1993; **1154**: 223-236.

43. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed Cell Death Induced by Ceramide. Science 1993; **259**: 1769-1771.

44. Okazaki T, Bell RM, Hannun YA. Sphingomyelin Turnover Induced by Vitamin D_3 in HL-60 Cells. Role in Cell Differentiation. J Bio Chem 1989; **264**: 19076-19080.

45. Okazaki T, Bielawska A, Bell RM, Hannun YA. Role of Ceramide as a Lipid Mediator of 1α, 25-dihydroxyvitamin B_3-Induced HL-60 Cell Differentiation. J Bio Chem 1990; **265**:15823-15831.

46. Hazen SL, Loeb LA, Gross RW. Purification and Characterization of Cytosolic Phospholipase A_2 Activities from Canine Myocardium and Sheep Platelets. Methods Enzymol 1991; **197**: 400-411.

47. Hirashima Y, Mills JS, Yates AJ, Harrocks LA. Phospholipase A_2 Activities with a Plasmalogen Substrate in Brain and Neural Tissue Cells: A Sensitive and Specific Assay Using Pyrenesulfonyl-labeled Plasmenylethanolamine. Biochim Biophys Acta 1990; **1047**: 35-40.

48. Farooqui AA, Yang HC, Harrocks LA. Plasmalogen Phospholipase A_2 and Signal Transduction. Brain Res Rev 1995; **21**: 152-161.

49. Yang HC, Farooqui AA, Horrocks LA. Plasmalogen Selective Phospholipase A_2 and its Role in Signal Transduction. J Lip Res 1996; **14**: 9-13.

50. Murakami M, Masuda S, Shimbara S, Bezzine S, Lazdunski M, Lambeau G, Gelb MH, Matsukura S, Kokubu F, Kudo I. Cellular Arachidonate-Releasing Function of Novel Classes of Secretory Phospholipase A_2s (Groups III and XII)*. J Bio Chem 2003; **278**: 10657-10667.

51. Sharp JD, White DA, Chiou XG, Goodson T, Gamboa GC, McClure D, Burgett S, Hoskins J, Skatrud PI, Sportsman JR, Becker GW, Kang UG, Roberts EF, Kramer RM. Molecular Cloning and Expression of Human Ca^{++}-Sensitive Cytosolic Phospholipase A_2. J Biol Chem 1991; **266**: 14850-14853.

52. Dennis EA. Diversity of group Types, Regulation, and Function of Phospholipase A_2. J Bio Chem 1994; **269**: 13057-13060.

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