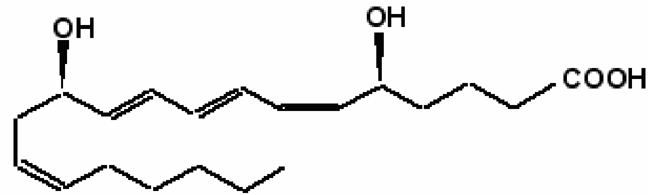
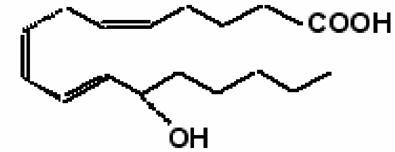
LEUKOTRIENLABORATION

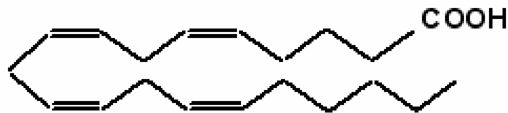
Biomedicinarlinjen
Medicinsk Biokemi





LEUKOTRIEN B4

12-HHT



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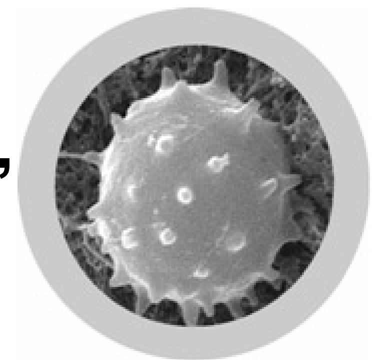
Inflammatory Processes

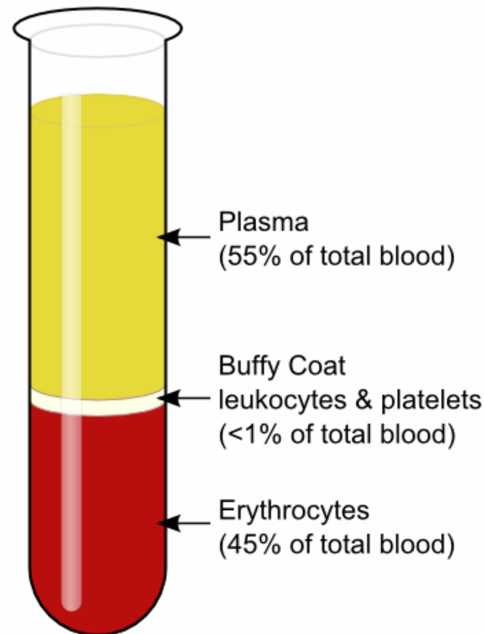
- **Inflammation (Latin, *inflammatio*, to set on fire) body's reaction to cell/tissue damage or pathogens**
- **Protective attempt by the body to remove the source of the injury and initiate healing**
- **Cells involved in the inflammatory process are mainly white blood cells or leukocytes**
- **Leukocytes are cells of the immune system that defend the body against both infectious disease and foreign materials**
- **Leukocytes are found throughout the body, including the blood & lymphatic system**



White Blood Cell (WBCs) → Leukocytes

Granulocytes: leukocytes with differently staining granules
Include: neutrophils, basophils, eosinophils

Agranulocytes: leukocytes with granule absence
Include: lymphocytes, monocytes, & macrophages



- after centrifugation, WBCs located in the Buffy Coat
- a layer of nucleated cells between the RBCs & plasma

Neutrophils → defend against bacterial or fungal infection

Eosinophils → parasitic infections, inflammatory cells in allergic reactions

Basophils → responsible for allergic & antigen response by histamine release

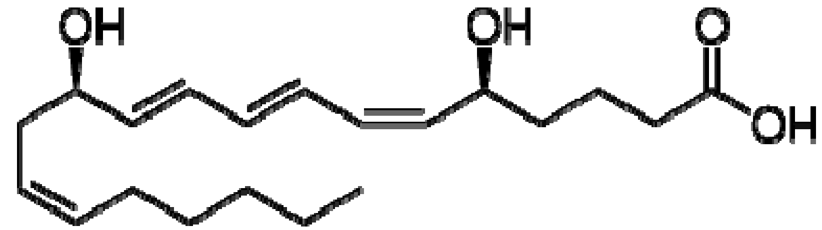
Lymphocytes → involved in immune response (B cells and T cells)

Monocytes → involved in phagocytosis and antigen presentation

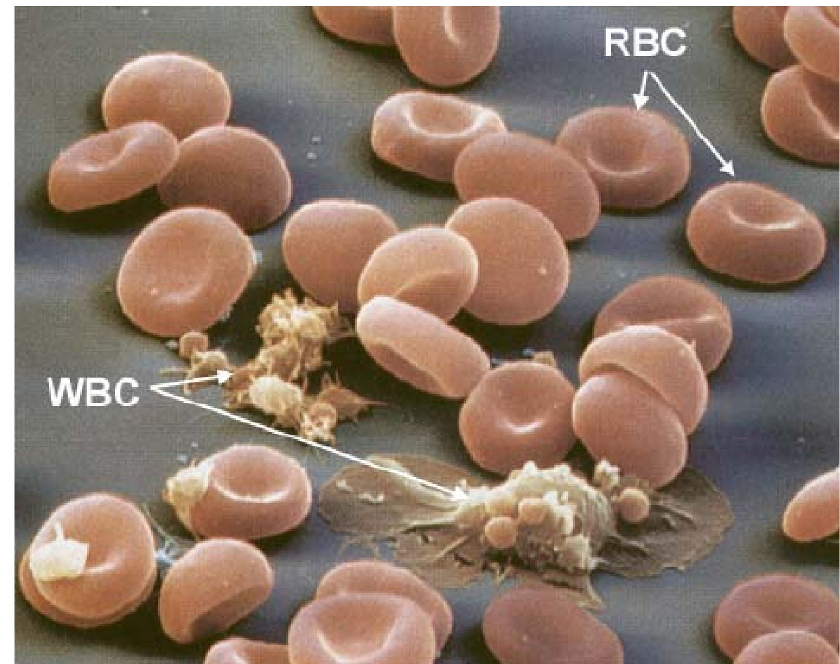
Macrophages → long lived cells involved in phagocytosis

Leukocytes produce inflammatory mediators

- leukocytes produce leukotriene B4, a leukotriene involved in inflammation



- LTB_4 produced in response to inflammatory mediators
- induces adhesion & activation of leukocytes on the endothelium, allowing them to bind to & cross into the tissue
- can induce formation of reactive oxygen species

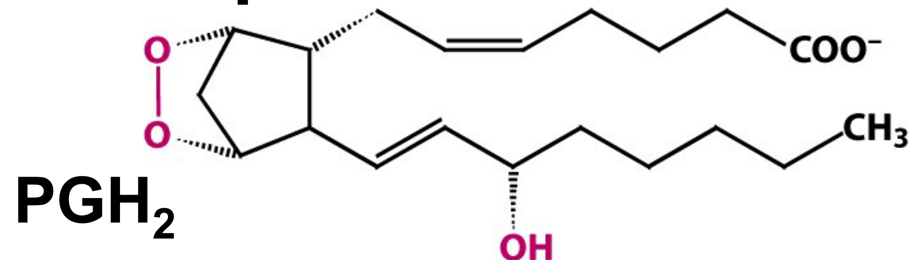


Eicosanoids (LTB_4 and friends) are derived from PUFAs

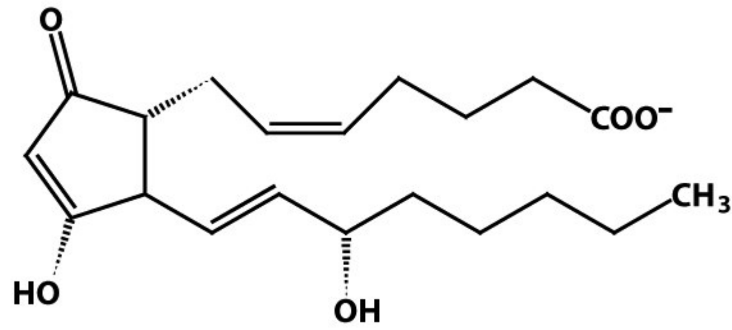
- **Arachidonic acid (20:4n6) is major precursor of multiple signal molecules:**

  prostaglandins (PG), prostacyclins, thromboxanes (TX) and leukotrienes (LT)

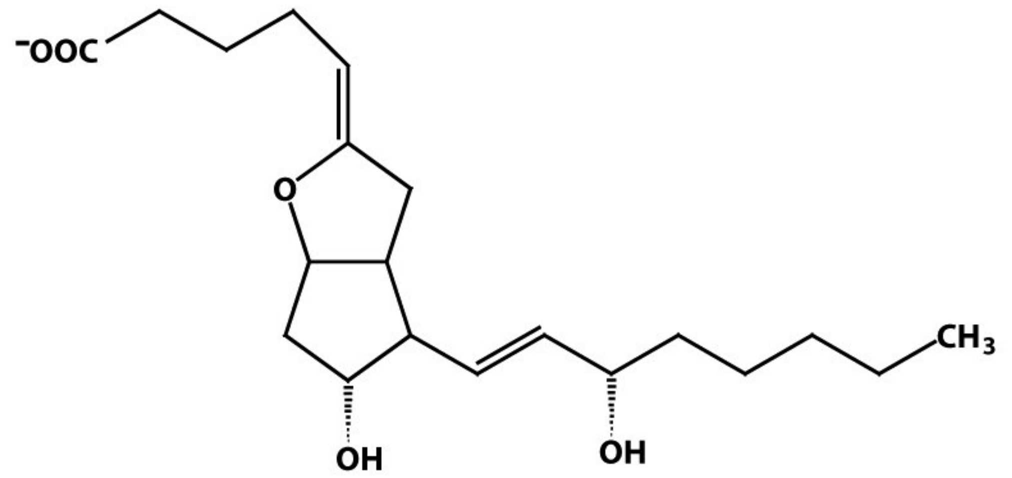
- **PGs = 20 carbon fatty acids containing a 5-carbon ring**
- **PGs stimulate inflammation, regulate blood flow, control ion transport, modulate synaptic transmission & induce sleep**



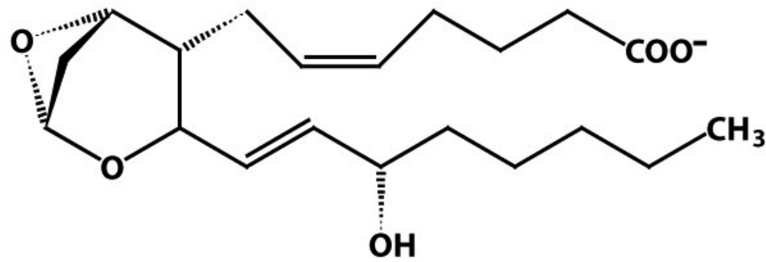
PGH_2



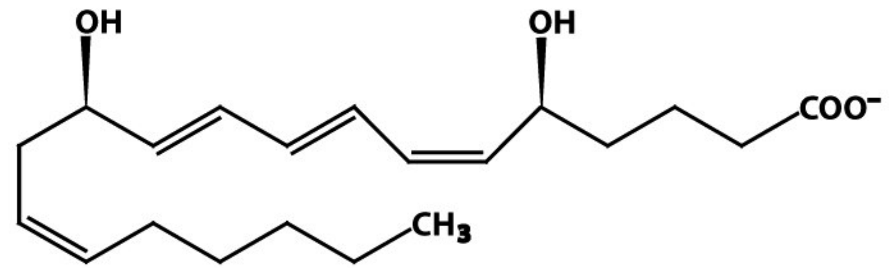
Prostaglandin E_2



Prostacyclin (PGI_2)



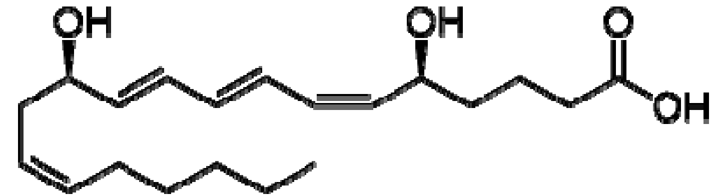
Thromboxane A_2 (TXA_2)



Leukotriene B_4

Biological effects of arachidonic metabolites

- **LTs**
  - LTB_4
    - Chemotaxic for phagocyte cells
    - Stimulate cytoadherence
    - Increase vascular permeability
  - Cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4)
    - Induce muscle contraction
    - Induce bronchial contraction
    - Increase vascular permeability



- **PGs**
  - Depend upon type and tissue, but generally induce vasodilation and bronchialdilation
- **TXA_2**
  - Induce vasoconstriction and bronchial constriction
  - Stimulate inflammatory cells (thrombocytes)

Eicosanoid-based medicines

Type	Medical condition
PGI_1 analog	Pulmonary hypertension, avoiding reperfusion injury
PG analog	Glaucoma, ocular hypertension
PG analog	Labor induction
PGE_2	Labor induction
PGI_2 analog	Pulmonary arterial hypertension
PGE_1 analog	Stomach ulcers, labor induction
LT receptor antagonist	Asthma, seasonal allergies
PGI analog	Pulmonary hypertension

20:4n6 (arachidonic acid)

↑ Δ5

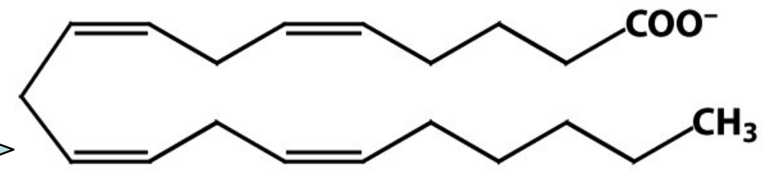
20:3n6 (homo-γ-linolenic acid)

↑ Elongase

18:3n6 (γ-linolenic acid)

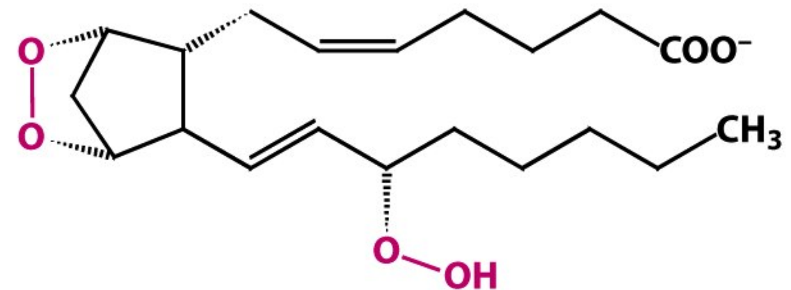
↑ Δ6

18:2n6 (linoleic acid)



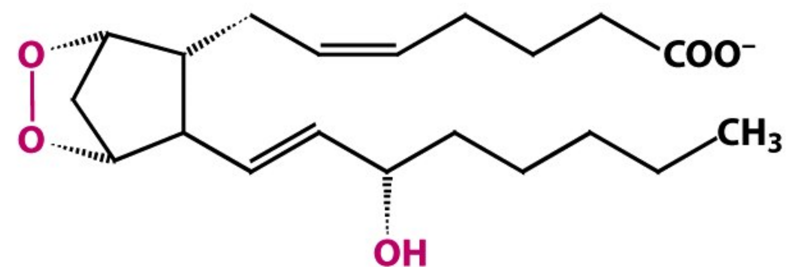
Arachidonate

Cyclooxygenase
$2\ O_2$



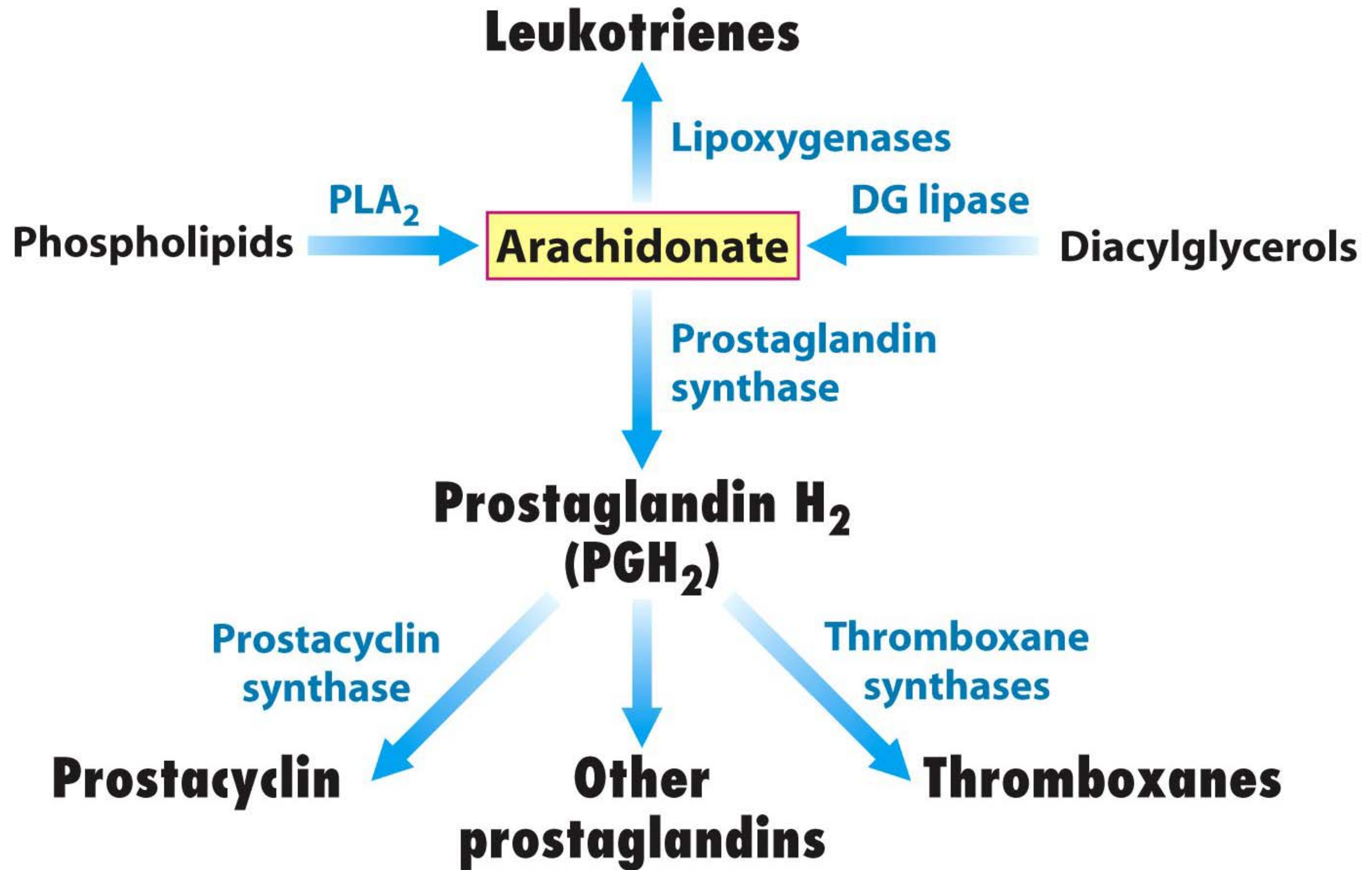
Prostaglandin G_2

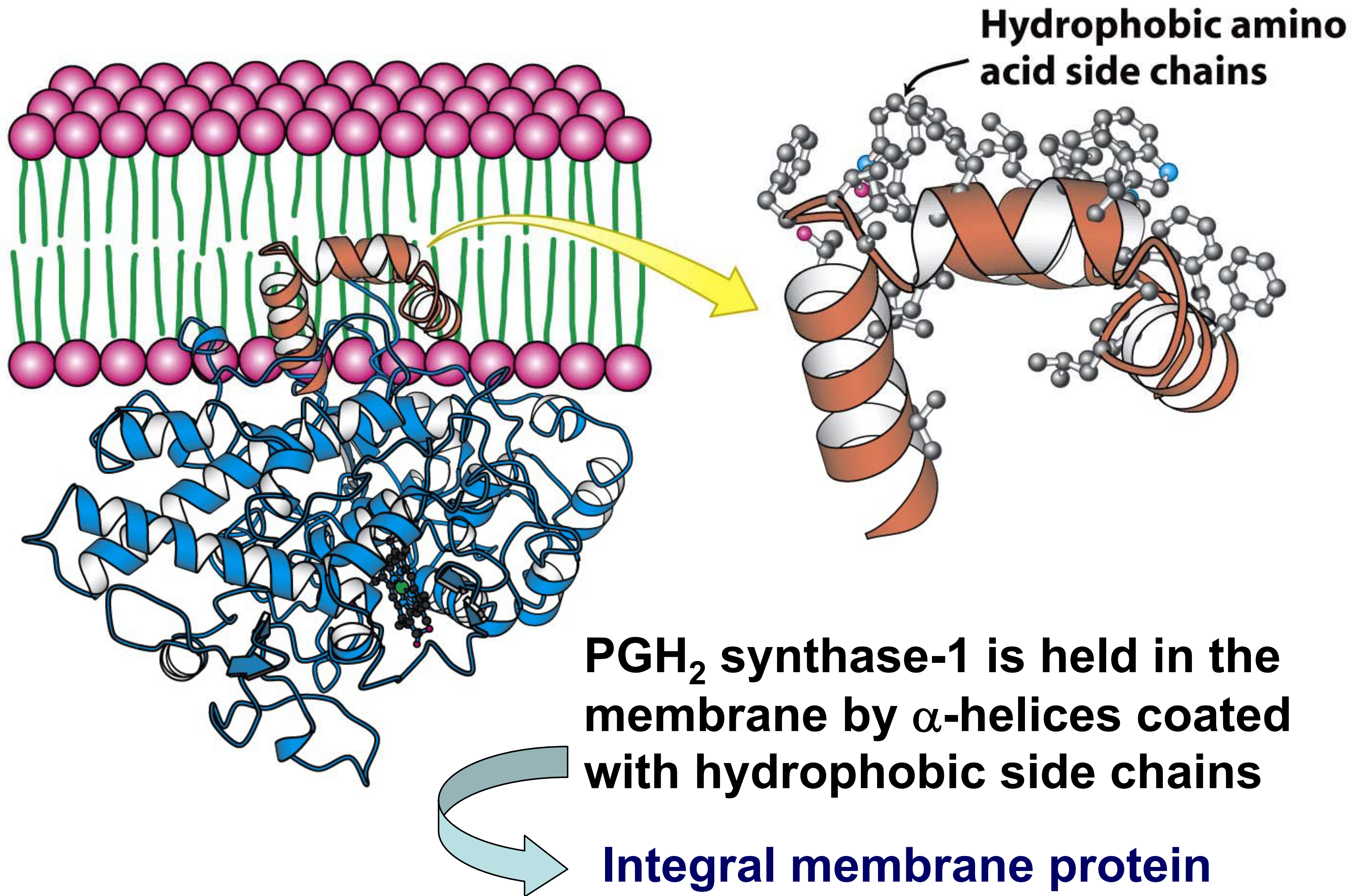
Peroxidase
$2\ H^+ + 2\ e^-$
H_2O



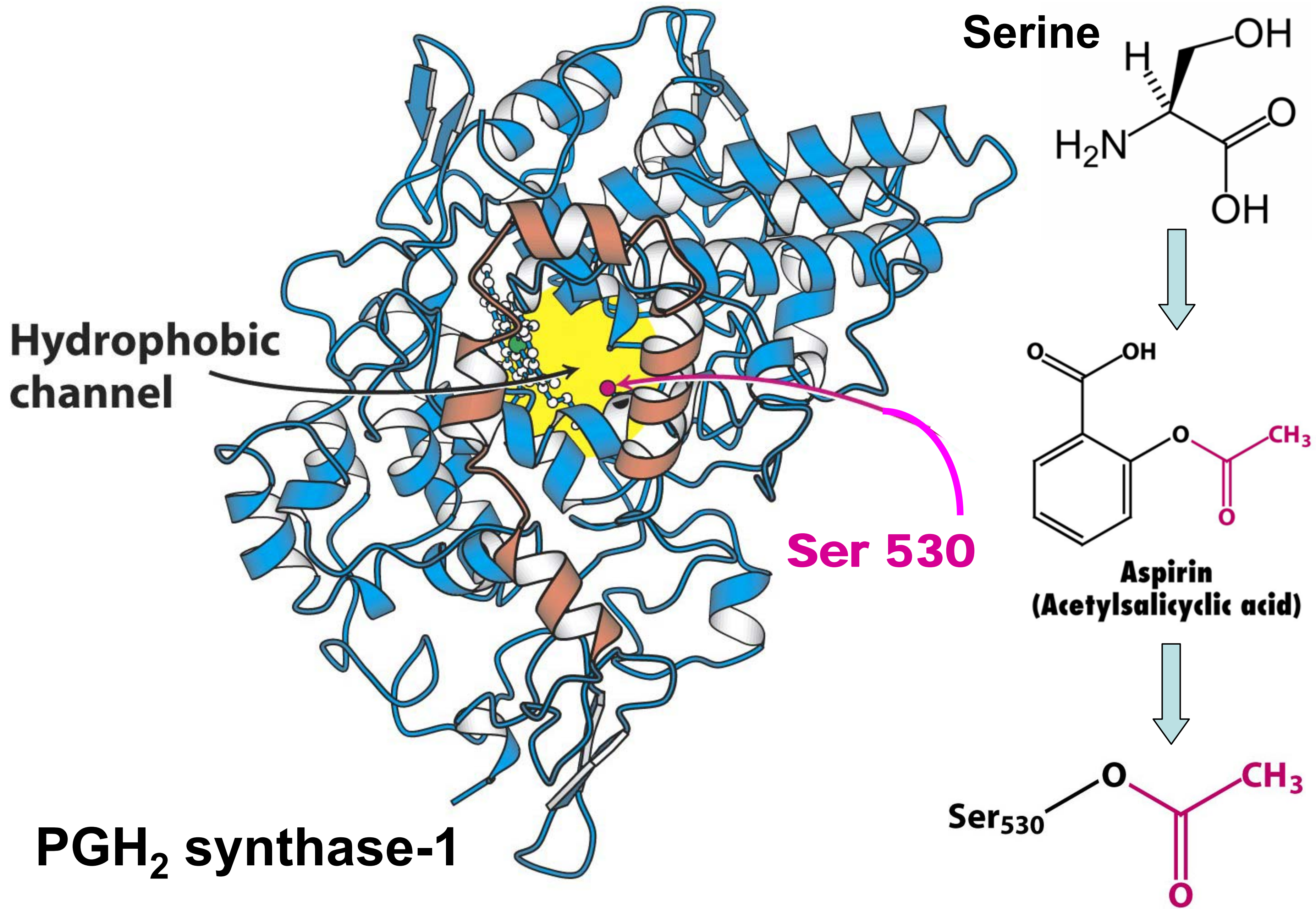
Prostaglandin H_2

PGH_2 promotes inflammation and modulates gastric acid secretion


Leukotrienes
Lipoxygenases
PLA$_2$
DG lipase
Phospholipids
Arachidonate
Diacylglycerols
Prostaglandin synthase
Prostaglandin H$_2$ (PGH$_2$)
Prostacyclin synthase
Thromboxane synthases
Prostacyclin
Other prostaglandins
Thromboxanes



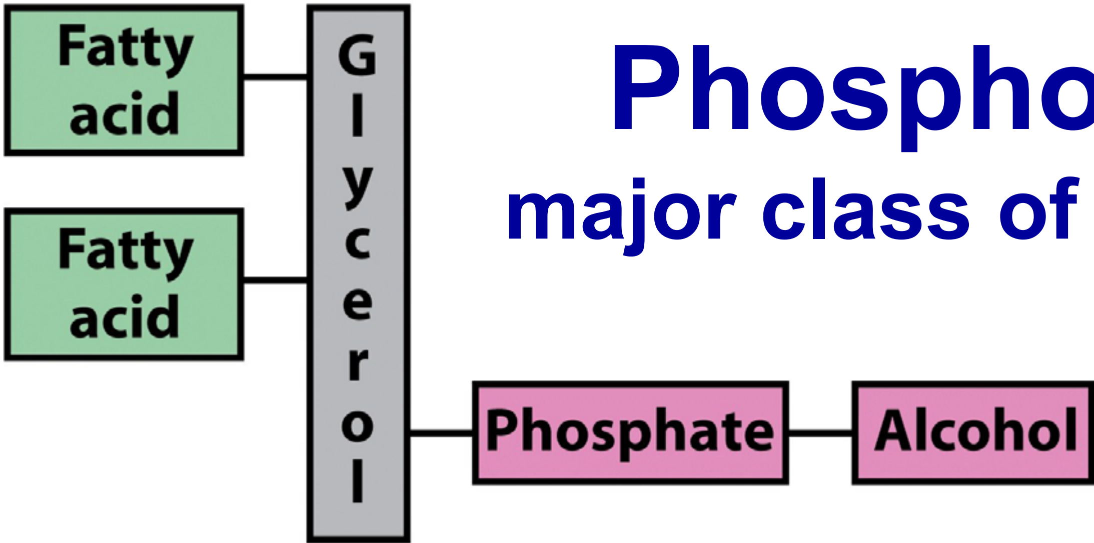
AA is hydrophobic and is funneled thru protein channel

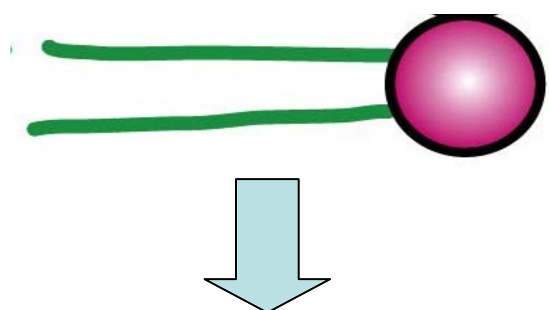


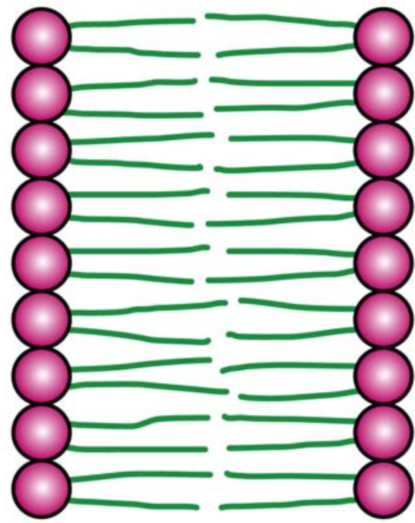
Serine
OH
H
H2N
O
OH
Hydrophobic channel
O
OH
O
CH3
O
Ser 530
Aspirin
(Acetylsalicyclic acid)
Ser530
O
CH3
O
PGH2 synthase-1

Phospholipids (PL)

major class of membrane lipids







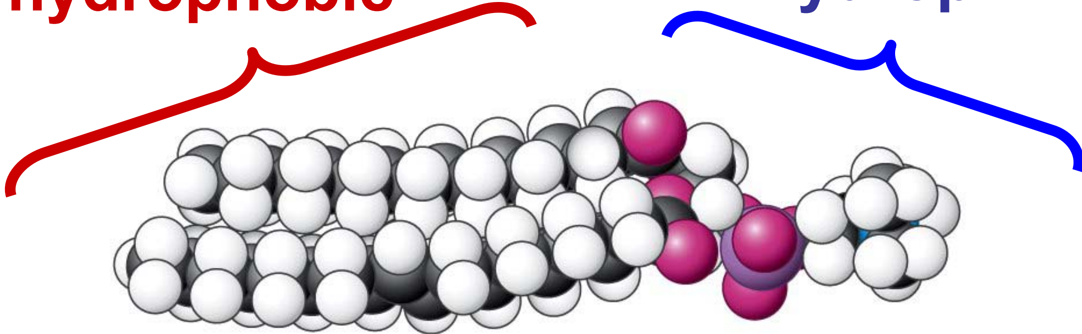
Membrane

Glycerophospholipid

constructed of 4 components

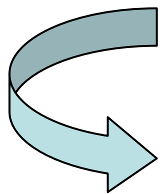
hydrophobic

hydrophilic

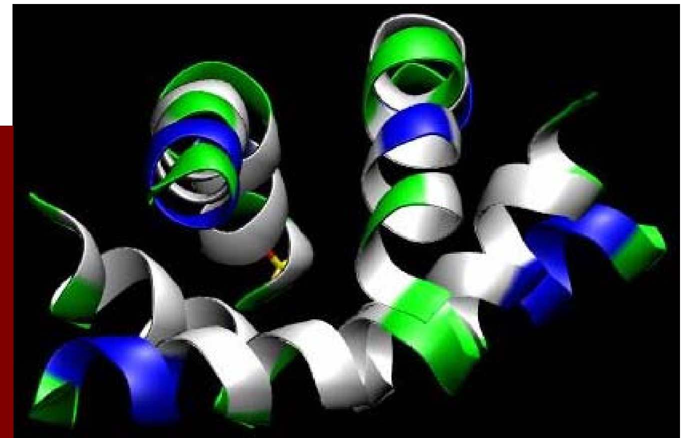


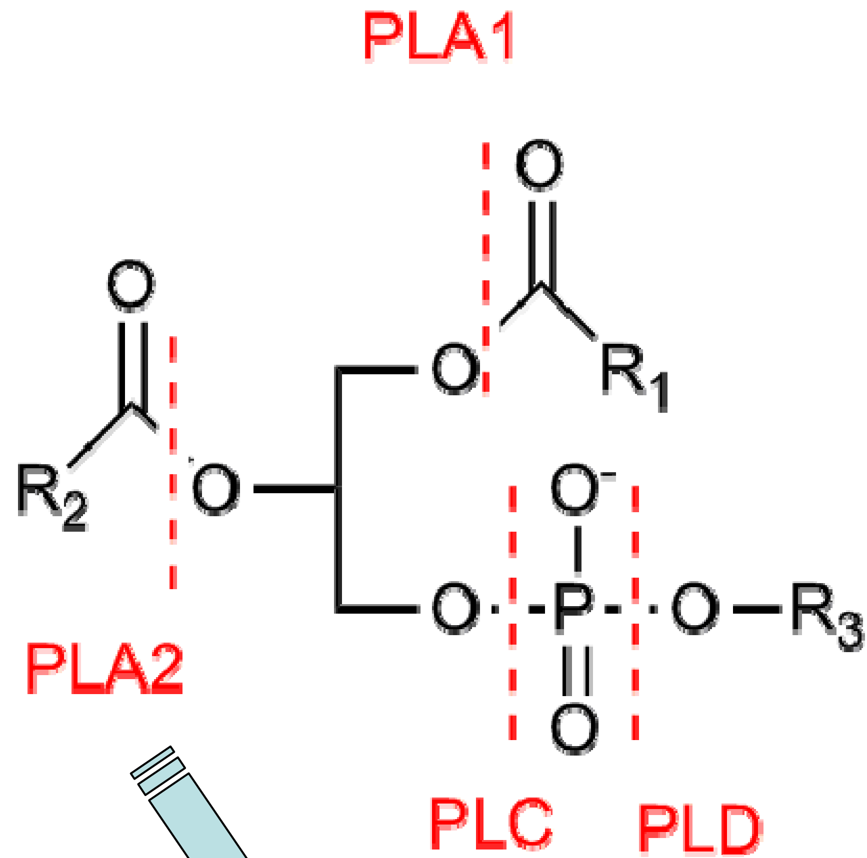
Phospholipase A_2 (PLA_2)

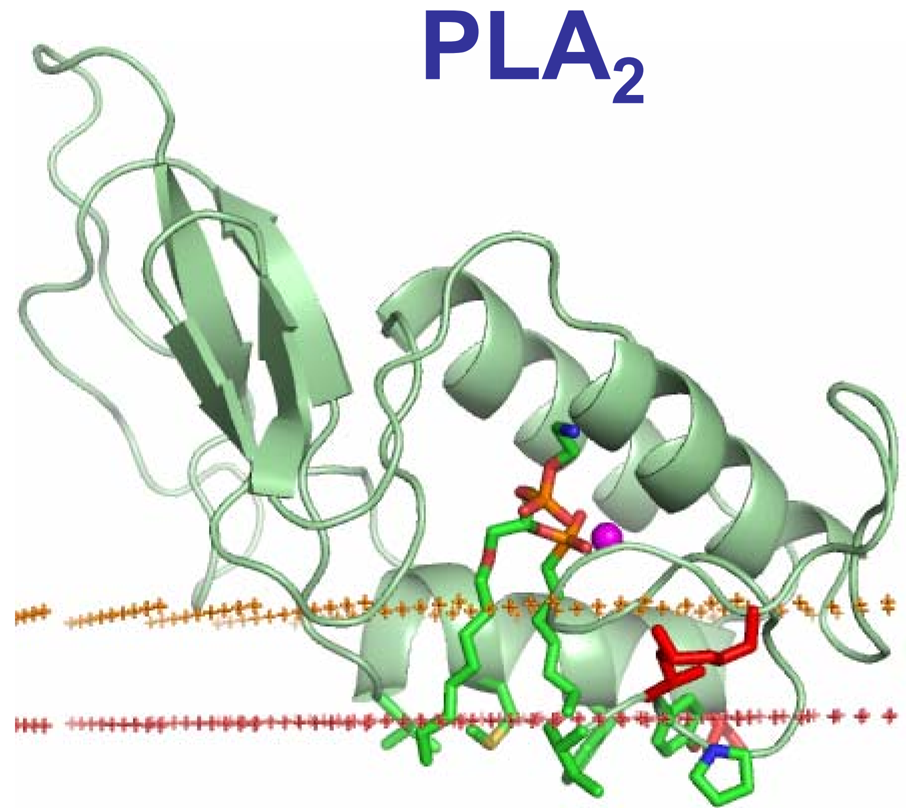
- specifically hydrolyzes the sn-2 acyl bond of PLs releasing AA & lysophospholipids
- upon downstream modification by COX, AA is modified into the active eicosanoids
- venom from snakes & insects contains melittin (26 aa peptide) that stimulates PLA_2
- AA is disproportionately released from the PL membrane after snake or insect bite due to increase in PLA_2 activity/levels

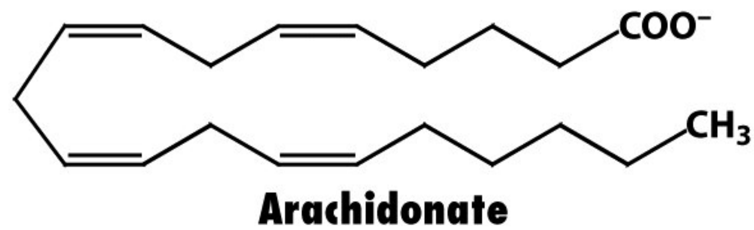


Results in inflammation & pain at the site of injury



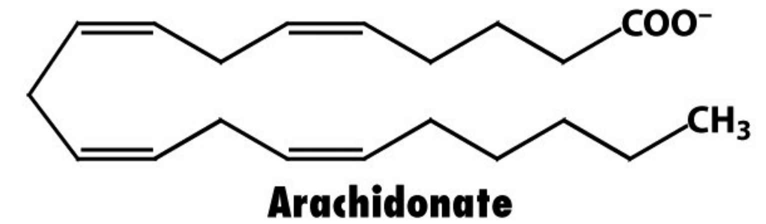

PLA1
O
O
R1
R2
O
PLA2
O-
O
P
O
R3
O
PLC
PLD

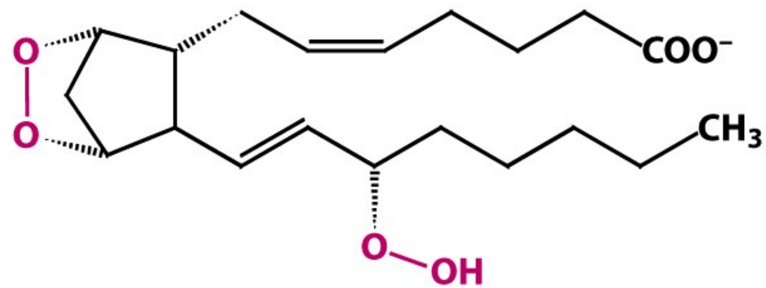

PLA2

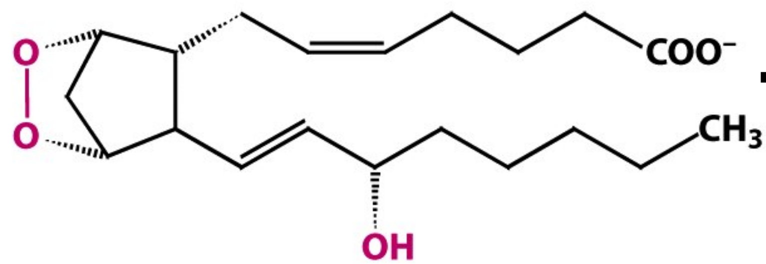

COO-
CH3
Arachidonate

Eicosanoid
pathways

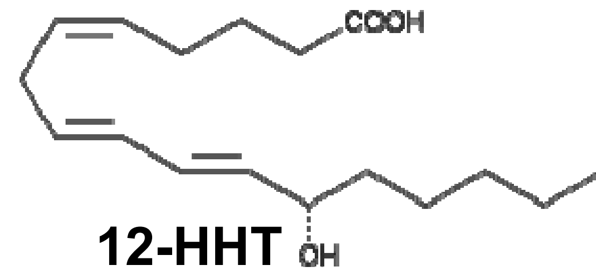
- 12-HHT is a product of the COX pathway
- synthesized by thromboxane synthase from PGH_2 concurrently with TXA_2

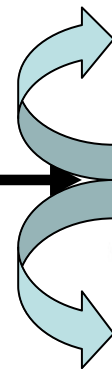


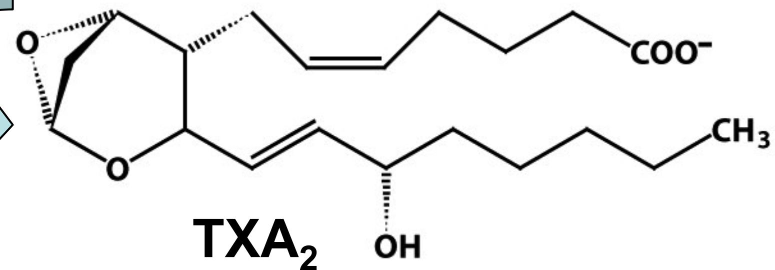




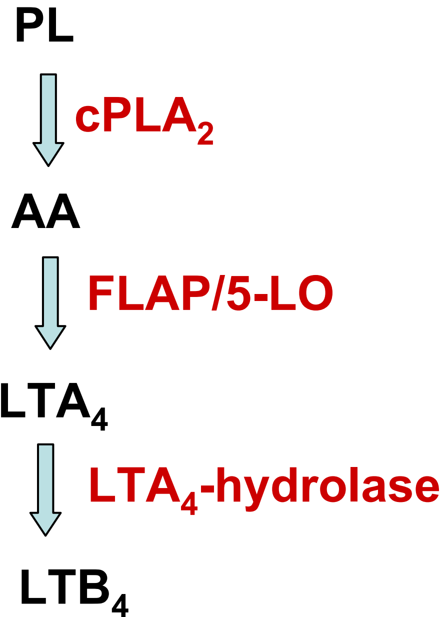
12-hydroxy-heptadecatrienoic acid

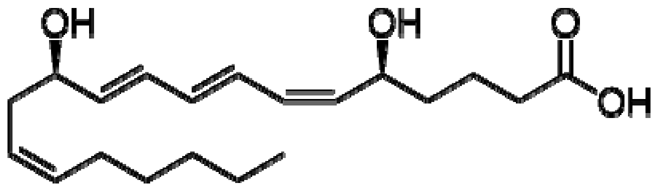


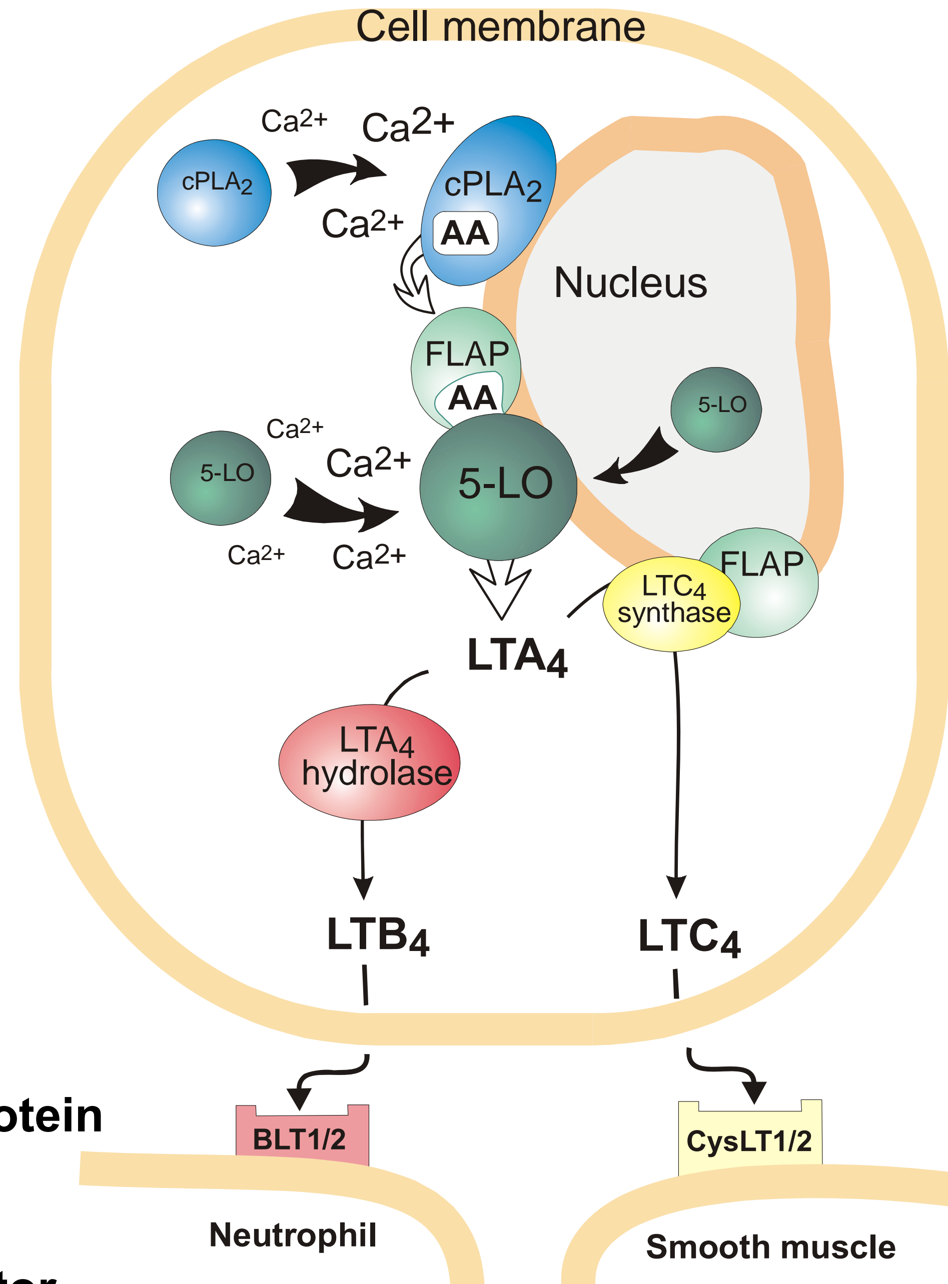




LTB_4 synthesis

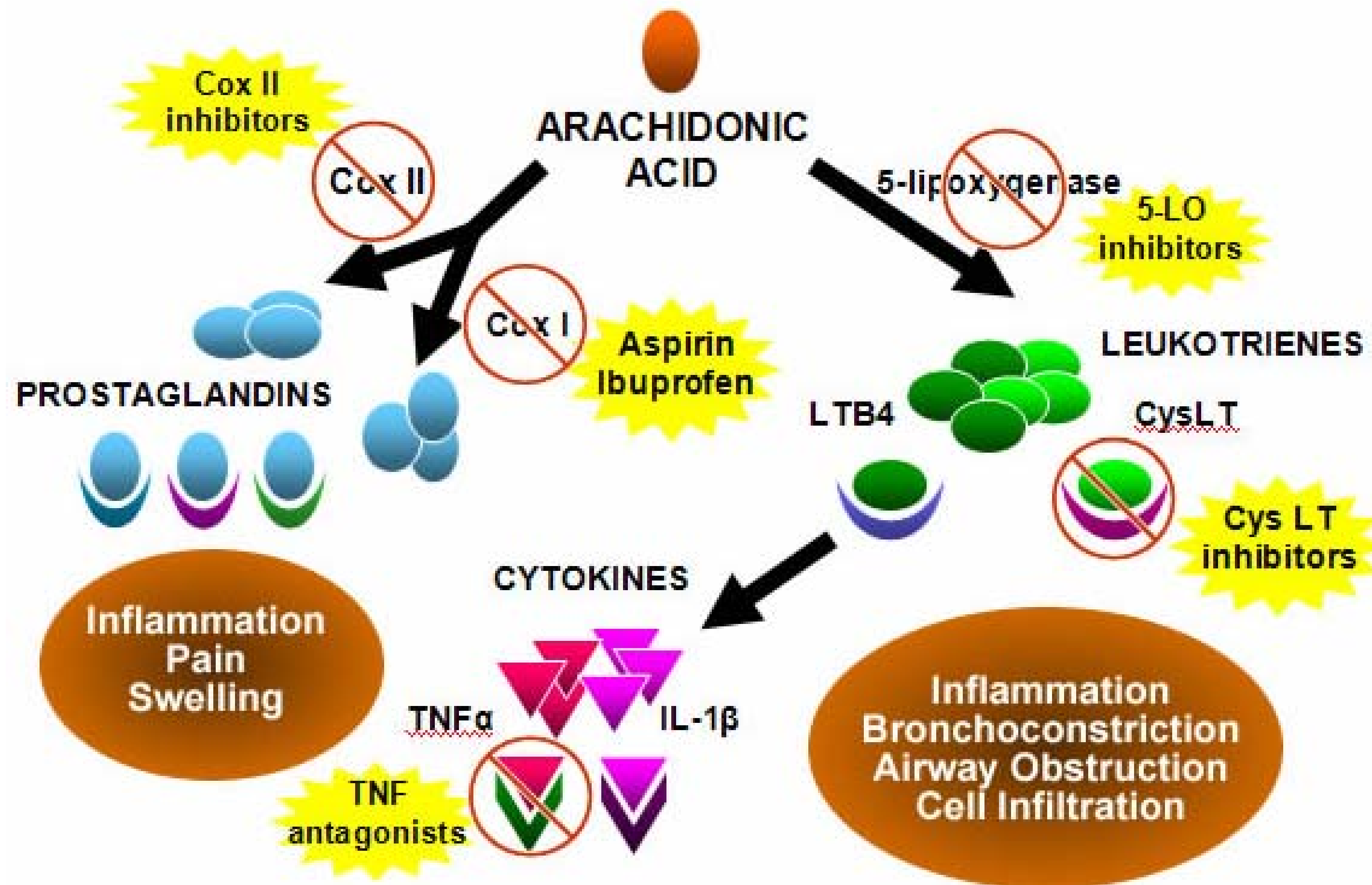




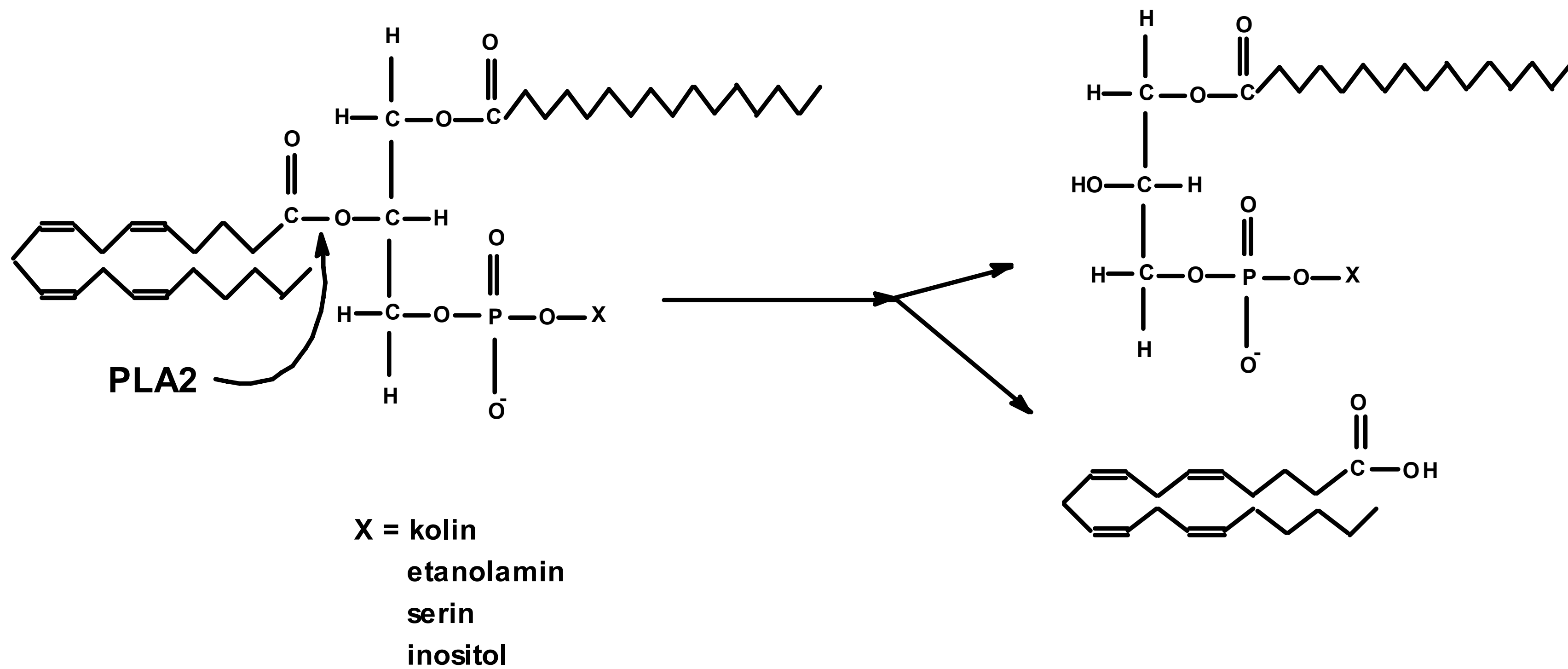


$cPLA_2$=cytosolic PLA_2
FLAP=five-lipoxygenase activating protein
5-LO=five-lipoxygenase
BLT1/2=LTB4 receptor
CysLT1/2=cysteinyl leukotriene receptor

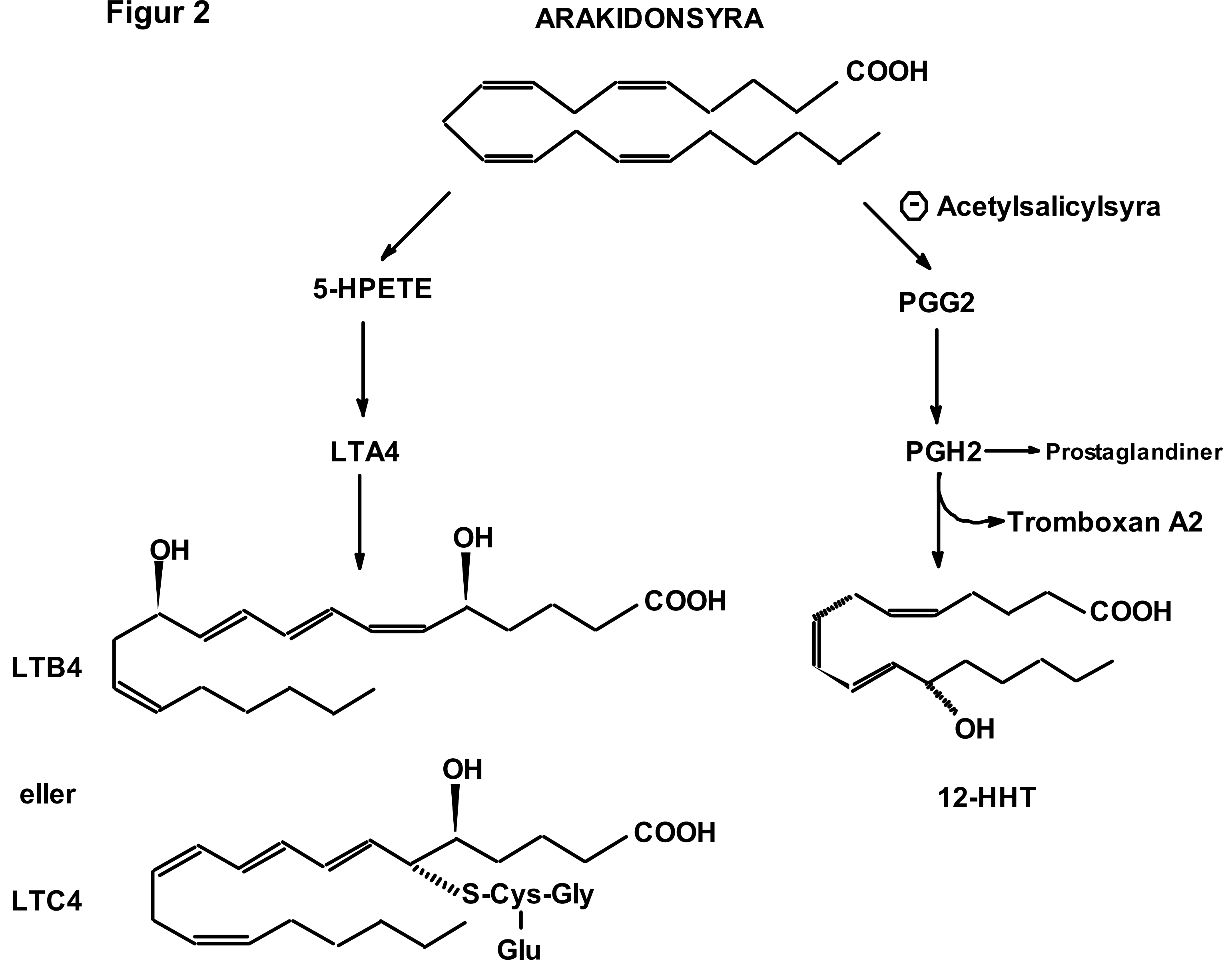
- AA is metabolized to produce inflammatory mediators
- Many current anti-inflammatory and pain medicines inhibit some portion of the AA pathway



Figur 1



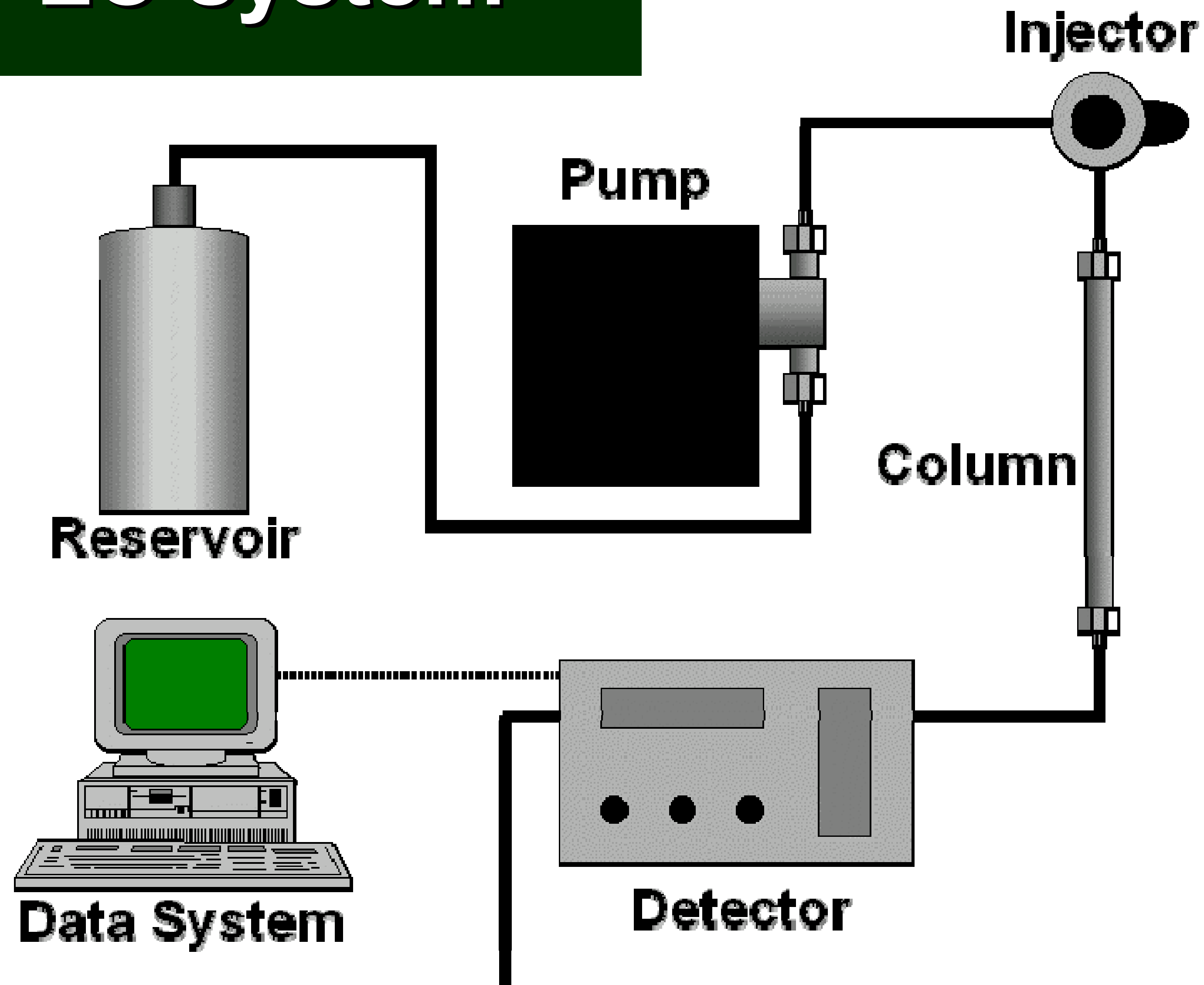
Fosfolipid → Fri fettsyra + Lysofosfolipid


Figur 2
ARAKIDONSYRA
COOH
Acetylsalicylsyra
5-HPETE
PGG2
LTA4
PGH2
Prostaglandiner
Tromboxan A2
OH
OH
COOH
COOH
LTB4
OH
eller
OH
12-HHT
COOH
LTC4
S-Cys-Gly
Glu

High Performance Liquid Chromatography

- The term High Performance Liquid Chromatography (HPLC) was coined to describe the separation of molecules under high pressure in a column filled with a matrix
- very widely used analytical technique
- Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary & mobile phases
- Separates compound based upon physical properties

HPLC system

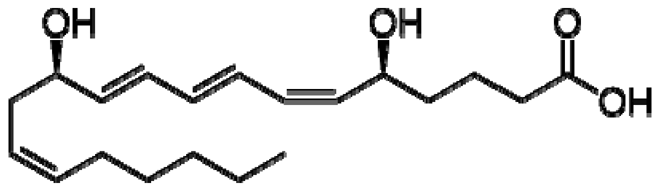


Stationary Phase

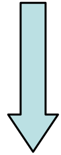
The stationary phase is defined as the immobile packing material in the column

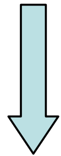
Mobile Phase

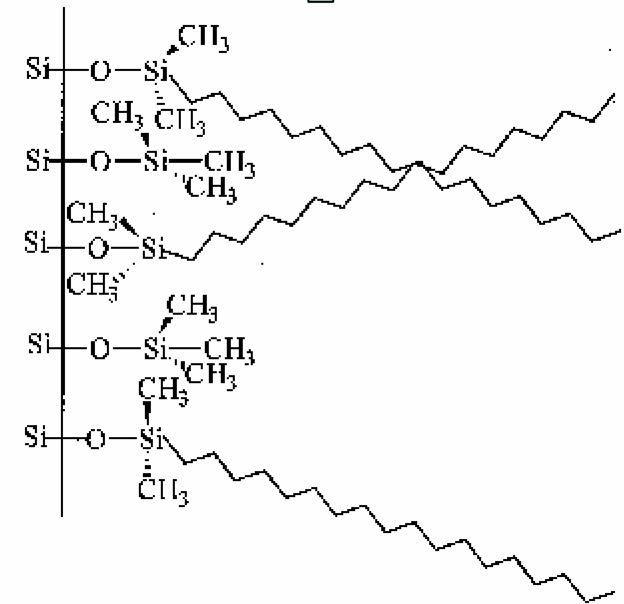
HPLC utilizes a liquid mobile phase to separate the components of a mixture

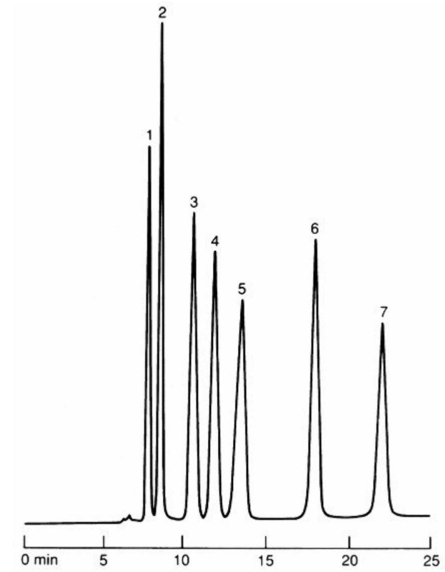


HPLC

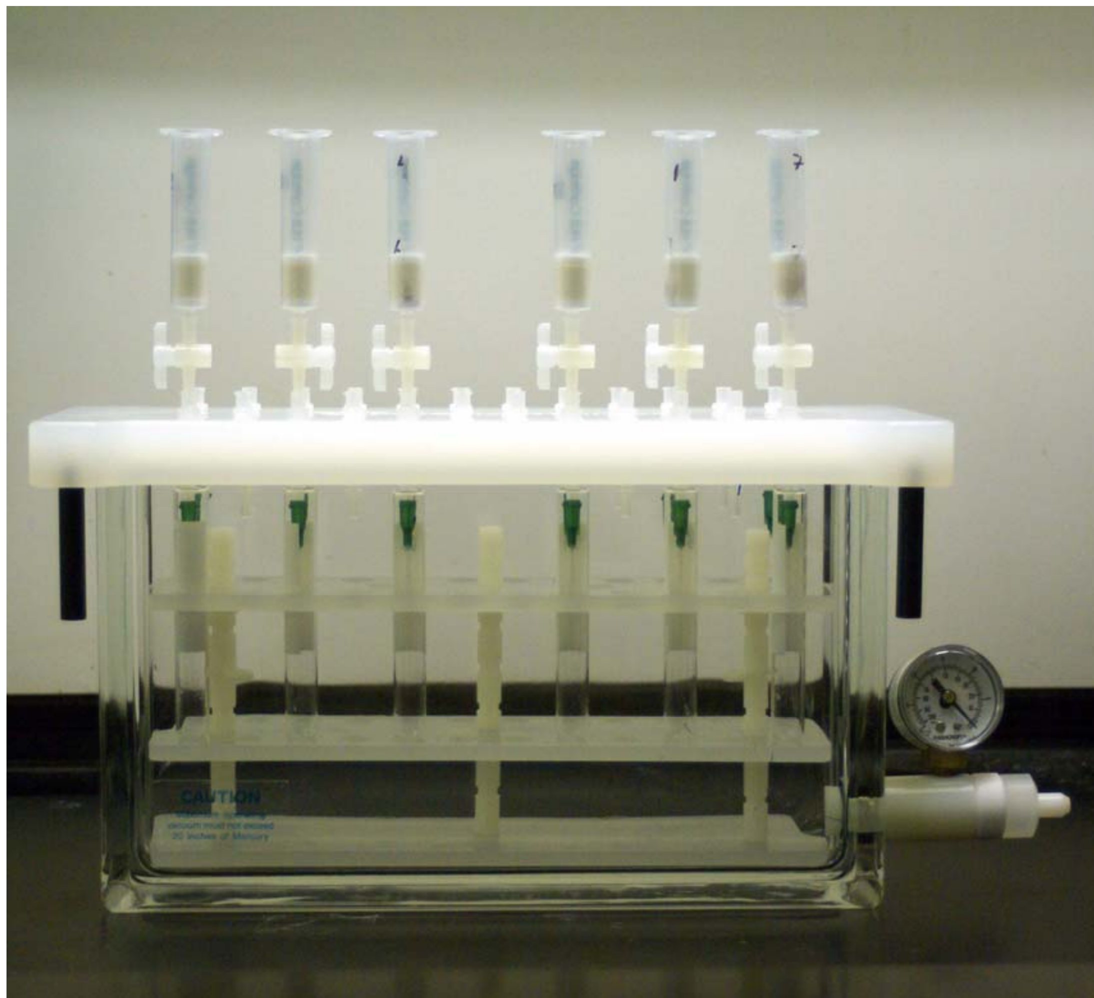








Solid-phase extraction (SPE) → Reverse phase



- separates analytes based upon polarity
- stationary phase consists of hydrocarbon chains
- retains hydrophobic compounds
- analyte is eluted by washing with a non-polar solvent

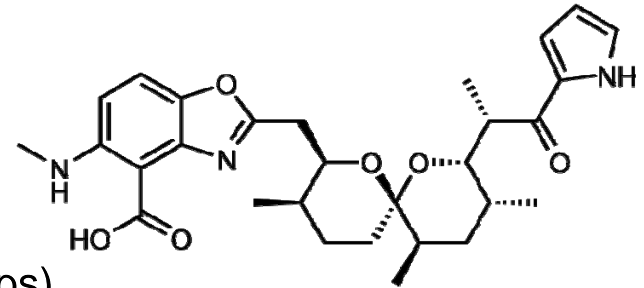
Lab protocol….

Method

1. Take a Vaccutainer-tube with blood.
   (~5 ml from the *human test subject*). This is tube **number 1** (or No ASA). The blood draws will be performed by a trained nurse in the lab.
   Discuss this with your partner - **NOW**

2. The *human test subject* takes an aspirin tablet, waits 90 min (record the time!!) and takes another blood sample. During this time, work-up the blood from tube 1 according to the method below. . . .

Method – step 1

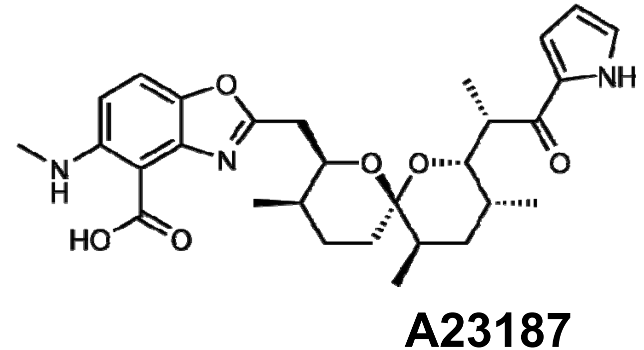


A23187

- **Sample number 1:**

3. Set the water-bath to 37°C (may be shared by several groups).
4. Transfer 5 ml blood in a plastic centrifuge tube (the middle line on the tube).
5. Add 5 ml 2% dextran solution with a plastic pipette (the top line on the tube)
6. Replace the stopper & mix thoroughly by inverting the tube carefully a couple of times.
7. Take out the stopper and let stand for 30 min (the sample should separate such that two phases of approximately the same volume form).
8. Remove the upper phase (~5 ml) with a plastic Pasteur pipette & place into a new plastic centrifuge tube.
9. Centrifuge on speed III for 15 min, with a lid (do not forget to balance the centrifuge).
10. Pour out the supernatant in the blood disposal unit and dissolve the pellet in 1 ml PBS.
11. Add (automated pipette): 5 µl 1 mM calcium ionophore & 5 µl 20 mM arachidonic acid
12. Replace the stopper & mix thoroughly, but gently. Incubate the tube for 20 min at 37°C.
13. Stop the reaction by adding 1 ml methanol, vortex carefully.
14. Place in ice water for at least 10 min.
15. Add 1 ml PBS. Add 20 µl PGB_2-standard (the teaching assistant will add the PGB_2).
16. Centrifuge the tube for 15 min at speed III (balance the centrifuge).
17. Transfer the supernatant to a new tube.

Method – step 1 cont.



- **Sample number 2:**

18. Sample **number 2** (includes ASA) is treated identical to sample **number 1** (No ASA).
19. Continue to step 2 with both samples. Do not forget to <u>label your tubes</u>!

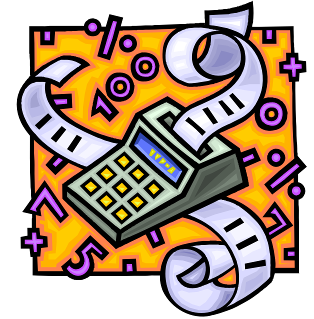
Method – step 2

1. Wash the C18-columns with 2 ml 100% methanol and then w/ 2 ml dest-water
2. Acidify the samples from point 16, step 1 by adding 3 drops of 20% acetic acid (control with pH-paper test strips, the pH should be 3-4).
3. Load the acidified samples onto the C18-columns with a syringe, the speed of injection should be **1 drop per second**.
4. Wash the columns with 2 ml dest-H2O by running the water through the columns.
5. Wash in the same way with 2 ml 25% methanol.
6. Elute the arachidonic acid metabolites from the columns carefully with 0.5 ml 100% methanol, **1 drop per second**. Collect the elution in 1.5 ml Eppendorf tubes marked with your group- and test number. ***Run air through the column after you have eluted the methanol to ensure that everything is removed from the column.***
7. Add 500 µl dest-H2O to each tube, mix carefully.
8. Mark 2 HPLC-autosampler vials with your group number and test number (1 container for each test, since LTB4 and 12-HHT will be analyzed with the same HPLC method but different wavelengths). *OBS!* Write directly on the container with a marker pen and do not use tape.
9. Transfer 500 µl to the marked HPLC-autosampler vials, cap and place the containers in group number order in the assigned test tube racks. Make sure that the racks are placed on ice.
10. Place the Eppendorf tubes with the remaining eluate in the assigned racks (we keep these just in case something goes wrong at the HPLC-analysis). *Should there be extra???*

Extra lab points . . . .

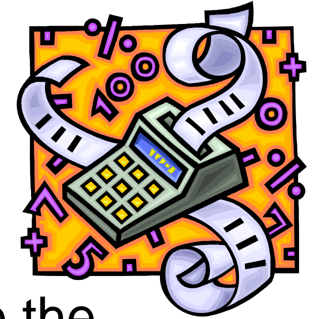
- Someone needs to donate blood. Decide with your partner in advance.
- If someone does not want to donate blood, tell me now
- Do not take any anti-inflammatory medicine before the lab (blood draw) . . .
- Pay extra attention to blood waste during lab, if you spill, notify T.A. and clean up immediately
- Wear goggles & lab coats at all times
- Don’t even think about bringing food into the lab
- Do not use automatic pipettes for blood
- We will add the PGB_2

Calculations



1. Identify LTB4, PGB2 and 12-HHT in the chromatogram using the retention times of the standards.
2. Calculate the amount of LTB4 in your samples using the area of the LTB4 peak in relation to the area of the PGB2 peak within the same chromatogram. You know the amount of PGB2 that was added to the samples. Use the following formula (where 1.67 is the ratio of the extinction coefficients for LTB4 and PGB2 at 270 nm):
3. Calculate the amount of 12-HHT in your samples using the area of the 12-HHT peak in the chromatogram where a known amount of 12-HHT was analyzed. Consider the total volume of sample that was injected.
4. Adjust the 12-HHT-values to account for possible losses during sample work-up. In point 2 above, an "internal standard method" was used to calculate the amount of LTB4. The internal standard method takes into account potential losses during the experimental work-up as well as technical variation in the instrumentation. When calculating 12-HHT in point 3, you used an "external standard method" that only gives the amount of 12-HHT in the final sample (in other words, the sample that was analyzed by HPLC). The external standard method does not provide the amount of 12-HHT in the sample prior to sample work-up and does not account for sample loss. Our interest is in knowing how much total 12-HHT was formed during the incubation and we therefore need to correct for losses in the sample work-up.

Calculations - cont



5. We do not have an internal standard for 12-HHT, but can use the recoveries for PGB2 in the LTB4 analysis as a “correction value” to estimate the loss in the sample work-up. We analyzed a standard of PGB2 by HPLC, and you have the area for an equivalent amount of PGB2 in the LTB4 samples (in other words, the amount of PGB2 that went through the sample work-up). Using this information, you can calculate the loss of PGB2 during the sample work-up. If you make the assumption that the losses were similar for 12-HHT, you can use this value (the mean value from the PGB2 loss in the two LTB4 samples) to “correct” the amount of 12-HHT calculated in point 3.

6. Finally, calculate the total amount of LTB4 and 12-HHT formed in the original blood samples taking into account all of the dilutions that were made and the quantities injected into the HPLC. Give the values in pmol/ml blood and present the results in a bar graph.

Lab Reports

- **Abstract** → **concise summary**
- **Introduction** → **theory and background**
- **Methods** → **concise, but thorough**
- **Results** → **What did you get? How much LTB_4 and 12-HHT?**
- **Discussion** → **critically analyze the experiment, what worked, what did not work? WHY? How does asprin work? Do your results ”make sense”? What is the significance of the experiment/results? What was the purpose of PGB_2?**

Lab Reports

- **Important changes to the method:**
  - **Inject 50 µl of your sample (NOT 200 µl)**
  - **Flow rate is 0.8 ml/min (NOT 1 ml/min)**

- **Scan in all chromatograms**
- **Submit as a MS Word document (as a .doc file, not a .docx file)**
- **Lab reports are due by Tuesday February 17th or will not be accepted**

Good luck, have fun, and don't hurt anyone…..