Knowledge that will change your world

3-11-19

Following pathways with isotopes

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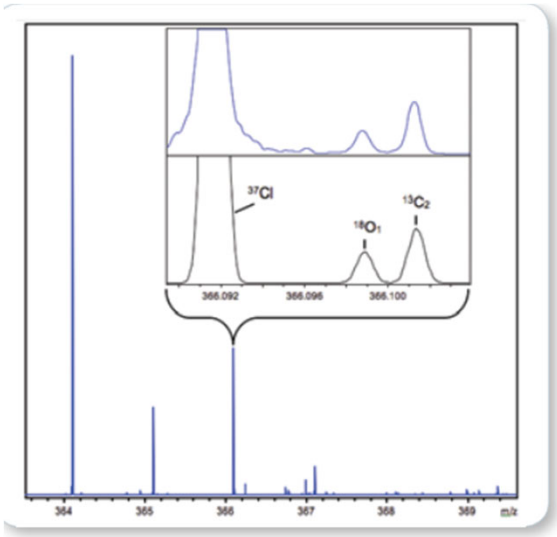
Synopsis

- **Extra value in the M+2 isotope peak**
- **Using isotopes in tracing metabolic and physiologic pathways**
  - Historical
  - Fluxomics
  - Physiology
- **Isotopes and enhanced chemical detection of metabolites**
- **Disturbing energy levels**

Value of natural isotopes

- The natural abundance of isotopes enables the investigator to determine the charge state of an ion
- The principal contribution to $[M+H]^+$ or $[M-H]^-$ isotope ions comes from ^{13}C (~1.1% of all carbon atoms)
- The intensity of the $[M+H]^+$ or $[M-H]^-$ ^{13}C isotope ion increases relative to the number of carbon atoms
- There is often an observable $^{13}C_2$ isotope peak

The importance of the M+2 ion



From Bruker

Value of the [M+2] peak

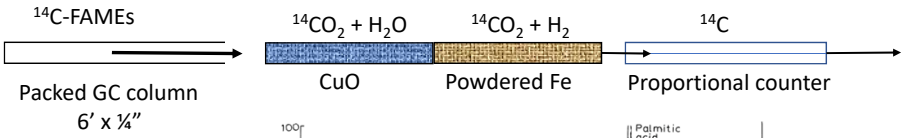
- The mass difference due to a nominal increase in mass of 2 contains a lot of information
  - These are isotopic mass differences for each of the common elements
    - $^{1}H_2/^{2}H_2$ 2 x 1.006277 = 2.012554 (0.012%)
    - $^{12}C_2/^{13}C_2$ 2 x 1.003355 = 2.006710 (1.078%)
    - $^{14}N_2/^{15}N_2$ 2 x 0.997035 = 1.994079 (0.364%)
    - $^{16}O_2/^{17}O_2$ 2 x 1.004217 = 2.008434 (0.038%)
    - $^{16}O_2/^{18}O_1$ 1 x 2.004246 = 2.004246 (0.205%)
    - $^{32}S_2/^{33}S_2$ 2 x 0.999387 = 1.998774 (0.752%)
    - $^{32}S_2/^{34}S_1$ 1 x 1.995796 = 1.995796 (4.252%)
- Needs the highest possible mass resolution
  - FT-ICR

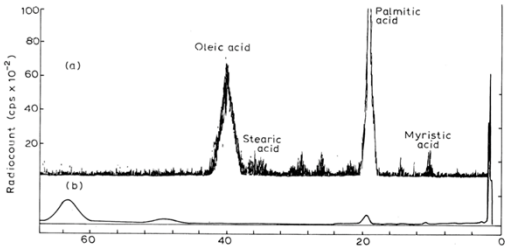
Using isotopes to trace a pathway

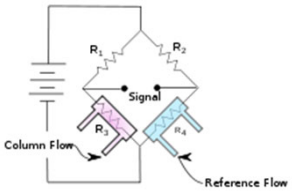
- Early studies (1930s) used ^{2}H, ^{13}C and ^{15}N labeling to map pathways
  - Limited to 1-200 *m/z* mass range
- 1950s/60s ^{14}C-radiotracers
  - 2D-Paper or thin layer chromatography
  - Radio gas chromatography
    - labeling of specific carbon atoms

Radio-gas chromatography of FAMEs

In Tony James' lab







Katharometer to detect H_2 by thermal conductivity

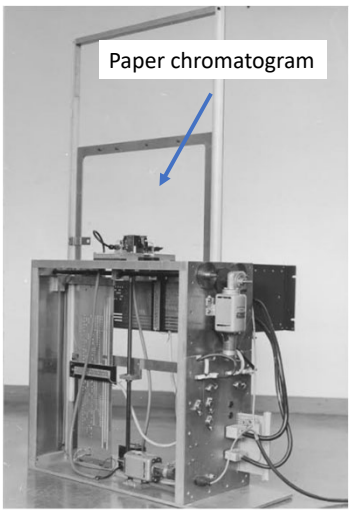
We were studying an isomer of palmitoleic acid – from β-oxidation of oleic acid, or direct desaturation? Collected the 16:1 peak using a gas density balance (no degradation) in **ether-soaked cotton wool**. Subjected to oxidation with permanganate-periodate – identified C_{11} monobasic acid and C_7 dibasic acid, i.e., 16:1Δ^7

Early beginnings of metabolomics in London

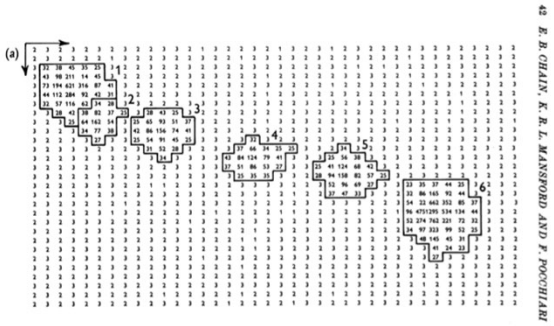
- **Sir Ernst Chain (1945 Nobel Laureate – the biochemist who characterized penicillin)**
  - **Also renown for his work on microanalysis**
- **Used 2D-paper chromatography to resolve glycolytic, Krebs cycle and amino acids derived from ^{14}C-glucose**
  - **Geiger counter mounted on a typewriter frame**
  - **Digitized the collected data and prepared computer-generated figures**

METABOLOMICS

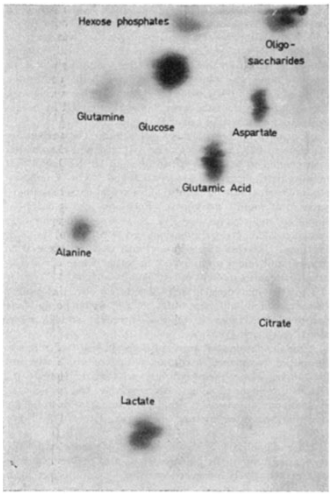
Keith Mansford



Radiochromatography examples



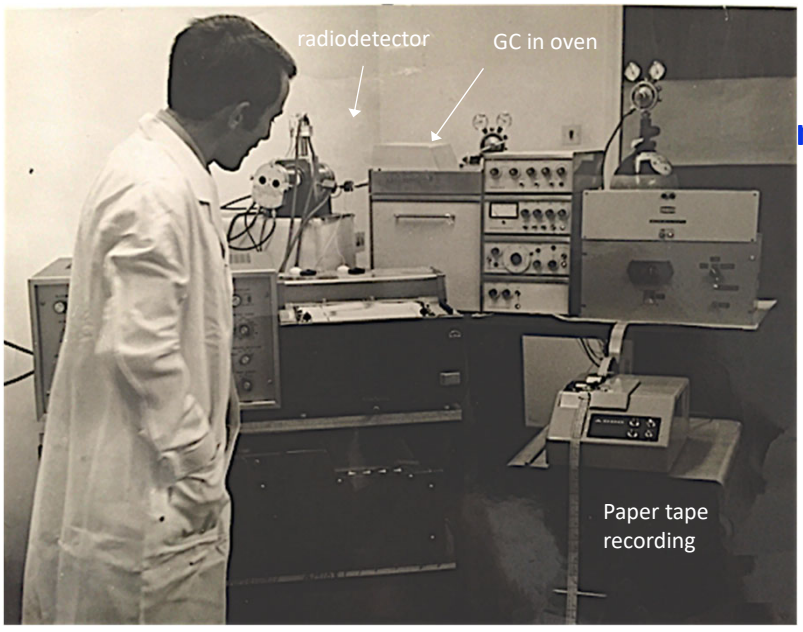
J Physiol (1960) 154:39
E.B. Chain, K.R.L. Mansford and F. Pocchiari



Autoradiogram of ^{14}C-glucose metabolites from an isolated perfused Langendorff rat heart preparation. The metabolites were separated by 2D-paper chromatography.

The conditions were:
1st dimension: butan-l-ol-acetic acid-water (40:11:25, by vol.) for 16hr.;
2nd dimension: (-) phenol-aq. NH_3 (sp.gr. 0.88)-water (80:1:20, by vol.) for 24hr.

Biochem. J. (1969) 115, 537
E.B. Chain, K.R.L. Mansford and L.H. Opie



Radio-GC analysis
metabolomics in its infancy

Radio gas-liquid chromatography with digitization of collected data

Developed this for my PhD work (1967-1970) to study glucose metabolism in acellular slime mold, *Physarum polycephalum*

Physarum polycephalum

The many headed slime mold

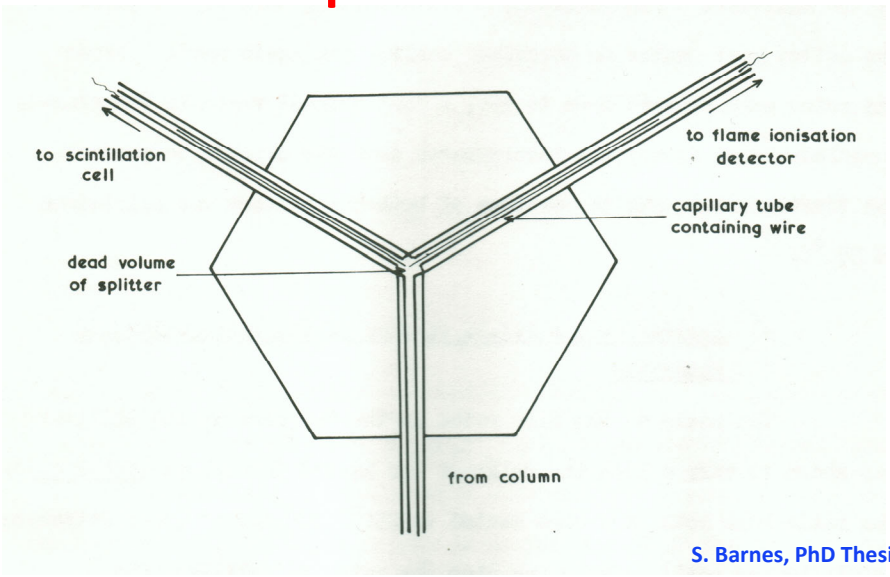
This is a single cell that spreads out to cover a petri dish

The cytoplasm is pushed to one end and back over a 30-45 s period

The cytoplasm has properties of a liquid and a solid

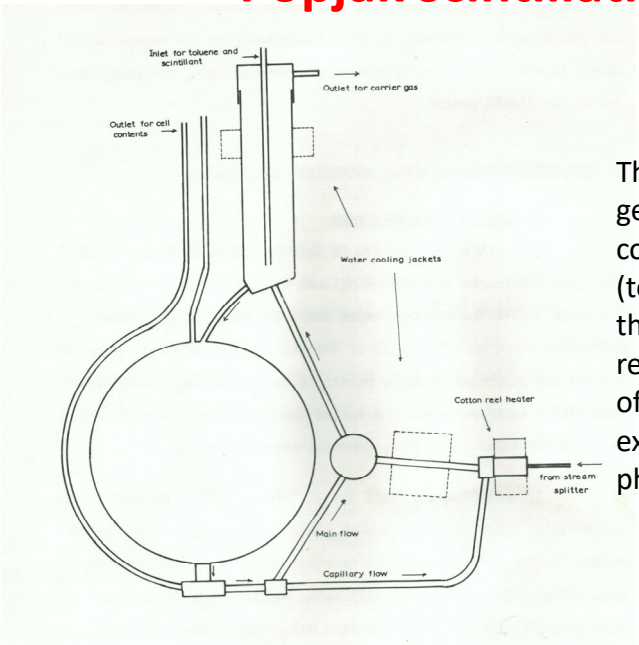
https://www.youtube.com/watch?v=Ia3kWlS_OZU

Stream splitter for radio GC



S. Barnes, PhD Thesis

Popjak scintillation cell



The key to this device was the mixing generated by the gas from the GC column causing (scintillation) fluid (toluene) to flow out of the bottom of the scintillation chamber, both to aid recirculation and to provide a source of solvent vapors that more efficiently extracted the compounds in the gas phase.

Application to the discovery of a new intermediate in squalene biosynthesis

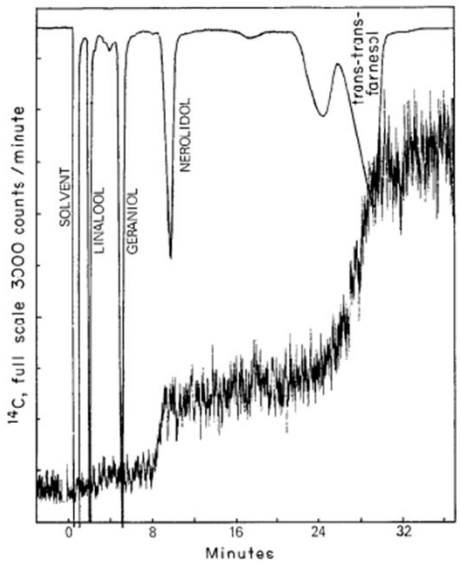
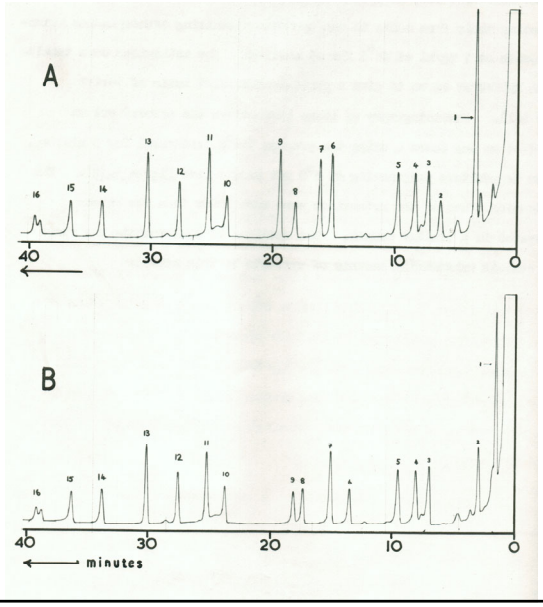


FIG. 19. Gas-liquid radiochromatogram of F_1 (*cf.* Fig. 4) from the chromatography on DEAE-cellulose of the butanol extract of an incubation of liver microsomes with farnesyl pyrophosphate. Nearly 30% of the total radioactivity in the specimen was accounted for by ^{14}C in nerolidol. Cochromatography with added markers of linolool, geraniol, nerolidol, *cis-trans*- and *trans-trans*-farnesol; simultaneous recording of mass and radioactivity detector.

It's worth reading this 1969 article in J Biol Chem for the depth of analysis that was undertaken to prove the identity of this intermediate

GC of glycolytic and Krebs cycle intermediates

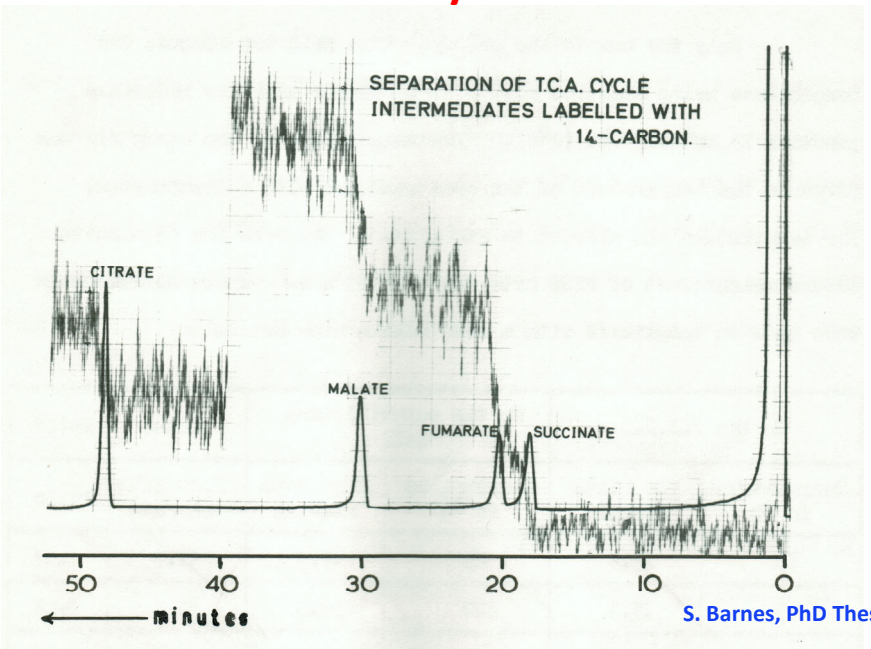


Temperature programming of TMS ester/ethers on a 5' x ¼ inch packed column of Chromosorb W coated with OV-1 liquid phase

1=pyruvate , 2=?? , 3=phosphate , 4=succinate , 5=fumarate, 6=oxaloacetate, 7=malate, 8=αKG, 9=hexadecane, 10=αGP, 11=citrate, 12=α–D-glucose, 13=β–D-glucose, 14=docosane, 15=F6P, 16=G6P

S. Barnes, PhD Thesis

Radio-GC of Krebs Cycle intermediates

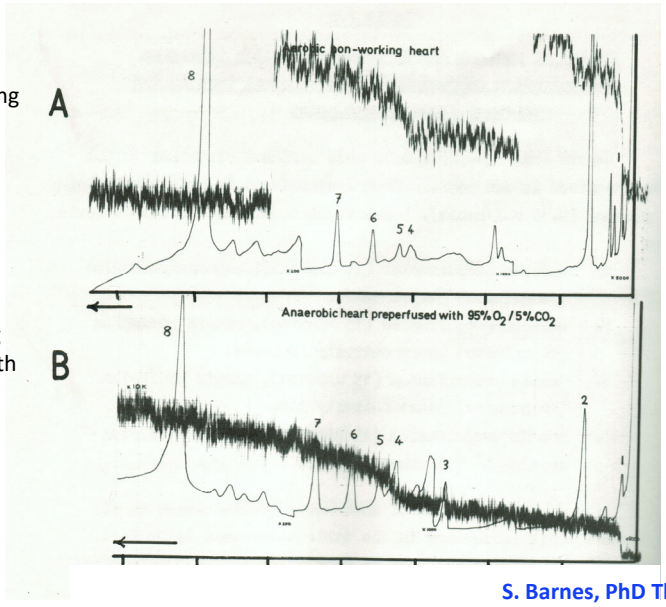


S. Barnes, PhD Thesis

Radio GC analysis of beating heart

Aerobic non-working heart

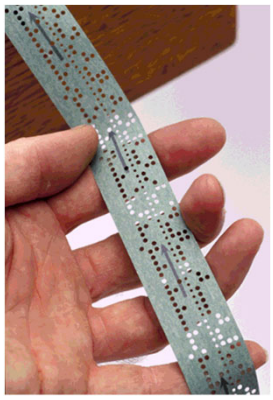
Anaerobic beating heart perfused with 95% O_2/5% CO_2



S. Barnes, PhD Thesis

Software for data analysis on a PDP9 computer

```
DIMENSION IBUFF(1000), IDATA(725,2)
COMMON IBUFF,IDATA,ITIME,INT,ISIG
5       ITIME=0
7       CALL TAPE(ISIG,INTA)
8       IWRITE(1,1001)INTA
        INT=INTA/100
10      NPOINT=1
11      CALL TAPE(ISG,IDATA(NPOINT,1))
        IF (ISIG.EQ.1) GO TO 16
        IF (ISIG.EQ.2) GO TO 13
        WRITE(1,1001)NPOINT
GO TO 11
13      IF (IDATA(1,1).GT.940) GO TO 11
        IF (IDATA(NPOINT,1).EQ.0) GO TO 11
        IF (NPOINT.EQ.725) GO TO 16
15      NPOINT=NPOINT+1
        GO TO 11
```



Punched tape data
1 data point/sec

Digital PDP computer
Had a screen

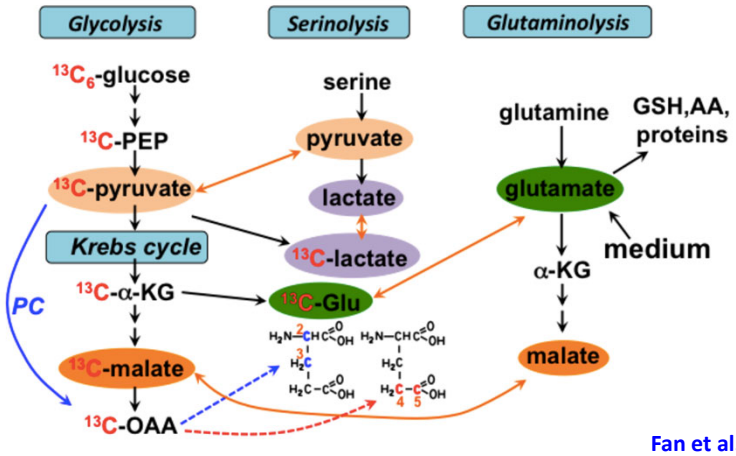
Fluxomics

See talk by Teresa Fan

https://www.uab.edu/proteomics/metabolomics/workshop/2018/videos/fan_day3.html

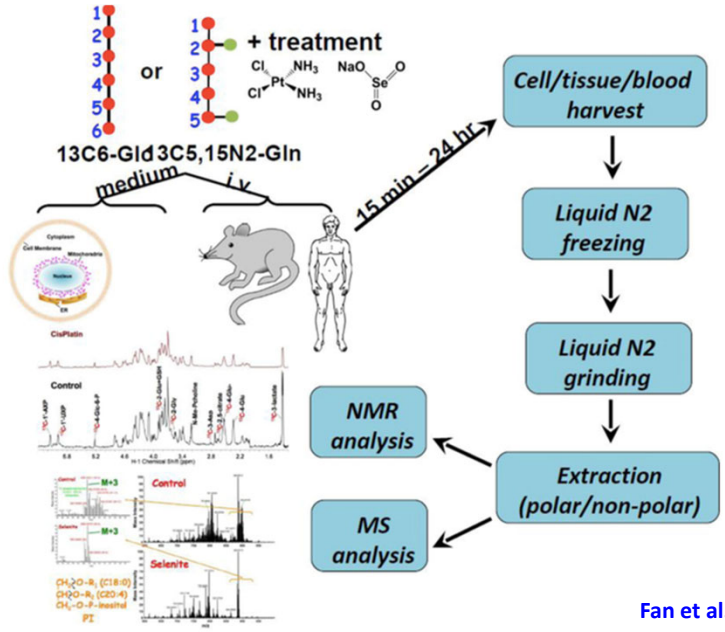
Fluxomics with stable isotopes

- **A feature of many metabolites is that they have multiple origins**

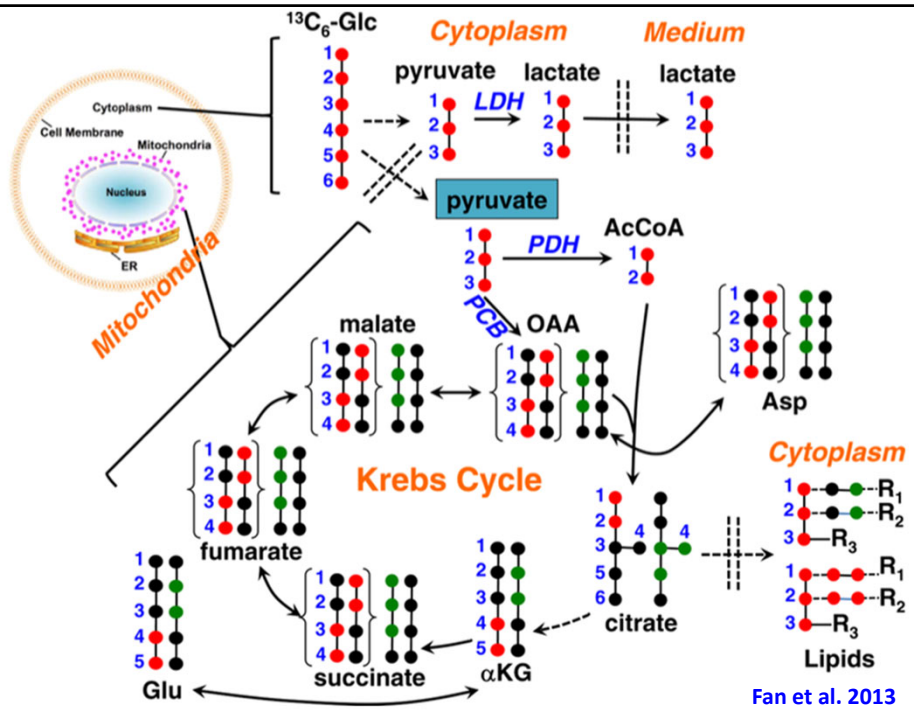


Fan et al. 2013

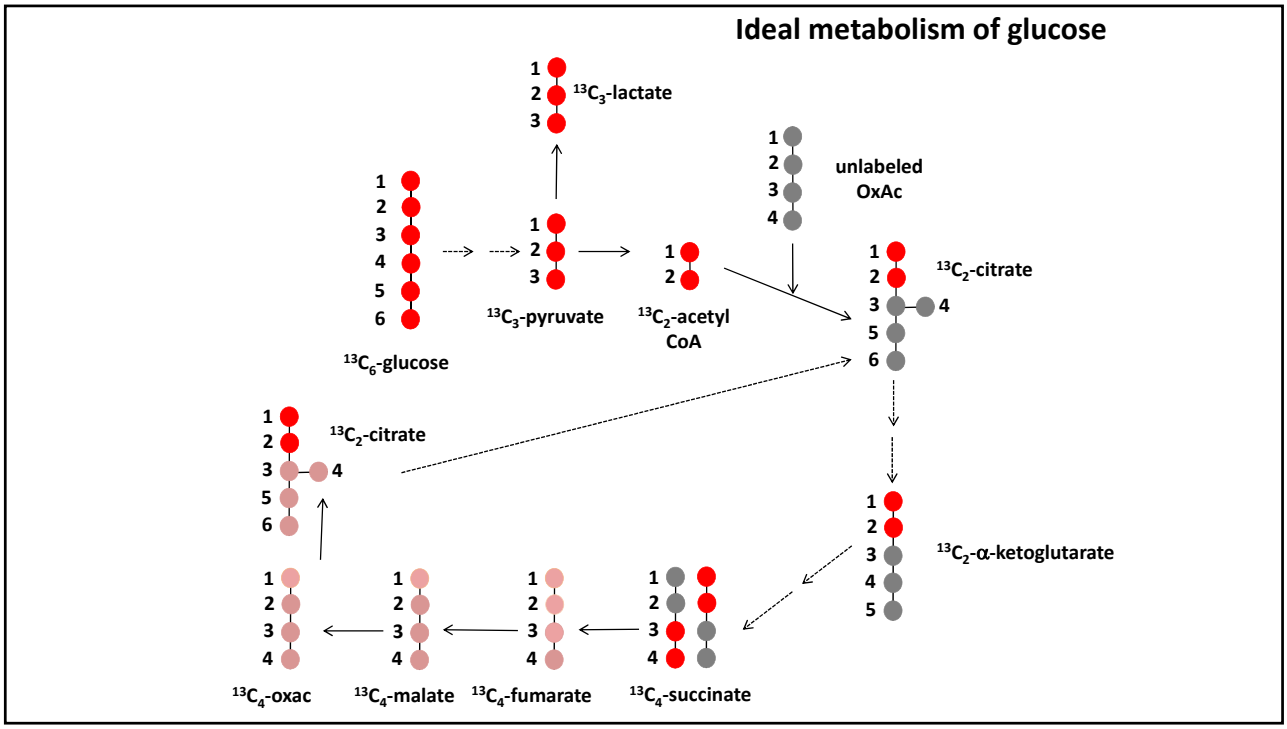
Stable isotope resolved metabolomics

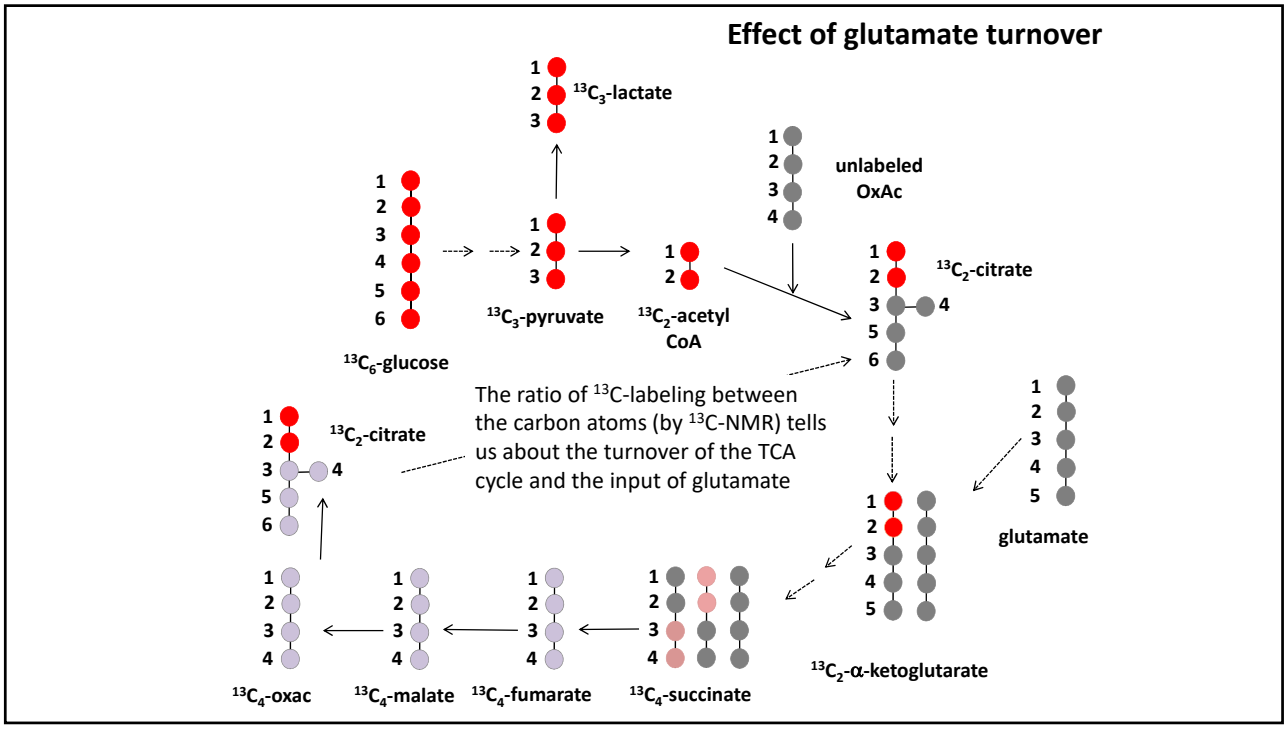


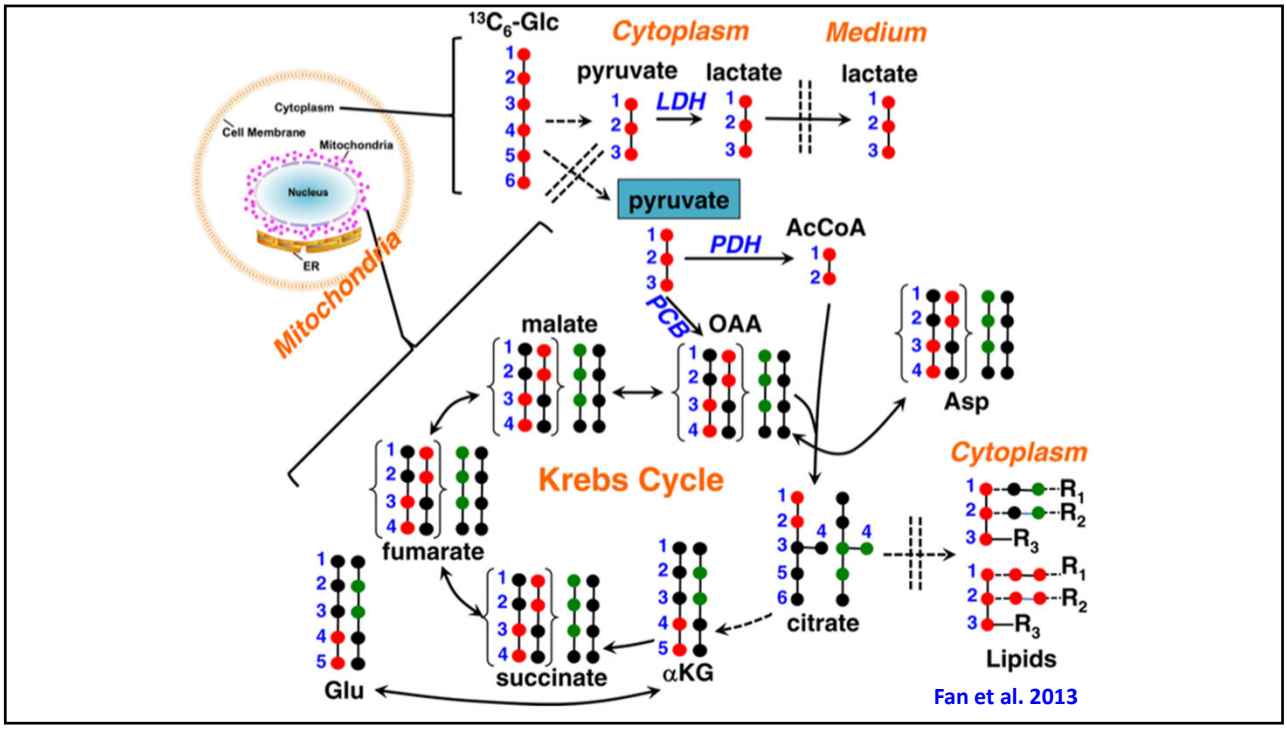
Fan et al. 2013



Fan et al. 2013

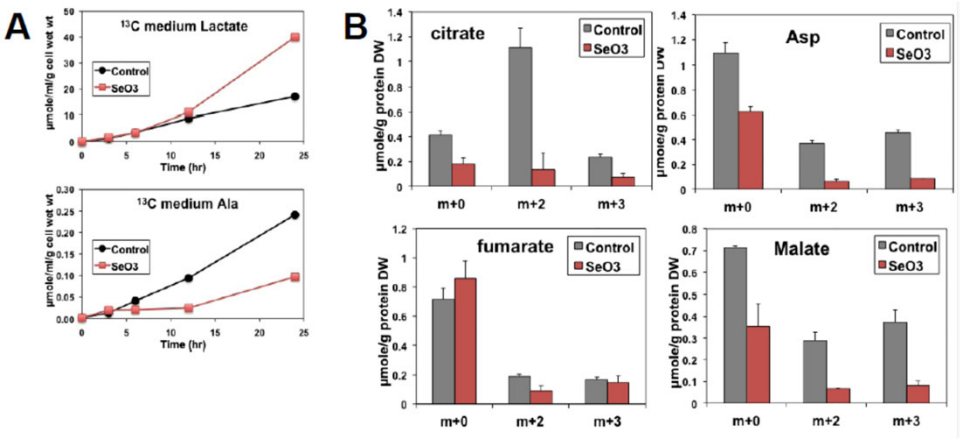

Ideal metabolism of glucose
$^{13}C_3$-lactate
unlabeled OxAc
$^{13}C_6$-glucose
$^{13}C_3$-pyruvate
$^{13}C_2$-acetyl CoA
$^{13}C_2$-citrate
$^{13}C_2$-citrate
$^{13}C_2$-α-ketoglutarate
$^{13}C_4$-oxac
$^{13}C_4$-malate
$^{13}C_4$-fumarate
$^{13}C_4$-succinate


Effect of glutamate turnover
$^{13}C_3$-lactate
unlabeled OxAc
$^{13}C_6$-glucose
$^{13}C_3$-pyruvate
$^{13}C_2$-acetyl CoA
$^{13}C_2$-citrate
$^{13}C_2$-citrate
The ratio of ^{13}C-labeling between the carbon atoms (by ^{13}C-NMR) tells us about the turnover of the TCA cycle and the input of glutamate
glutamate
$^{13}C_2$-α-ketoglutarate
$^{13}C_4$-oxac
$^{13}C_4$-malate
$^{13}C_4$-fumarate
$^{13}C_4$-succinate



Fan et al. 2013

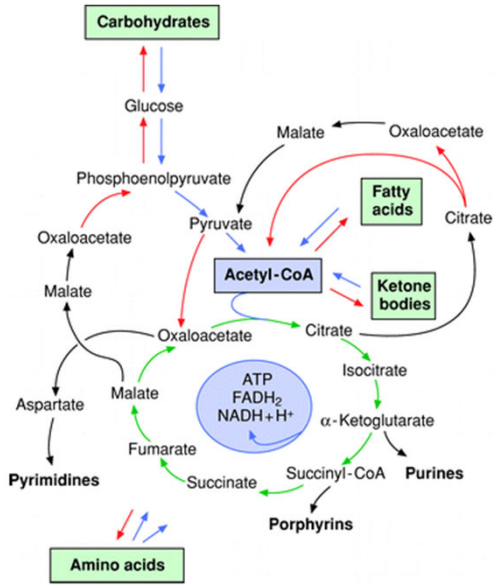
Effect of selenite on pools of intermediates



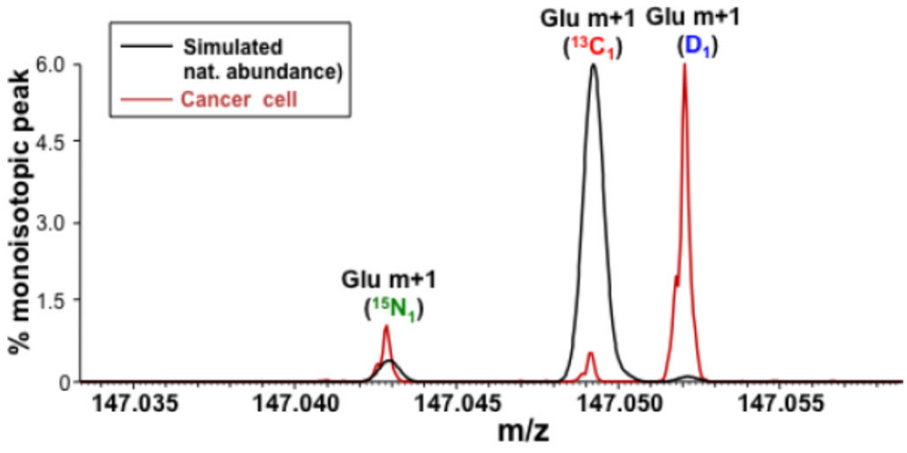
Pyruvate carboxylase converts pyruvate to oxaloacetate and by-passes the early steps in the Krebs cycle. Treatment of the cells with selenite blocks this step and the ^{13}C-content of citrate sharply decreases

Fan et al. 2013

Anaplerotic reactions

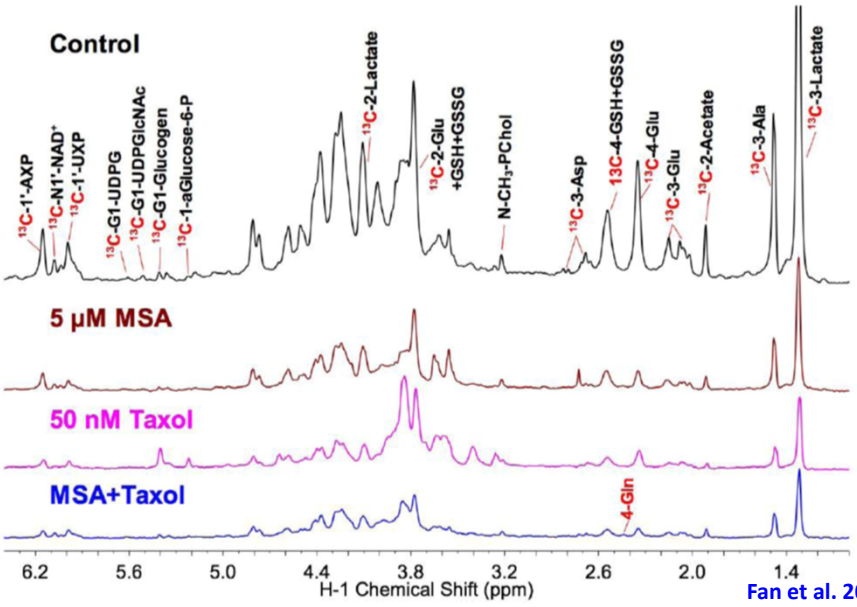


High resolution FT-ICR-MS



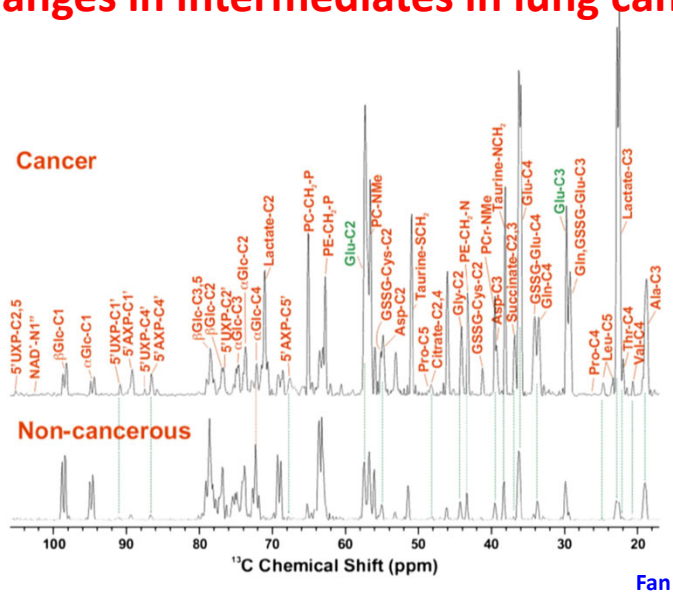
Fan et al. 2013

Use of ^{1}H-^{13}C-NMR



Fan et al. 2013

Changes in intermediates in lung cancer

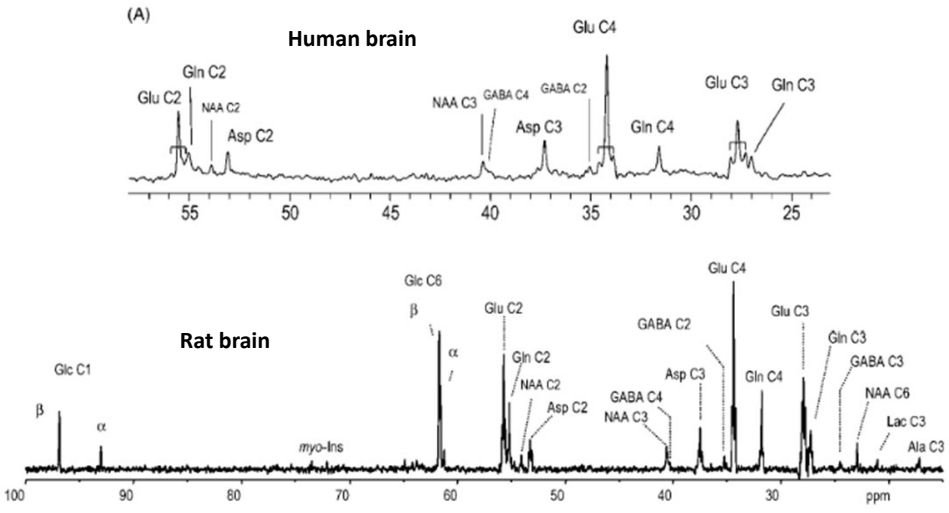


Fan et al. 2013

Biological NMR

- If ^{13}C-labeled precursors are used, there is a very much enhanced set of ^{13}C NMR resonances
- You have a choice between analysis of a biological extract (have all the time you need)
- And direct analysis in tissue:
  - Surface coil technology in the living animal
  - Magic Angle Spinning on a piece of tissue

NMR analysis of metabolites from ^{13}C-labeled precursors using pulse sequences



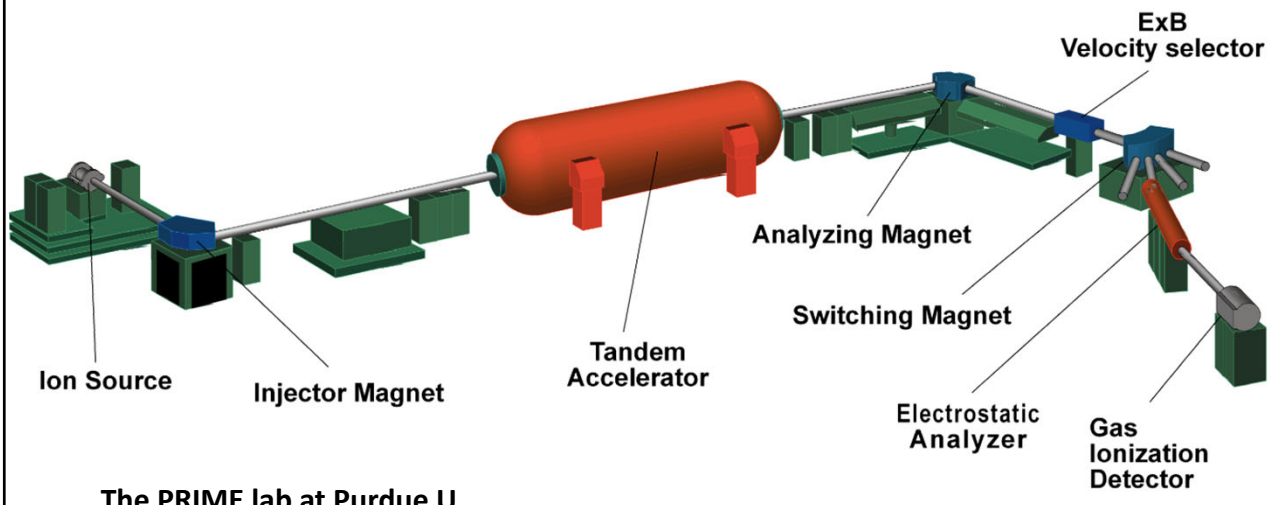
Probing the depths of metabolite penetration into tissues

Ultimate sensitivity by sacrificing metabolite identity at physiologic sites by ^{14}C-labeling the precursor of interest

Technology the same as the one used for radiocarbon dating

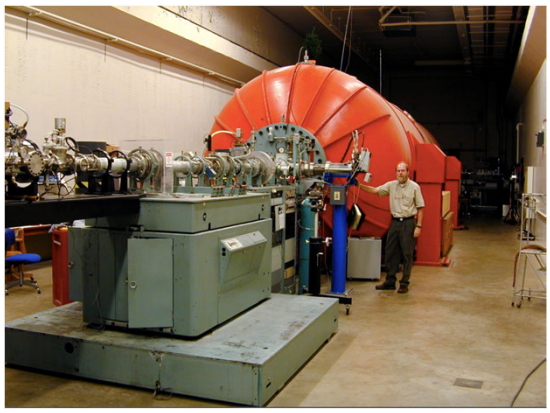
Accelerator mass spectrometry (AMS)

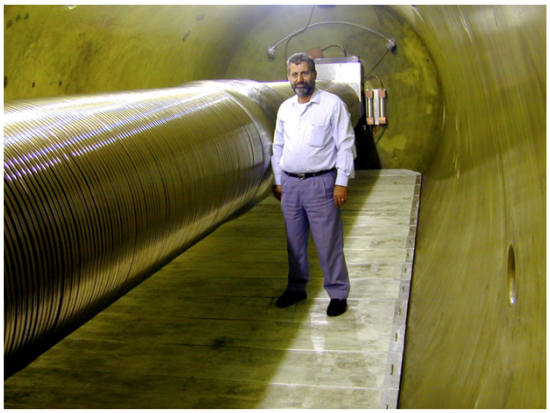
The ultimate mass spectrometer

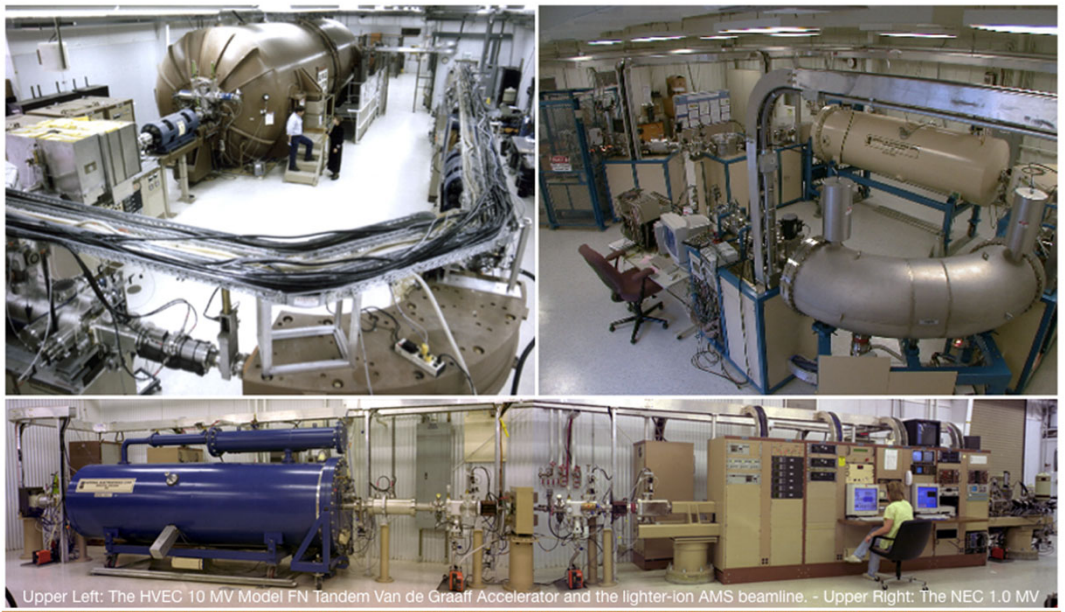


The PRIME lab at Purdue U

The Van der Graaf accelerator – PRIME lab



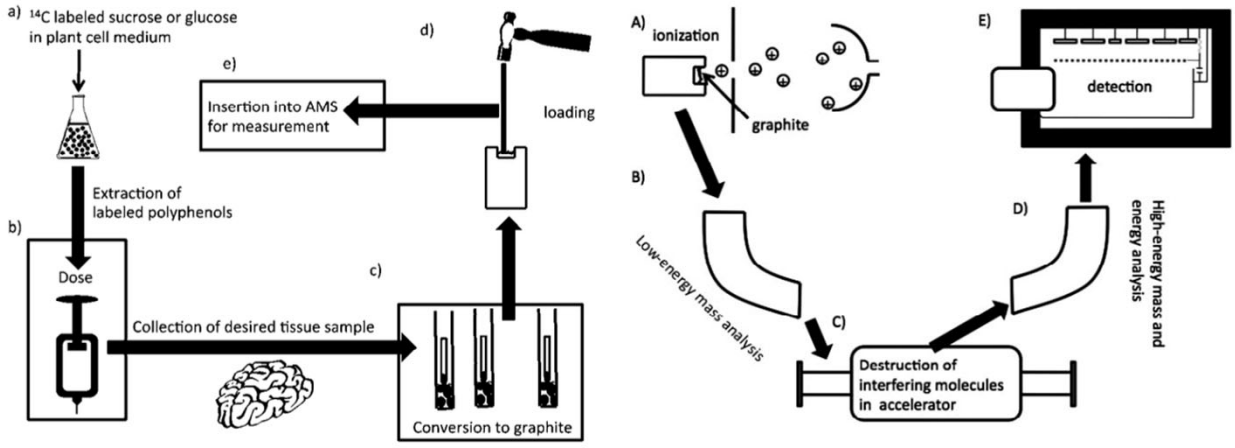




Upper Left: The HVEC 10 MV Model FN Tandem Van de Graaff Accelerator and the lighter-ion AMS beamline. - Upper Right: The NEC 1.0 MV Model 3SDH-1 Tandem Accelerator for BioAMS. - Lower: The NEC 1.7 MV Model 5SDH-2 Tandem Accelerator for Ion Beam Analysis and neutron production.

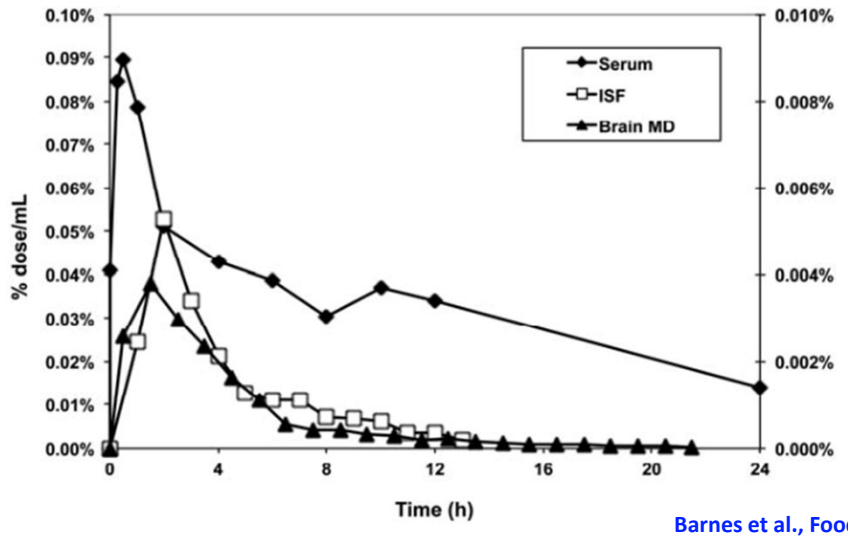
The AMS facility at the Lawrence Livermore National Laboratory

Tracing the appearance of a ^{14}C-labeled precursor



Barnes et al., Food Funct 2011

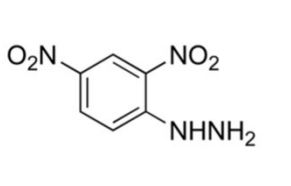
Tracing the movement of ^{14}C-intermediate in tissues



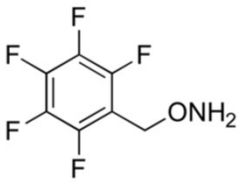
Barnes et al., Food Funct 2011

Using chemical reagents in metabolomics

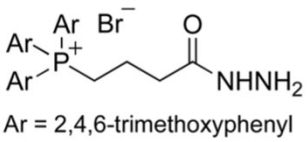
Carbonyl derivatization reagents



DNPH

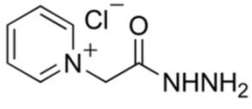


PFBHA

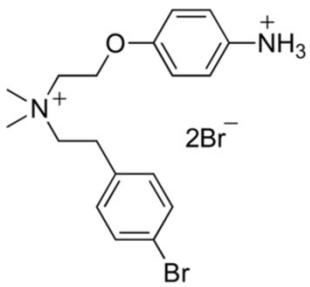


Ar = 2,4,6-trimethoxyphenyl

TMPP-PrG

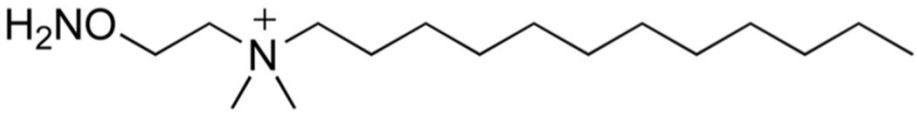


Girard-P reagent

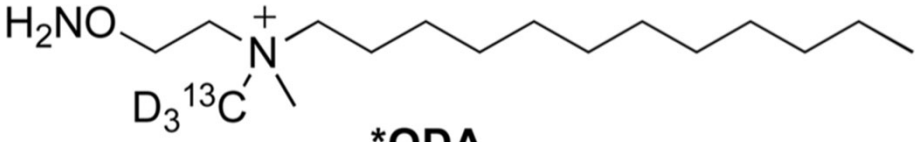


4-APEBA

Isotopic carbonyl reagents

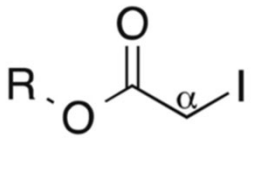


QDA

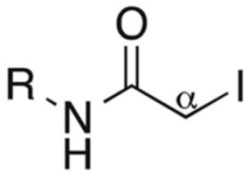


***QDA**

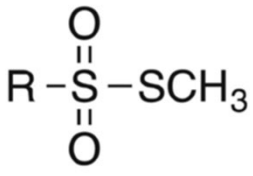
Thiol derivatization reagents



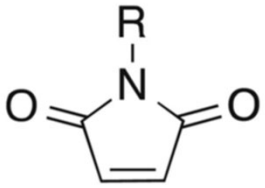
IAA



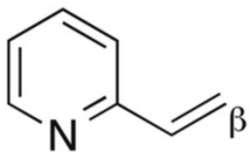
IAM



R = CH_3, **MMTS**

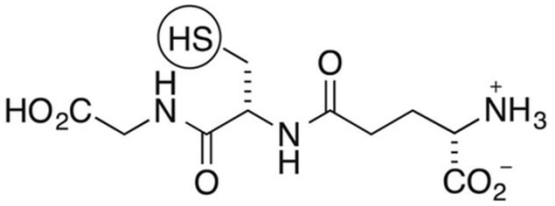


R = CH_3CH_2, **NEM**

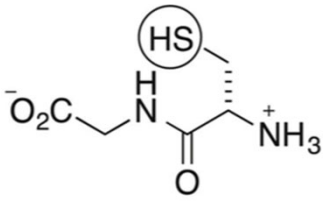


VP

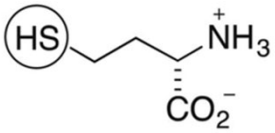
Detectable thio-metabolites



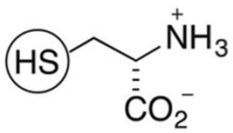
L-glutathione



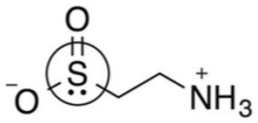
L-cysteinylglycine



L-homocysteine

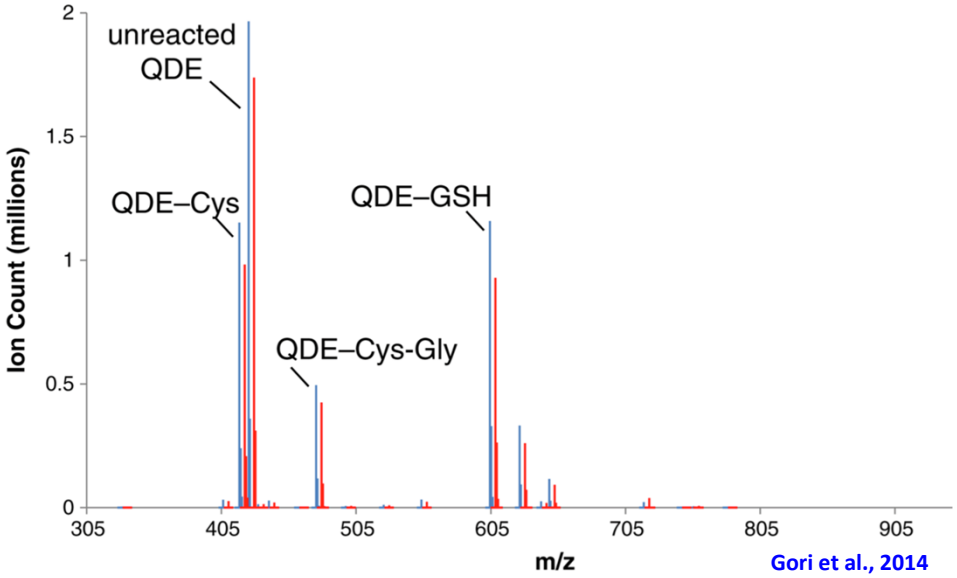


L-cysteine



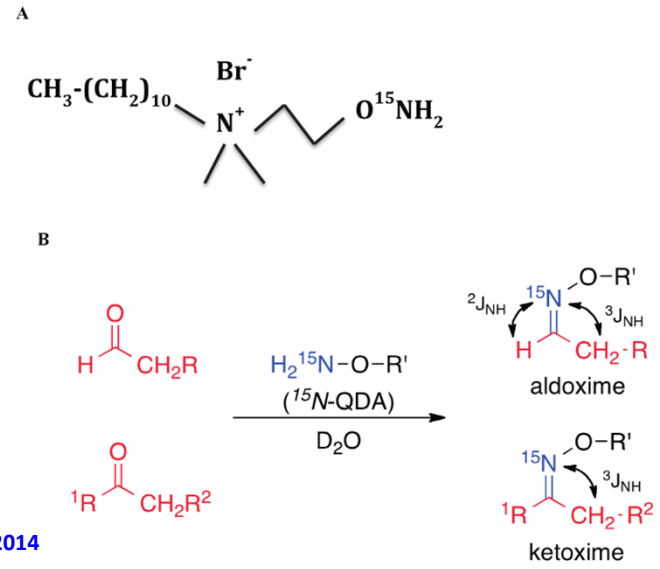
hypotaurine

Thiol metabolites in A459 cell extract

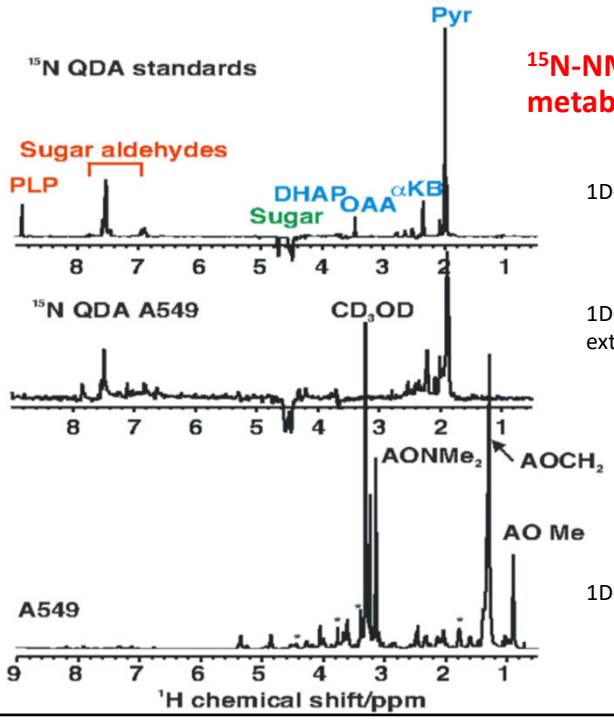


Gori et al., 2014

^{15}N-labeled derivatization reagent



Lane et al., 2014



^{15}N-NMR of derivatized metabolites

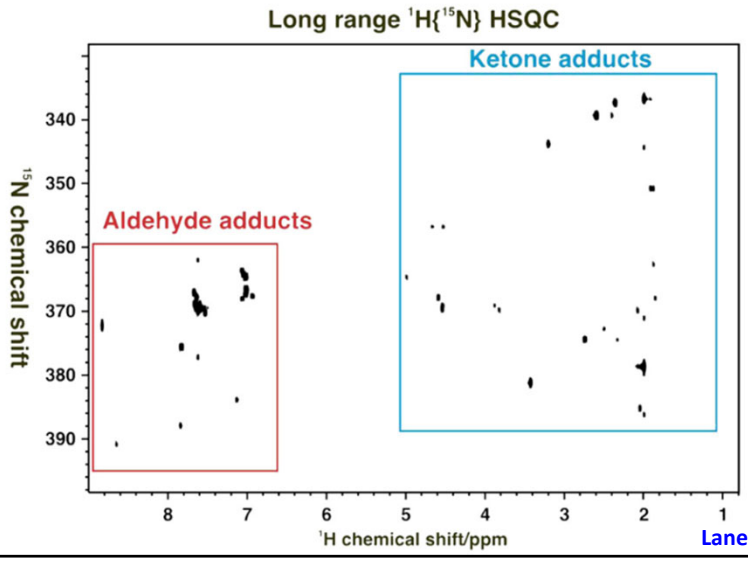
1D-HSQC spectrum of stds

1D-HSQC spectrum of polar extract of A549 cells

1D-presat spectrum of A549 cells

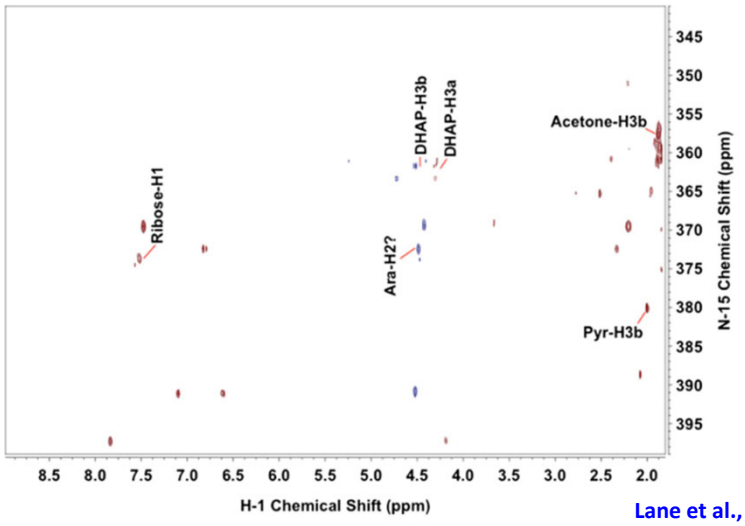
Lane et al., 2014

2D-^{1}H, ^{15}N-NMR of standards



Lane et al., 2014

2D-^{1}H, ^{15}N-NMR of A459 cell extract



Lane et al., 2014

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