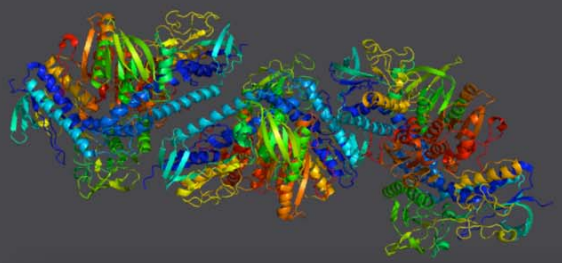
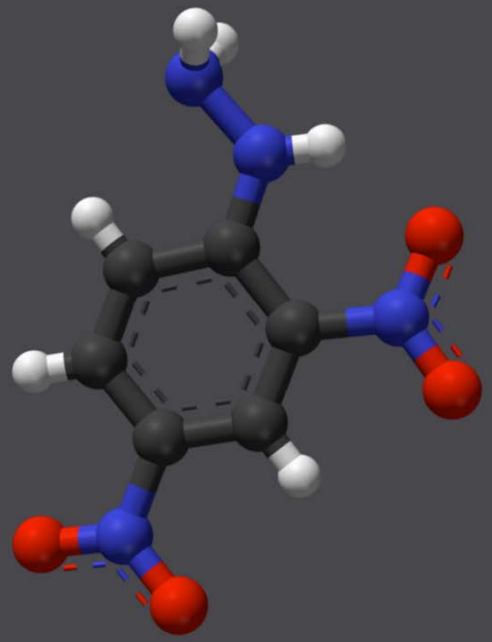
Removal of Unreacted Dinitrophenyl Hydrazine from Carbonyl Derivatives





Amira Barkal
Mentored by Dr. Gary Merrill
Biochemistry and Biophysics

Carbonyls and Disease

Alzheimer's Disease

- Degenerative brain disease
  - Irreversible tissue damage
- 7th leading cause of death among Americans
  - Affects 5.1 million Americans

- Cytotoxic carbonyls in higher amounts in Alzheimer's patients[1]

↓

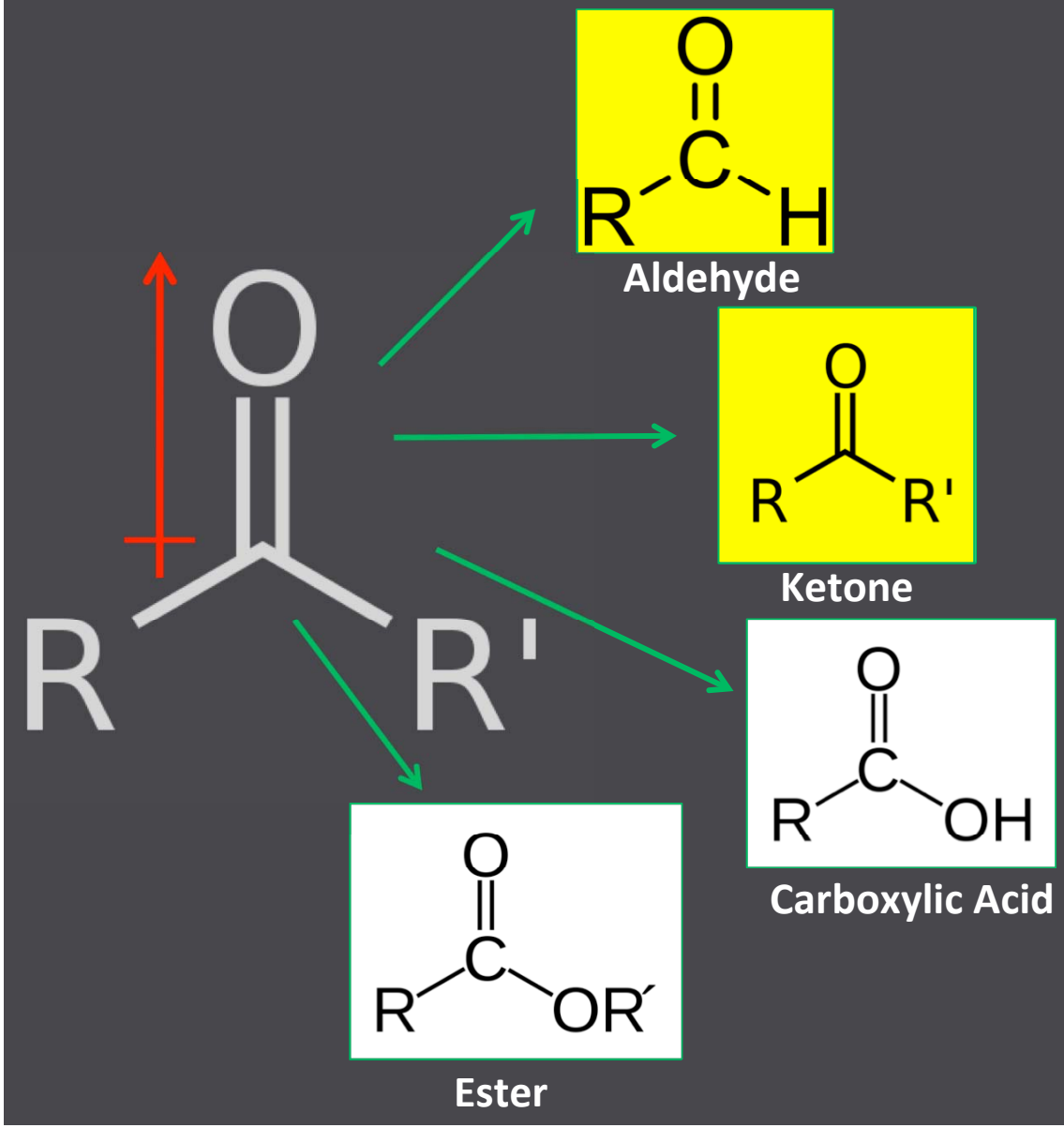
Oxidative stress

↓

Tissue damage

[1]Toxicol Appl Pharmacol 2002 Nov 1;184(3):187-97.

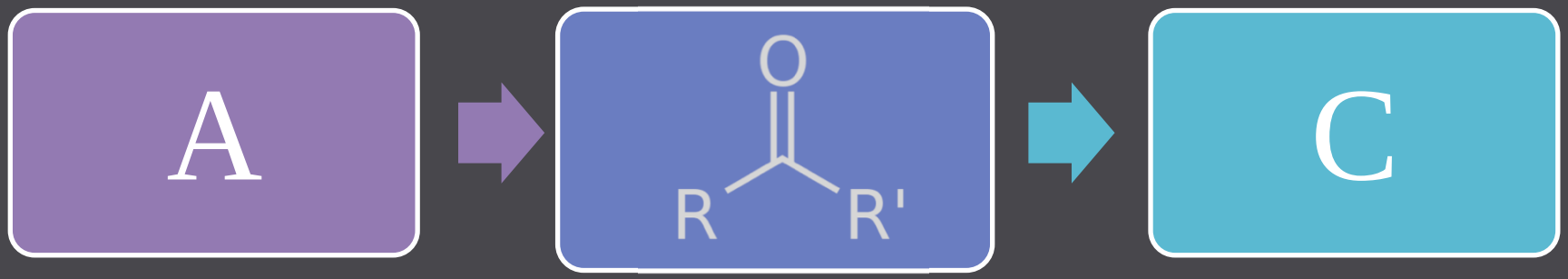
Carbonyls: Definition



- **Reactive**
- **Only aldehyde and ketone compounds**
  - **Not** esters in triglycerides
  - **Not** carboxylic acids in amino acids

Carbonyl Function

- Reactive intermediates

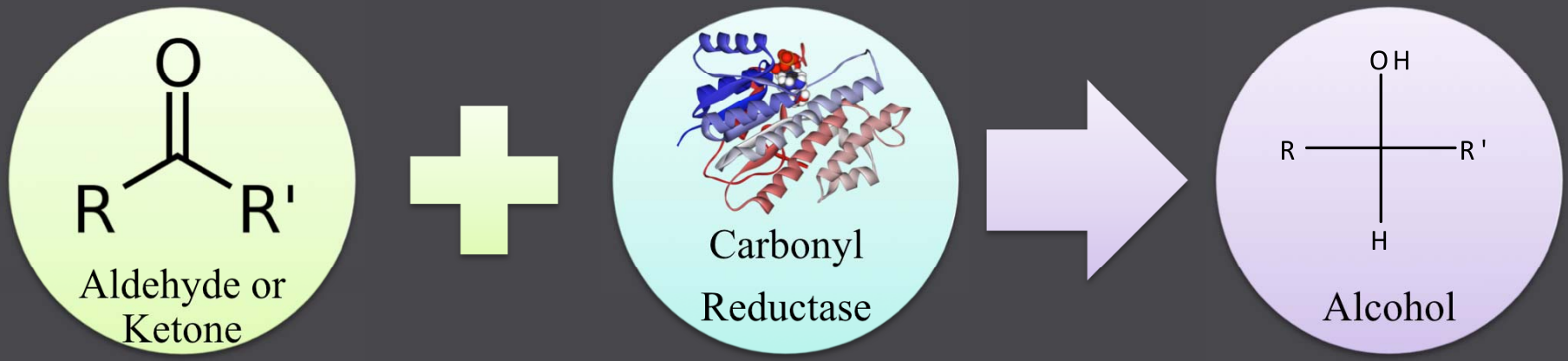


- Easy for the reaction to proceed

How do organisms control these carbonyl intermediates to prevent damage?

Carbonyl Regulation

- Normally in very small amounts
  - 0.1 % of the cytosol
- Reducing agents
  - Carbonyl reductases



Reactive

More stable

Studying Carbonyls

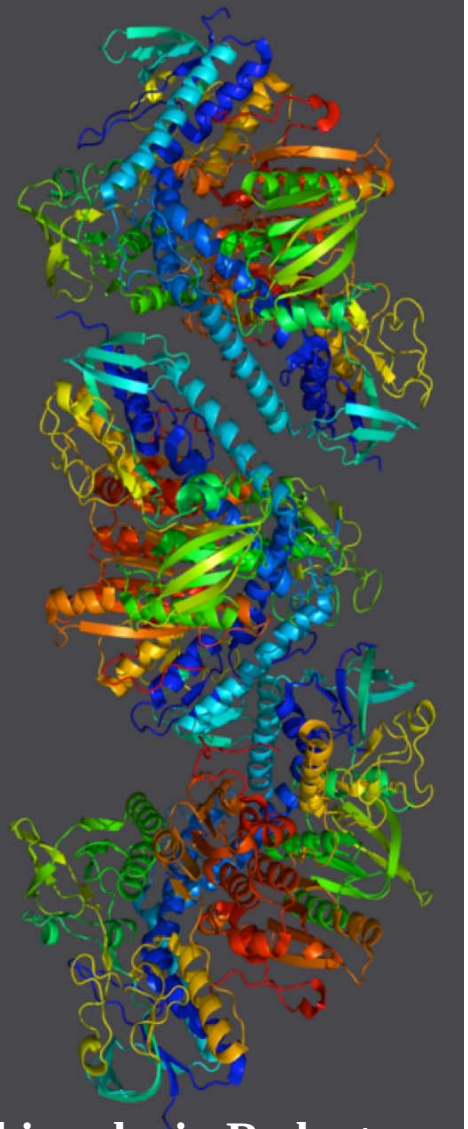
- Very hard to study
  - Small amounts
  - Reactive

- Many carbonyl metabalomics pathways undiscovered

- Not a determined protocol to study carbonyls from tissues

Merrill Lab and Carbonyls

- **Thioredoxin reductase (TR1)**
  - **Reduces thioredoxin**
  - **Part of thioredoxin pathway**
    - **Reduces disulfide bonds**
- **Discovered intrinsic carbonyl reducing capability**
  - ***In vitro* analysis[2]**



Thioredoxin Reductase

[2]*In vitro* analysis performed by Cameron Long in Merrill Lab

Merrill Lab and Carbonyls

- **Studies mice lacking TR1 in liver**

VS.

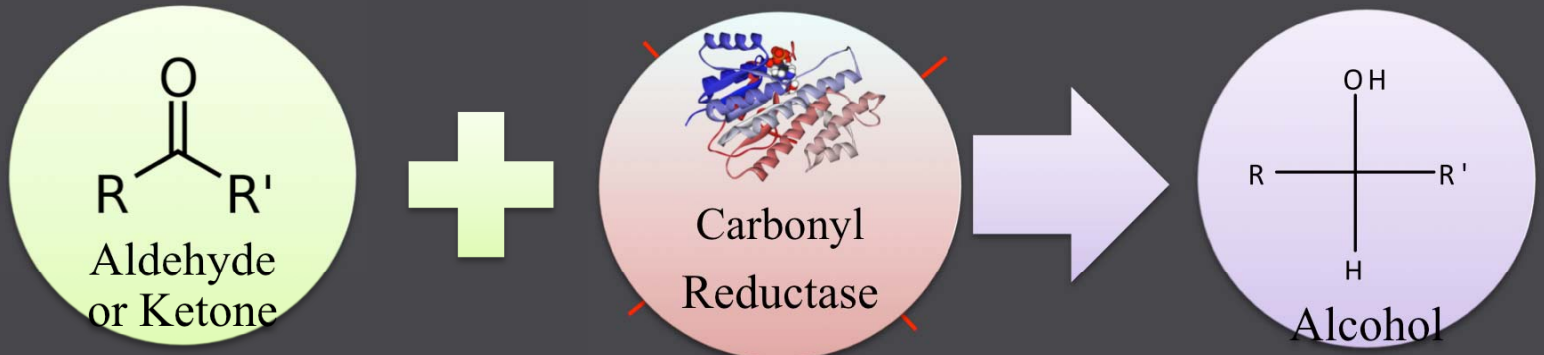
- Wildtype TR levels
- Wildtype carbonyl reductase 3 (Cbr3) mRNA levels

- No TR in livers
- 60-fold higher levels Cbr3 mRNA levels

Merrill Lab and Carbonyls

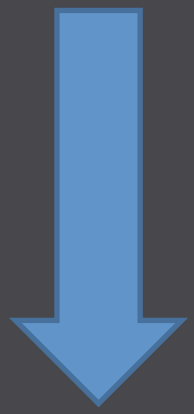
Wildtype— +TR

Mutant— -TR



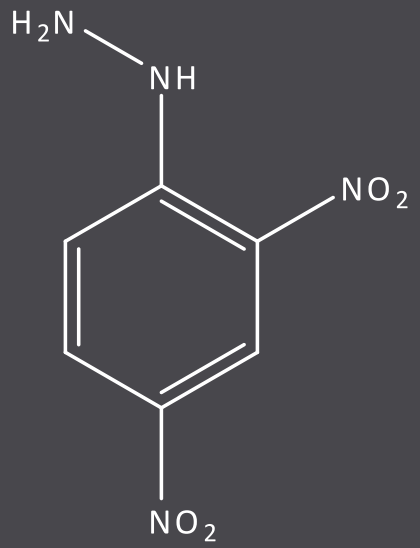
Goal

- Identify carbonyl compounds that accumulate in mutant mice because TR1 is missing

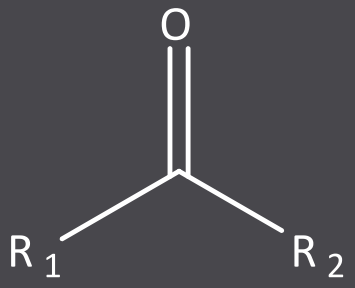


- Determine a general method by which carbonyls can be collected and identified from tissues

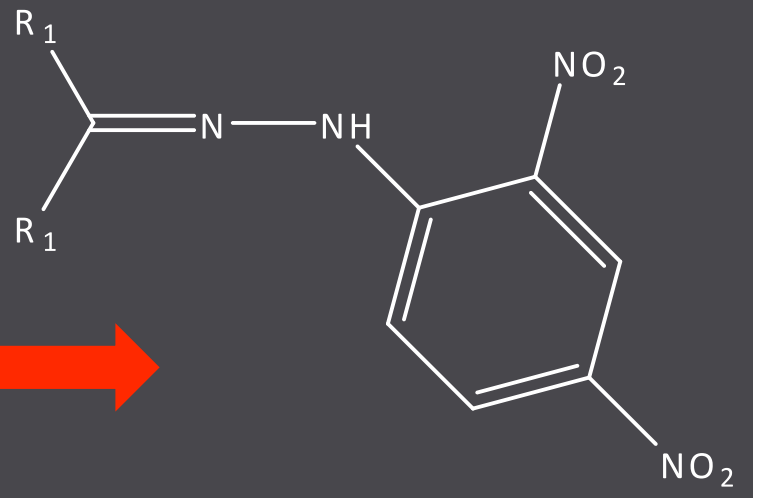
2,4-Dinitrophenylhydrazine (DNPH)



2,4-Dinitrophenylhydrazine



Aldehyde or Ketone



Stable Hydrazone

- Acidic conditions—1 M HCl
- Must be used in great excess with tissues to ensure all carbonyls react

Experimental Protocol

Wildtype or Mutant Liver

Flash freeze

Pulverize under liquid nitrogen

Methanol extract

Centrifuge

Methanol Insoluble Material

Methanol Soluble Material

Treat with excess DNPH

Spin

Soluble Hydrazones

Insoluble Hydrazones

Remove Excess DNPH

Resolubilize in New Solvent

Mass Spectrometry Analysis

Removing Unreacted DNPH

Why?

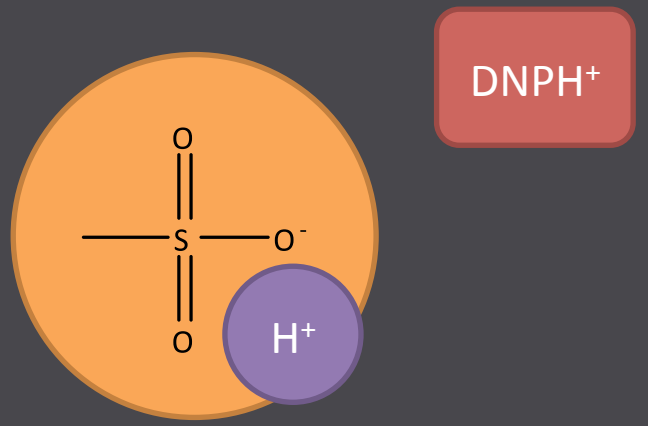
- Excess DNPH complicates mass spectrometry results because DNPH breakdown products would be identical to hydrazone breakdown products

Hypothesis: A resin treatment of soluble hydrazones will bind unreacted DNPH and allow the passage of hydrazones.

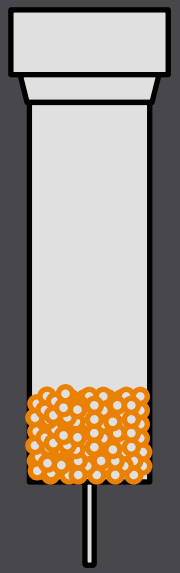
Dowex 50-X8 Ion Exchange Resin

- Research[3] indicates DNPH binds to ion exchange resins
  - Acidic conditions—DNPH positively charged

- Resin has sulfonates on bead with H^+ bound
  - Resin exchanges DNPH cation for H^+



Dowex 50-X8 Resin Bead



[3] *Journal of Chromatography*, 626 (1992) 284-288
Elsevier Science Publishers B.V., Amsterdam

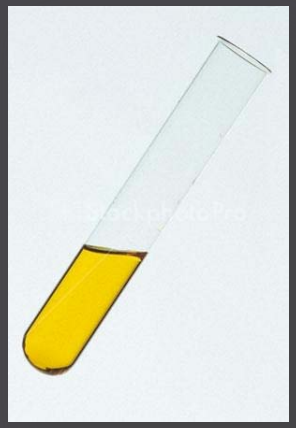
Criteria for Resin Removal Technique

1. Must bind DNPH under acidic conditions.

2. Must not bind hydrazones under acidic conditions.

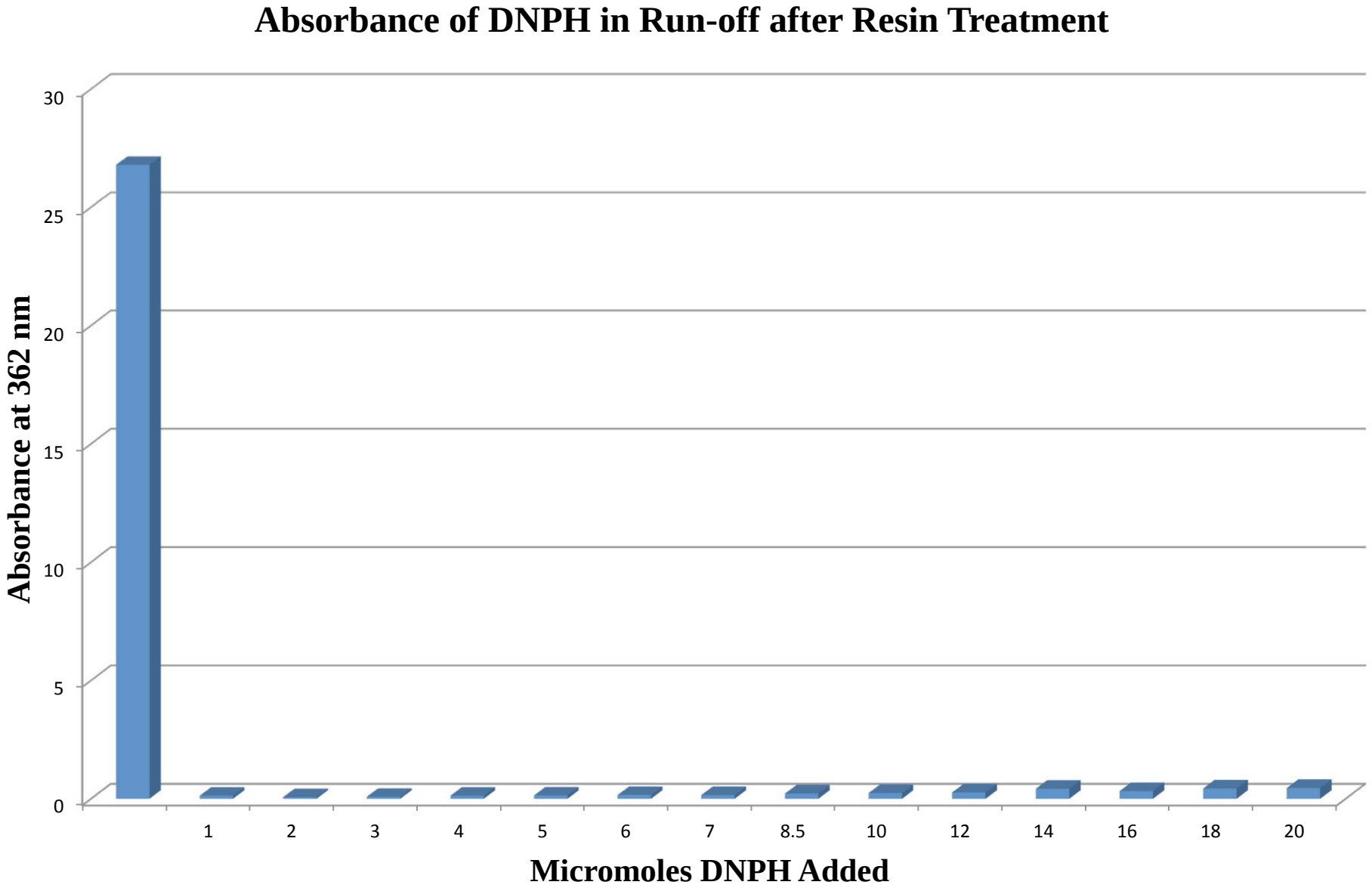
Resin Binding DNPH

- Testing binding affinity
  - 2.5 mM solution of DNPH in EtOH and 1 M HCl
  - Passed through column of 0.5 mL activated Dowex resin
  - DNPH is yellow
    - Peak absorbance at 362 nm



- Volumes (mL) of 2.5 mM DNPH solution passed through resin
  - Absorbance of run-off measured at 362 nm in spectrophotometer

Dowex Resin DNPH Binding Affinity



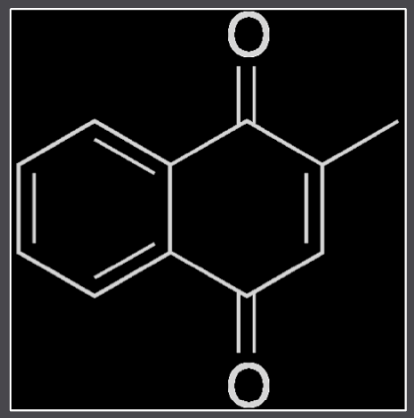
Criteria for Resin Removal Technique

1. Must bind DNPH under acidic conditions.

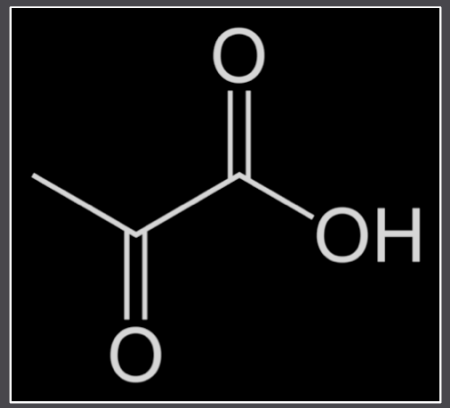
2. Must not bind hydrazones under acidic conditions.

Resin Binding of Hydrazone

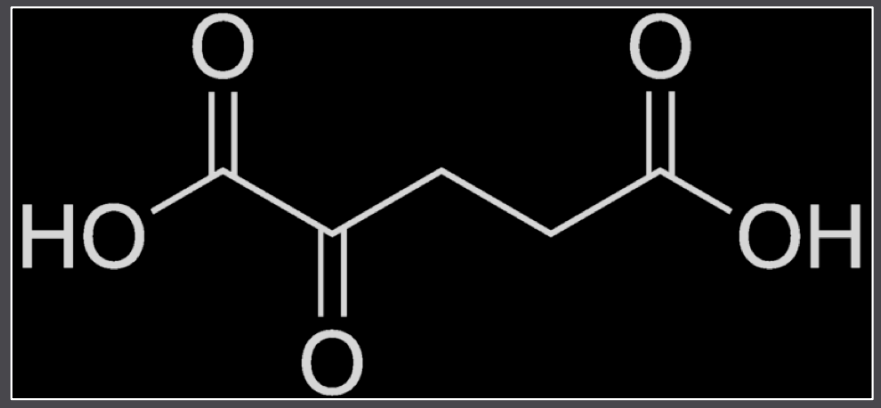
- Test the resin binding affinity for hydrazone
- Hydrazones of three model compounds formed and resolubulized in respective solvents



Menadione



Pyruvate



α-Ketoglutarate

Resin Binding of Hydrazone

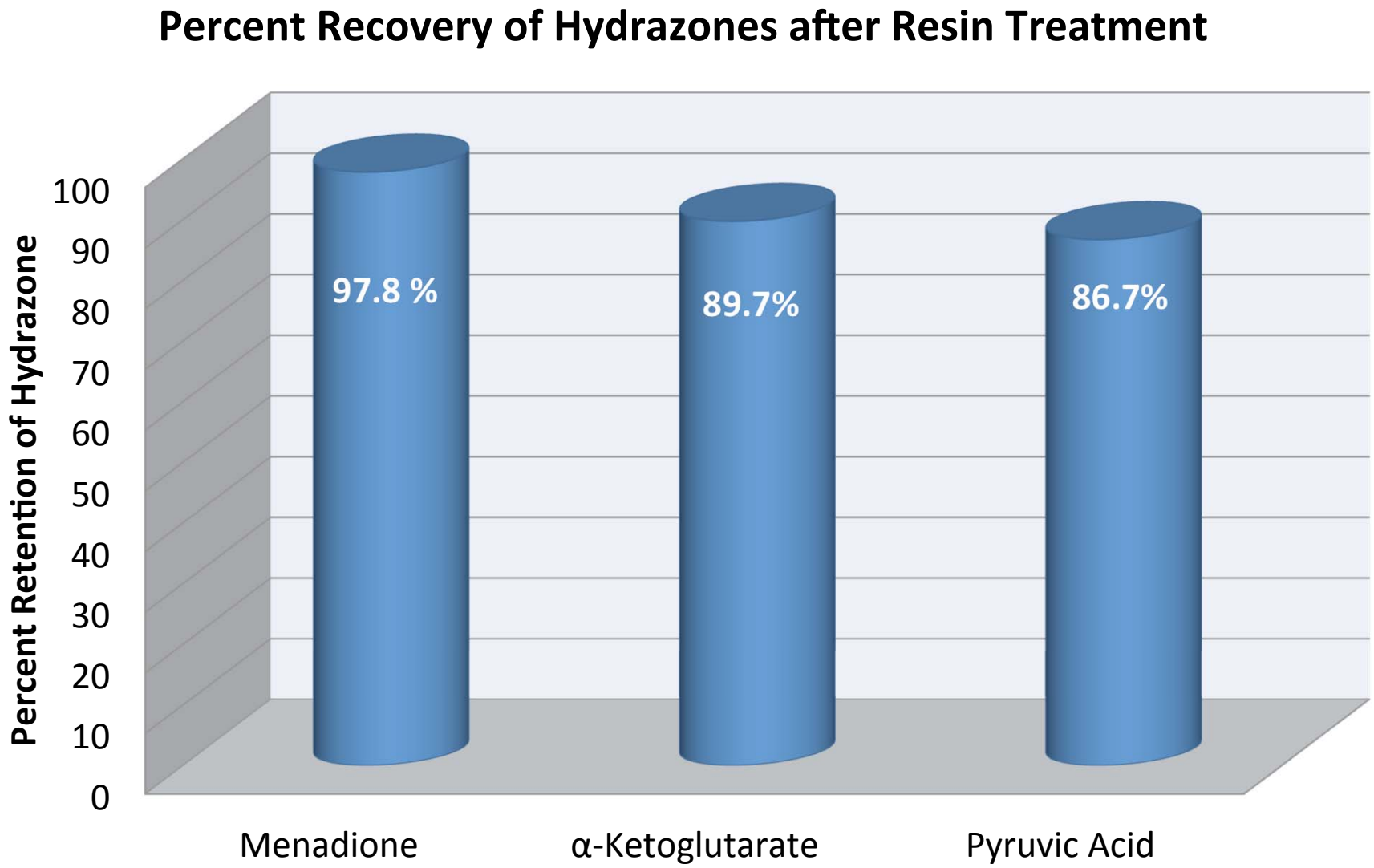
Hydrazone Staining:

- Hydrazones turn a deep blue or brown in the presence of base (a full volume of 2 M NaOH)

- Hydrazones were stained with 2 M NaOH, and absorbances of 1:40 dilutions measured at peak wavelengths before and after resin treatment
  - **Percent recovery** was calculated

Hydrazone	Color in Presence of Base	Peak Wavelength
Menadione	Blue	592 nm
Pyruvic Acid	Brown	395 nm
α-Ketoglutarate	Brown	419 nm

Recovery of Hydrazones after Resin Treatment



Criteria for Resin Removal Technique

1. Must bind DNPH under acidic conditions.

2. Must not bind hydrazones under acidic conditions.

Conclusions and Looking Ahead

- A viable solution for the removal of unreacted DNPH was determined
- Mass spectrometry results obtained explained by Thi Nguyen
- The method for harvesting carbonyls from mutant and wildtype livers is still being tested

Looking ahead to:

- identifying a piece of the thioredoxin reductase pathway.
- determining best method for measuring carbonyl levels and identities from tissues.

Acknowledgements

HHMI

Cripps Undergraduate Research

URISC

Dr. Gary Merrill

Dr. Fred Stevens

Thi Nguyen

The Mass Spectrometry Lab

Dr. Kevin Ahern