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THE ROLE OF OXIDANT STRESS AND GENDER IN THE ERYTHROCYTES ARGININE METABOLISM AND AMMONIA MANAGEMENT IN PATIENTS WITH TYPE 2 DIABETES

PLOS One

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1 Works for me dx.doi.org/10.17504/protocols.io.q5qdy5w

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ABSTRACT

The erythrocytes are not simply gas transporting cells, but they have a very important role in the regulation and transport of metabolites related to nitrogen metabolism both in healthy subjects and in patients with type 2 DM. The different levels of metabolites are associated with gender differences.

We have two particular objectives: 1) To study the differences in the levels of nitrogen metabolites, such ammonium and nitric oxide, and the correlations that exist between them in RBC and serum, in healthy subjects and patients with type 2 DM. And 2) to determine the differences in the levels of nitrogen metabolites, such ammonium and nitric oxide, and the correlations that exist between them in relation to gender in healthy subjects and patients with type 2 DM.

This cross-sectional study included 80 patients diagnosed with type 2 DM (40 female and 40 male patients) and their corresponding controls paired by gender (n = 80). We separated serum and red blood cells (RBC) and determined metabolites mainly through colorimetric and spectrophotometric assays. We evaluated changes in the levels and correlations of the main catabolic by-products of blood nitrogen metabolism, as ammonium, nitric oxide (NO), malondialdehyde (MDA), among others.

Healthy female and male controls showed a differential blood metabolites distribution involved in arginine metabolism, as ornithine, ammonium, urea and . NO. Patients with DM had increased ammonia, citrulline, urea, uric acid, and ornithine, mainly in the RBC, while the level of arginine was significantly lower in men with type 2 DM. These findings were associated with hyperglycemia, glycosylated hemoglobin (Hb A_{1c}), as well as with levels of RBC's MDA. Most of the DM-induced alterations in nitrogen-related metabolites seem to be associated with a different RBC capacity for the release of these metabolites and resulting in an abrogation of the gender-related differential management of nitrogen metabolites in healthy subjects.

This study provides the first documented findings giving strong evidence of a putative role of RBC as an extra-hepatic mechanism for controlling serum levels of nitrogen-related metabolites, which differs according to gender in healthy subjects. Type 2 DM promotes higher ammonia, citrulline, and MDA blood levels, which culminate in a loss of the differential management of nitrogen-related metabolites seen in healthy women and men and a characteristic pattern of metabolic disturbances that culminates in a loss of the differential management of nitrogen-related metabolites seen in healthy women and men. Therefore, it is not unlikely that these characteristic patterns of blood metabolites elicited by type 2 DM might be involved in the specific physiopathology of this disease, and changes in the oxidative status, i.e., increased lipid peroxidation in RBC membranes from diabetic patients, are playing a role in the metabolic alterations found in these blood cells.

EXTERNAL LINK

<https://doi.org/10.1371/journal.pone.0219481>

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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MATERIALS

NAME ▾

CATALOG # ▾

VENDOR ▾

| NAME ▾ | CATALOG # ▾ | VENDOR ▾ |
|---|---------------|-----------------|
| Citrulline | C7629 | Sigma – Aldrich |
| Arginine | A5006 | Sigma – Aldrich |
| Urea | U5378 | Sigma – Aldrich |
| HEPES | RDD002 | Sigma – Aldrich |
| Uric | U2625 | Sigma – Aldrich |
| Glucose | 158968 | Sigma – Aldrich |
| Potassium dihydrogen phosphate | NIST200B | Sigma – Aldrich |
| Monobasic potassium phosphate | PHR1330 | Sigma – Aldrich |
| Sodium bicarbonate | S6014 | Sigma – Aldrich |
| Sodium carbonate | 223484 | Sigma – Aldrich |
| Sodium barbiturate | 11715-100G | Sigma – Aldrich |
| Sodium chloride | 746398 | Sigma – Aldrich |
| Perchloric acid | 244252 | Sigma – Aldrich |
| Potassium nitrite | 310484 | Sigma – Aldrich |
| Potassium nitrate | 1548349 | Sigma – Aldrich |
| Tris base | T1503 | Sigma – Aldrich |
| Hydrochloric acid | 320331-500ML | Sigma – Aldrich |
| Ninhydrin | 151173 | Sigma – Aldrich |
| Sulfuric acid | 258105 | Sigma – Aldrich |
| Acetic acid | 320099-6X2.5L | Sigma – Aldrich |
| alpha-Ketoglutaric acid sodium salt | K1875 | Sigma – Aldrich |
| Adenosine 5-diphosphate sodium salt | A2754 | Sigma – Aldrich |
| Tetraethyl ammonium chloride monohydrate | 86605 | Sigma – Aldrich |
| Beta-nicotinamide adenine dinucleotide, reduced disodium salt hydrate | N8129 | Sigma – Aldrich |
| Glutamate dehydrogenase (Gldh) | 10197734001 | Sigma – Aldrich |
| Trichloroacetic acid (TCA) | T6399 | Sigma – Aldrich |
| Potassium bicarbonate | 237205 | Sigma – Aldrich |
| Succinic acid | 398055 | Sigma – Aldrich |
| Urease from Canavalia ensiformis (Jack bean) | U7752 | Sigma – Aldrich |
| Ninhydrin | N4876 | Sigma – Aldrich |
| Sodium hydroxide | S8045 | Sigma – Aldrich |
| Phenazone | P0800000 | Sigma – Aldrich |
| 2,3-Butanedione monoxime | 31550 | Sigma – Aldrich |
| N-(1-Naphthyl)ethylenediamine dihydrochloride | 222488 | Sigma – Aldrich |

| NAME ▾ | CATALOG # ▾ | VENDOR ▾ |
|--|-------------|-----------------|
| Hemoglobin human | H7379 | Sigma – Aldrich |
| Potassium ferricyanide (III) | 702587 | Sigma – Aldrich |
| Potassium phosphohate dibasic | 60356 | Sigma – Aldrich |
| Phosphoric acid | 7664-38-2 | Meyer |
| Acetic Acid, Glacial | 0040 | Meyer |
| Potassium cyanide | 207810 | Sigma – Aldrich |
| 1,1,3,3-Tetraethoxypropane | 9889 | Sigma – Aldrich |
| Copper (II) sulfate pentahydrate | 209198 | Sigma – Aldrich |
| Folin & Ciocalteus phenol | F9253 | Sigma – Aldrich |
| Sulfanilamide | S9251 | Sigma – Aldrich |
| Sodium nitrite | 237213 | Sigma – Aldrich |
| Potassium sodium tartrate tetrahydrate | 217255 | Sigma – Aldrich |
| Ethanol absolute | 13225483 | JT Baker |
| Bovine serum albumin | A7906 | Sigma – Aldrich |
| Manganese (II) chloride tetrahydrate | 221279 | Sigma – Aldrich |
| L-Ornithine dihydrochloride | 75440 | Sigma – Aldrich |
| Pyridine | 360570 | Sigma – Aldrich |
| Butyl alcohol | W217808 | Sigma – Aldrich |
| Potassium Chloride | 746436 | Sigma – Aldrich |
| Potassium hydroxide | P1767 | Sigma – Aldrich |
| 2-Thiobarbituric acid | | Sigma – Aldrich |
| Ammonium chloride | 254134 | Sigma – Aldrich |
| Triethanolamine | 90279 | Sigma – Aldrich |
| L-Ornithine monohydrochloride | 02375 | Sigma – Aldrich |
| Iron (III) sulfate hydrate | 307718 | Sigma – Aldrich |
| Sodium nitrate | S5506 | Sigma – Aldrich |

STEPS MATERIALS

| NAME ▾ | CATALOG # ▾ | VENDOR ▾ |
|-----------------------|------------------|-----------------|
| Perchloric acid | 244252 | Sigma – Aldrich |
| Sodium hydroxide | SB0617.SIZE.500G | Bio Basic Inc. |
| Potassium hydroxide | P1767 | Sigma – Aldrich |
| 2-Thiobarbituric acid | | Sigma – Aldrich |
| Perchloric acid | 244252 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |

| NAME ▾ | CATALOG # ▾ | VENDOR ▾ |
|---|-------------|-----------------|
| Potassium hydroxide | P1767 | Sigma – Aldrich |
| 2-Thiobarbituric acid | | Sigma – Aldrich |
| 1,1,3,3-Tetraethoxypropane | 9889 | Sigma – Aldrich |
| Manganese (II) chloride tetrahydrate | 221279 | Sigma – Aldrich |
| Sodium carbonate | 222321 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| Ninhydrin | N4876 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| Phosphoric acid | 7664-38-2 | Meyer |
| Manganese (II) chloride tetrahydrate | 221279 | Sigma – Aldrich |
| Sodium carbonate | 223484 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| Ninhydrin | N4876 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| Phosphoric acid | 7664-38-2 | Meyer |
| Potassium ferricyanide (III) | 702587 | Sigma – Aldrich |
| Potassium cyanide | 207810 | Sigma – Aldrich |
| Monobasic potassium phosphate | PHR1330 | Sigma – Aldrich |
| Sodium carbonate | 223484 | Sigma – Aldrich |
| Copper (II) sulfate pentahydrate | 209198 | Sigma – Aldrich |
| Potassium sodium tartrate tetrahydrate | 217255 | Sigma – Aldrich |
| Folin & Ciocalteus phenol | F9253 | Sigma – Aldrich |
| Bovine Serum Albumin (BSA) | A7906 | Sigma Aldrich |
| Adenosine 5-diphosphate sodium salt | A2754 | Sigma – Aldrich |
| alpha-Ketoglutaric acid sodium salt | K1875 | Sigma – Aldrich |
| Beta-nicotinamide adenine dinucleotide, reduced disodium salt hydrate | N8129 | Sigma – Aldrich |
| Triethanolamine | 90279 | Sigma – Aldrich |
| Glutamate dehydrogenase (Gldh) | 10197734001 | Sigma – Aldrich |
| Triethanolamine | 90279 | Sigma – Aldrich |
| Tris base | T1503 | Sigma – Aldrich |
| Succinic acid | 398055 | Sigma – Aldrich |
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| NAME ▾ | CATALOG # ▾ | VENDOR ▾ |
|---|-------------|-----------------|
| Urease from Canavalia ensiformis (Jack bean) | U7752 | Sigma – Aldrich |
| Ammonium chloride | 254134 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| Ninhydrin | N4876 | Sigma – Aldrich |
| Phosphoric acid | 7664-38-2 | Meyer |
| Ethanol absolute | 13225483 | JT Baker |
| L-Ornithine monohydrochloride | O2375 | Sigma – Aldrich |
| Sulfuric acid | 258105 | Sigma – Aldrich |
| Iron (III) sulfate hydrate | 307718 | Sigma – Aldrich |
| 2,3-Butanedione monoxime | 31550 | Sigma – Aldrich |
| Phenazone | P0800000 | Sigma – Aldrich |
| Acetic Acid, Glacial | 0040 | Meyer |
| N-(1-Naphthyl)ethylenediamine dihydrochloride | 222488 | Sigma – Aldrich |
| Sulfanilamide | S9251 | Sigma – Aldrich |
| Phosphoric acid | 7664-38-2 | Meyer |
| Sodium nitrate | S5506 | Sigma – Aldrich |

Biochemical Measurements

- Whole blood was poured directly into ice-cold perchloric acid (8% w/v, final concentration). After centrifugation, we obtained perchloric acid-extracts of serum and RBC.
 - Whole blood was centrifugated to separate RBC and serum samples

[M] 8 Mass/Volume Percent



We extract proteins from blood



Perchloric acid

by Sigma – Aldrich

Catalog #: 244252



Be carefull, avoid contact with skin, eyes and do not inhale

4 °C on ice

- The perchloric acid-extracts obtained in the previous step were neutralized: we added sodium hydroxide until we reach a pH of 7. These preparations were used to determine the following metabolites: ammonia, urea, arginine, citrulline, ornithine, nitrites, malondialdehyde (MDA), uric acid.



Sodium hydroxide

by Bio Basic Inc.

Catalog #: SB0617.SIZE.500G

- 3 For the determination of glucose, insulin, HbA1C, triglycerides, cholesterol, alanina aminotransferasa (ALT), and aspartato aminotransferasa (AST), we only used serum samples (1b).

Glucose determination

- 4 Glucose was determined in serum samples with the glucose oxidase reaction using the Glucosa-LQ GOD-POD kit (SPINREACT). The color developed was read at 450 nm using Biotek instruments.



Biotek instruments ELx800

USA

Insulin determination

- 5 Insulin was determined in serum samples with the Human Insulin ELISA kit (ELH-Insulin-2 RayBio, USA). The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800

USA

HbA1C determination

- 6 HbA1C was determined in serum samples with the glycosylated HbA1c kit (BoiSys-Kovalent, Brazil; 4190045K R1 2). The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800

USA

Quantitative determination of triglycerides

- 7 Triglycerides were determined in serum samples with the GPO-POD enzymatic colorimetric kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800

USA

Uric acid determination

- 8 Uric acid was determined in serum samples with the Uricase-POD Enzymatic colorimetric kit (Spinreact, Spain). The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800
USA

Aspartate aminotransferease (AST) determination

- 9 AST was determined in serum samples with the NADH.Cinetic UV.IFCC kit (Spinreact, Spain).
The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800
USA

Alanine aminotransferase (ALT) determination

- 10 ALT was determined in serum samples with the NADH.Cinetic UV.IFCC kit (Spinreact, Spain).
The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800
USA

Cholesterol determination

- 11 Cholesterol was determined in serum samples with the CHOD-POD lit (Spinreact,Spain).
The color developed was read at 450 nm using Biotek instruments.



Biotek instruments EL x 800
USA

Malondialdehyde (MDA) determination (Hernandez-Munoz et al. (1984); Biochem Pharmacol 33(16); pp 2599-2604)

- 12 MDA was quantified by the thiobarbituric acid method using acid extracts from RBC and serum (6% perchloric acid w/v).
Samples of these extracts (0.5ml) were mixed with 1.5 ml of 20% acetic acid (adjusted to pH 2.5 with KOH) and with 1.5 ml of 8% thiobarbituric acid.


[M]8 Mass/Volume Percent 8% 2-thiobarbituric acid




Potassium hydroxide
by Sigma – Aldrich
Catalog #: P1767




2-Thiobarbituric acid
by Sigma – Aldrich




Perchloric acid
by Sigma – Aldrich
Catalog #: 244252



Acetic Acid, Glacial
by Meyer
Catalog #: 0040



Potassium hydroxide
by Sigma – Aldrich
Catalog #: P1767



2-Thiobarbituric acid
by Sigma – Aldrich

[M]6 Mass/Volume Percent 6% perchloric acid w/v

[M]20 Volume Percent 20% acetic acid

Malondialdehyde (MDA) determination

- 13 The samples were kept for 45 minutes a boiling water bath and 1 ml of 2% KCL was added at the end of the incubation to each sample.

 **00:45:00 boiling water bath**


[M]2 Mass/Volume Percent 2% KCL

 **1 ml of 2% KCL**


- 14 The color complex formed at the end of the incubation (in the last step) was extracted with the mix butanol-pyridin (15:1, v/v) and detected at 532 nm in a spectrophotometer (Beckman Coulter).

The extinction coefficient of malonaldehyde color complex was $1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$, as determined using a standard curve with tetraethoxypropane.

[M]15 Volume Percent mix butanol-pyridin (15:1, v/v)



Beckman Coulter DU 640
Spectrophotometer



1,1,3,3-Tetraethoxypropane
by Sigma – Aldrich
Catalog #: 9889

Arginine determination (Colombo, JP and Konarska L.; Arginase in Bergmeyer HU and Bergmeyer J, Grassi M., Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie; 1984)

- 15 Content of arginine was determined in neutralized perchloric extracts of RBC and serum as arginine converts to ornithine using rat liver cytosol-containing activated arginase.

Liver cytosol enzyme was activated by a 20 min-incubation with 10 mM Manganese chloride (MnCl_2) at 55 °C.

 **00:20:00 20 minutes incubation**

 **55 °C for incubation**

 **10 Molarity (m)** 10mM Manganese chloride (MnCl₂)



Manganese (II) chloride tetrahydrate

by [Sigma – Aldrich](#)

Catalog #: 221279

Arginine determination

16 To a sample of 0.5 ml acid-extract, 0.3 ml of sodium carbonates buffer (pH = 9.5) was added and the reaction started with 10 µg of cytosolic protein.

The mixture was incubated by 40 min at 37 °C, and stopped by addition of 1.5 ml of acetic acid.

 **37 °C incubation**


 **00:40:00 incubation**



Sodium carbonate

by [Sigma – Aldrich](#)

Catalog #: 222321

 **10 µg** 10 µg of cytosolic protein.



Acetic Acid, Glacial

by [Meyer](#)

Catalog #: 0040

17 The color resulting from the reaction was developed through a ninhydrin solution (140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v) in boiling water during 45 min.

The colored was detected at 515 nm, contrasting with a standard curve for ornithine (30, 50, 100, 200 500, 1000 nmoles in the reaction mixture).



Ninhydrin

by [Sigma – Aldrich](#)

Catalog #: N4876



Acetic Acid, Glacial

by [Meyer](#)

Catalog #: 0040



Phosphoric acid

by [Meyer](#)


Catalog #: 7664-38-2

 **140 Molarity (m)** 140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v

 **00:45:00**

- 18 Arginase was determined in RBC. Five mg of hemoglobin from RBC lysate were activated for arginase activity by a 20 min-incubation with 10 mM MnCl_2 .


 **00:20:00 incubation time**

 **Manganese (II) chloride tetrahydrate**
by [Sigma – Aldrich](#)
Catalog #: 221279


 **5 mg hemoglobin from RBC**

- 19 Samples were now incubated in 0.3 ml sodium carbonate buffer (pH = 9.5) by 10 min at 37 °C, and stopped by addition of 1.5 ml of acetic acid.


 **00:10:00 incubation**


 **Sodium carbonate**
by [Sigma – Aldrich](#)
Catalog #: 223484


 **37 °C incubation temperature**

 **Acetic Acid, Glacial**
by [Meyer](#)
Catalog #: 0040

- 20 The color was developed through a ninhydrin solution (140 mM dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v) in boiling water by 45 min.
The colored was detected at 515 nm in a spectrophotometer (Beckman Coulter), contrasting with a standard curve for ornithine (30,50, 100, 200 500, 1000 nmoles in the reaction mixture).
Activity expressed as nmoles·min⁻¹ ·mg of hemoglobin protein.

 **Ninhydrin**
by [Sigma – Aldrich](#)
Catalog #: N4876

 **Acetic Acid, Glacial**
by [Meyer](#)
Catalog #: 0040

 **Phosphoric acid**
by [Meyer](#)
Catalog #: 7664-38-2

 **00:45:00 duration in boiling water**

[M] **140 Molarity (m) mM** ninhydrin solution dissolved in acetic acid:6 M phosphoric acid, 6:4 v/v

[M] **6 Volume Percent v/v** acetic acid:6 M phosphoric acid



Beckman Coulter DU 640
spectrophotometer

Hb determination (Colombo, JP and Konarska L.; Arginase in Bergmeyer HU and Bergmeyer J, Grassi M., Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie; 1984)

- 21 Add 1ml of any type of sample (an extract, RBC or serum) 2 ml Drabkin solution.
Drabkin's solution:
Dissolve 200 mg $K_3[(CN)_6]$, 50 mg KCN, 140 mg KH_2PO_4 in 1000 ml water. We omitted the addition of antifoam because it is not available (no supply).
The sample was centrifugated when needed. In this case we measured the absorbance of the supernatant at 546 nm. When centrifugation was not needed, the sample was directly measured in the spectrophotometer (Beckman Coulter).
Calculate the concentration of hemoglobin (gr/l) with a hemoglobin cyanide-standard or using the molar absorbtion coefficient $E=44 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.
Molecular weight of Hb is 64 458.



Potassium ferricyanide (III)
by Sigma – Aldrich
Catalog #: 702587



Potassium cyanide
by Sigma – Aldrich
Catalog #: 207810



Monobasic potassium phosphate
by Sigma – Aldrich
Catalog #: PHR1330

200 mg $K_3[(CN)_6]$

50 mg KCN

140 mg KH_2PO_4

1000 ml water

2 ml Drabkin solution




Beckman Coulter DU 640
Spectrophptometer


Protein determination (Lowry OH et al. (1951); Protein measurement with the Folin phenol reagent. J of Biol Chem. Nov; 193(1):265-75.)

- 22 We prepared the following reagents:
Reagent A, 2 per cent $NaCO_3$ in 0.10 N NaOH
Reagent B, 0.5 per cent $CuSO_4 \cdot 5 H_2O$ in 1 per cent potassium sodium tartrate.


Reagent C, alkaline copper solution. Mix 50 ml. of Reagent A with 1 ml. of Reagent B. Discard after 1 day. Reagent D, carbonate-copper solution, is the same as Reagent C except for omission of NaOH.
 Reagent E, diluted Folin reagent (Sigma-Aldrich) diluted 1:1 in water.
 We prepared a solution from bovine serum albumine and used it as a standard curve.




Sodium carbonate
 by Sigma – Aldrich
 Catalog #: 223484




Copper (II) sulfate pentahydrate
 by Sigma – Aldrich
 Catalog #: 209198



Potassium sodium tartrate tetrahydrate
 by Sigma – Aldrich
 Catalog #: 217255



Folin & Ciocalteus phenol
 by Sigma – Aldrich
 Catalog #: F9253




Bovine Serum Albumin (BSA)
 by Sigma Aldrich
 Catalog #: A7906

- 23** Procedure for Proteins in Solution or Readily Soluble in Dilute Alkali- (Directions are given for a final volume of 1.1 to 1.3 ml., but any multiple or fraction of the volumes given may be employed as desired'.)
 To a sample of 5 to 100 ul of protein in 0.2 ml. or less in a 3 to 10 ml. test-tube, add 1 ml. of Reagent C.
 Mix well and allow to stand for 10 minutes or longer at room temperature.

🕒 00:10:00

- 24** Add 0.10 ml. of Reagent E very rapidly and mixed within a second or two (see below).
 After 30 minutes or longer, the sample is read in a calorimeter or spectrophotometer.
 For the range 5 to 25 ul of protein per ml. of final volume, it is desirable to make readings at or near 750 nm, the absorption peak.
 Calculate from a standard curve.

🕒 00:30:00



Beckman DU 640
 Spectrophotometer

Ammonia determination (Bergmeyer HU, Beutler HO. Ammonia, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie, 1984:454-461.)

- 25** Ammonia reacts with 2-oxoglutarate and NADH to form l-glutamate in the presence of de glutamate dehydrogenase. As a result we obtain oxidized NADH (NAD). The amount of NADH consumed in the reaction is stoichiometric with the amount of ammonia.
 We use neutralized perchloric extracts of RBC or serum samples. All operations are made at 0-4°C.

Pipette successively into the cuvettes:

-1 ml of reaction buffer (2-oxoglutarate 35 mM; ADP: adenine diphosphate 1.82 mM; TEA: triethanolamine 0.5M. pH 8).

- 0.10 ml NADH (6 mM)

-0.10 - 0.5 ml sample

Final volume with water to 3.2 ml.

Mix and monitor change in absorbance until constant. Read absorbance (0).

4 °C at all time before incubation



Adenosine 5-diphosphate sodium salt

by Sigma – Aldrich

Catalog #: A2754



alpha-Ketoglutaric acid sodium salt

by Sigma – Aldrich

Catalog #: K1875



**Beta-nicotinamide adenine dinucleotide,
reduced disodium salt hydrate**

by Sigma – Aldrich

Catalog #: N8129



Triethanolamine

by Sigma – Aldrich

Catalog #: 90279

- 26 Add 0.02 ml GIDH (glutamate dehydrogenase, 3,000 U/ml in triethanolamine 150 mM pH 8 buffer; diluted 1:400)
Mix. After, read every 20 min until stability using a spectrophotometer.
Calculate from a standard curve with NH₄Cl solution.



Glutamate dehydrogenase (Gldh)

by Sigma – Aldrich

Catalog #: 10197734001



Triethanolamine

by Sigma – Aldrich

Catalog #: 90279



**Beckman Coulter DU 640
spectrophotometer**

Urea determination (Kerscher L, Ziegenhorn J. Urea, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. VII. Deerfield Beach, Florida, Verlag Chemie, 1984:444-453.)

- 27 Urea in the presence of urease is transformed to 2 ammonium molecules, which is determined by the aforementioned method.
Pipette successively into the cuvettes:
-1.75 ml of reaction buffer (Tris 150 mM, succinate 60 mM, 2-oxoglutarate 12.5 mM, ADP. Adenosine 5-diphosphate sodium salt 2 mM, pH

8)

-0.1 ml NADH 5 mM

- 0.66 ml GIDH (glutamate dehydrogenase, 3,000 U/ml Tris_HCl buffer 150 mM pH 8; diluted 1:400)

- 0.05 ml neutralized perchloric extracts of RBC or serum diluted 10-fold

Mix thoroughly alter 10 min read (339 nm) A(0)

🕒 00:10:00 mix thoroughly



Tris base

by Sigma – Aldrich

Catalog #: T1503



Succinic acid

by Sigma – Aldrich

Catalog #: 398055



alpha-Ketoglutaric acid sodium salt

by Sigma – Aldrich

Catalog #: K1875



Adenosine 5-diphosphate sodium salt

by Sigma – Aldrich

Catalog #: A2754



Glutamate dehydrogenase (Gldh)

by Sigma – Aldrich

Catalog #: 10197734001

28 Add 0.005 ml urease solution (2.5kU/L). Mix.

After 15 min read (339 nm) the final absorbance A using a spectrophotometer.

Used $A - A(0) = \Delta A$ for calculation.

Calculation The value thus obtained has to be multiplied by the factor of sample pre dilution and using a standard curve with NH₄Cl solution.

🕒 00:15:00 mix



Urease from Canavalia ensiformis (Jack bean)

by Sigma – Aldrich

Catalog #: U7752



Ammonium chloride

by Sigma – Aldrich

Catalog #: 254134



Beckman Coulter DU 640
spectrophotometer

Ornithine determination (A spectrophotometric Method for the direct determination of cysteine in the presence of other naturally occurring Amino Acids. M.K. Gaitonde. Biochem J, 1967, 104: 627.)

- 29 We added water to 0.3 0.5 ml acid-extracts of serum or RBC to have a final volume of 1 ml. Mix.
Add 0.5 ml acetic acid, glacial. Mix.
Add 0.5 ml ninhydrin *. Mix.
*ninhydrin solution: 250 mg ninhydrin in 6 ml glacial acetic, 4ml phosphoric acid 0.6 M.
Put in boiling water bath for 10 min.
Allow cooling to room temperature and add 3 ml EtOH 95%
Read at 515 nm using a spectrophotometer.
Calculation using a standard curve with 5, to 100 nmoles of ornithine.



Acetic Acid, Glacial
by Meyer
Catalog #: 0040



Ninhydrin
by Sigma – Aldrich
Catalog #: N4876



Phosphoric acid
by Meyer
Catalog #: 7664-38-2



Ethanol absolute
by JT Baker
Catalog #: 13225483

00:10:00 boiling water bath



L-Ornithine monohydrochloride
by Sigma – Aldrich
Catalog #: 02375





Beckman Coulter DU640
spectrophotometer


Citrulline determination (Ceriotti G. Ornithine Carbamoyltransferase, in: Bergmeyer HU, Bergmeyer J, Grassl M, Eds. Methods of Enzymatic Analysis, Vol. IV. Deerfield Beach, Florida, Verlag Chemie, 1984:319-334.)


The citrulline is formed from serum ornithine and carbamyl phosphate by ornithine carbamoyl transferase. This method is based on the


- 30 citrulline determination by the diacetylmonoxime-phenazone reaction.
For the colorimetric reaction, we add 0.2 - 0.5 ml of acid extracts of serum and RBC to a mixture* of 1.5 ml of diacetylmonoxime and phenazone, prepared immediately before use.
*Phenazone: 4 g are dissolved in 1 L of 40% v/v sulfuric acid solution ; 50 mg of ferric sulfate are added. Diacetylmonoxime solution: 0.5% in 5% acetic acid.

 **Sulfuric acid**
by Sigma – Aldrich
Catalog #: 258105

 **Iron (III) sulfate hydrate**
by Sigma – Aldrich
Catalog #: 307718


 **2,3-Butanedione monoxime**
by Sigma – Aldrich
Catalog #: 31550

 **Phenazone**
by Sigma – Aldrich
Catalog #: P0800000

 **Acetic Acid, Glacial**
by Meyer
Catalog #: 0040

- 31 We performed the colorimetric reaction in a 15 min boiling water bath incubation.
The color formed is read at 460 nm using a spectrophotometer against a blank with water treated in the same manner.
The absorbance at 460 nm is divided by 0.038 (the absorbance for 1 pg of citrulline, giving the amount of micrograms of citrulline contained).

 **00:15:00 boiling water bath**


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Nitrites determination (Green, L.C. et al. (1982). Analysis of Nitrate, nitrite, and [15N]Nitrate in Biological Fluids. Analytical Biochemistry, 126, pp. 131-138)


- 32 Based on the technique of Green, L.C. et al. (1982). Analysis of Nitrate, nitrite, and [15N]Nitrate in Biological Fluids. Analytical Biochemistry, 126, pp. 131-138, modified by Dr. Rolando Hernandez Munoz (IFC, UNAM, Mexico).


Content of nitrites was determined in neutralized perchloric extracts of RBC and serum.
A sample of 0.5 ml acid-extract was incubated 20 min at room temperature with the 0.5 ml of Griess reagent (0.1% naphthyl-ethylene diamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid).
After developing the color, assays were read at 550 nm using a spectrophotometer, contrasting with a standard curve for sodium nitrate in deionized water.


 **00:20:00 incubation time**

 **N-(1-Naphthyl)ethylenediamine dihydrochloride**
by [Sigma – Aldrich](#)
Catalog #: 222488

 **Sulfanilamide**
by [Sigma – Aldrich](#)
Catalog #: S9251

 **Phosphoric acid**
by [Meyer](#)
Catalog #: 7664-38-2

 **Sodium nitrate**
by [Sigma – Aldrich](#)
Catalog #: S5506

 **Beckman Coulter DU 640 spectrophotometer**



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