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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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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.

MATERIALS

MATERIALS

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

⊗	Ninhydrin	Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876
⊗	Sodium hydroxide	Sigma – Aldrich Catalog #S8045
⊗	Phenazone	Merck MilliporeSigma (Sigma-Aldrich) Catalog #P0800000
⊗	2,3-Butanedione monoxine	Merck MilliporeSigma (Sigma-Aldrich) Catalog #31550
⊗	N-(1-Naphthyl)ethylenediamine dihydrochloride	Merck MilliporeSigma (Sigma-Aldrich) Catalog #222488
⊗	Hemoglobin human	Sigma – Aldrich Catalog #H7379
⊗	Potassium ferricyanide (III)	Merck MilliporeSigma (Sigma-Aldrich) Catalog #702587
⊗	Potassium phosphate dibasic	Sigma – Aldrich Catalog #60356
⊗	Phosphoric acid	Meyer Catalog #7664-38-2
⊗	Acetic Acid, Glacial	Meyer Catalog #0040
⊗	Potassium cyanide	Merck MilliporeSigma (Sigma-Aldrich) Catalog #207810
⊗	1,1,3,3-Tetraethoxypropane	Merck MilliporeSigma (Sigma-Aldrich) Catalog #9889
⊗	Copper (II) sulfate pentahydrate	Merck MilliporeSigma (Sigma-Aldrich) Catalog #209198
⊗	Folin & Ciocalteu phenol	Merck MilliporeSigma (Sigma-Aldrich) Catalog #F9253
⊗	Sulfanilamide	Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9251
⊗	Sodium nitrite	Sigma – Aldrich Catalog #237213
⊗	Potassium sodium tartrate tetrahydrate	Merck MilliporeSigma (Sigma-Aldrich) Catalog #217255
⊗	Ethanol absolute	JT Baker Catalog #13225483
⊗	Bovine serum albumin	Sigma – Aldrich Catalog #A7906
⊗	Manganese (II) chloride tetrahydrate	Merck MilliporeSigma (Sigma-Aldrich) Catalog #221279
⊗	L-Ornithine dihydrochloride	Sigma – Aldrich Catalog #75440
⊗	Pyridine	Sigma – Aldrich Catalog #360570
⊗	Butyl alcohol	Sigma – Aldrich Catalog #W217808
⊗	Potassium Chloride	Sigma – Aldrich Catalog #746436
⊗	Potassium hydroxide	Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767
⊗	2-Thiobarbituric acid	Merck MilliporeSigma (Sigma-Aldrich)
⊗	Ammonium chloride	Merck MilliporeSigma (Sigma-Aldrich) Catalog #254134

- ⊗ Triethanolamine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #90279**
- ⊗ L-Ornithine monohydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #02375**
- ⊗ Iron (III) sulfate hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #307718**
- ⊗ Sodium nitrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5506**

STEP MATERIALS

- ⊗ Perchloric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #244252**
- ⊗ Sodium hydroxide **Bio Basic Inc. Catalog #SB0617.SIZE.500G**
- ⊗ Potassium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767**
- ⊗ 2-Thiobarbituric acid **Merck MilliporeSigma (Sigma-Aldrich)**
- ⊗ Perchloric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #244252**
- ⊗ Acetic Acid, Glacial **Meyer Catalog #0040**
- ⊗ Potassium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767**
- ⊗ 2-Thiobarbituric acid **Merck MilliporeSigma (Sigma-Aldrich)**
- ⊗ 1,1,3,3-Tetraethoxypropane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #9889**
- ⊗ Manganese (II) chloride tetrahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #221279**
- ⊗ Sodium carbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #222321**
- ⊗ Acetic Acid, Glacial **Meyer Catalog #0040**
- ⊗ Ninhydrin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876**
- ⊗ Acetic Acid, Glacial **Meyer Catalog #0040**
- ⊗ Phosphoric acid **Meyer Catalog #7664-38-2**
- ⊗ Manganese (II) chloride tetrahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #221279**
- ⊗ Sodium carbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #223484**
- ⊗ Acetic Acid, Glacial **Meyer Catalog #0040**
- ⊗ Ninhydrin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876**
- ⊗ Acetic Acid, Glacial **Meyer Catalog #0040**
- ⊗ Phosphoric acid **Meyer Catalog #7664-38-2**
- ⊗ Potassium ferricyanide (III) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #702587**

⊗	Potassium cyanide Merck MilliporeSigma (Sigma-Aldrich) Catalog #207810
⊗	Monobasic potassium phosphate Merck MilliporeSigma (Sigma-Aldrich) Catalog #PHR1330
⊗	Sodium carbonate Merck MilliporeSigma (Sigma-Aldrich) Catalog #223484
⊗	Copper (II) sulfate pentahydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #209198
⊗	Potassium sodium tartrate tetrahydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #217255
⊗	Folin & Ciocalteus phenol Merck MilliporeSigma (Sigma-Aldrich) Catalog #F9253
⊗	Bovine Serum Albumin (BSA) Merck MilliporeSigma (Sigma-Aldrich) Catalog #A7906
⊗	Adenosine 5-diphosphate sodium salt Merck MilliporeSigma (Sigma-Aldrich) Catalog #A2754
⊗	alpha-Ketoglutaric acid sodium salt Merck MilliporeSigma (Sigma-Aldrich) Catalog #K1875
⊗	Beta-nicotinamide adenine dinucleotide, reduced disodium salt hydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #N8129
⊗	Triethanolamine Merck MilliporeSigma (Sigma-Aldrich) Catalog #90279
⊗	Glutamate dehydrogenase (Gldh) Merck MilliporeSigma (Sigma-Aldrich) Catalog #10197734001
⊗	Triethanolamine Merck MilliporeSigma (Sigma-Aldrich) Catalog #90279
⊗	Tris base Merck MilliporeSigma (Sigma-Aldrich) Catalog #T1503
⊗	Succinic acid Merck MilliporeSigma (Sigma-Aldrich) Catalog #398055
⊗	alpha-Ketoglutaric acid sodium salt Merck MilliporeSigma (Sigma-Aldrich) Catalog #K1875
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⊗	Urease from Canavalia ensiformis (Jack bean) Merck MilliporeSigma (Sigma-Aldrich) Catalog #U7752
⊗	Ammonium chloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #254134
⊗	Acetic Acid, Glacial Meyer Catalog #0040
⊗	Ninhydrin Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876
⊗	Phosphoric acid Meyer Catalog #7664-38-2

⊗	Ethanol absolute JT Baker Catalog #13225483
⊗	L-Ornithine monohydrochloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #02375
⊗	Sulfuric acid Merck MilliporeSigma (Sigma-Aldrich) Catalog #258105
⊗	Iron (III) sulfate hydrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #307718
⊗	2,3-Butanedione monoxime Merck MilliporeSigma (Sigma-Aldrich) Catalog #31550
⊗	Phenazone Merck MilliporeSigma (Sigma-Aldrich) Catalog #P0800000
⊗	Acetic Acid, Glacial Meyer Catalog #0040
⊗	N-(1-Naphthyl)ethylenediamine dihydrochloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #222488
⊗	Sulfanilamide Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9251
⊗	Phosphoric acid Meyer Catalog #7664-38-2
⊗	Sodium nitrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5506
⊗	Perchloric acid Merck MilliporeSigma (Sigma-Aldrich) Catalog #244252
⊗	Sodium hydroxide Bio Basic Inc. Catalog #SB0617.SIZE.500G
⊗	Potassium hydroxide Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767
⊗	2-Thiobarbituric acid Merck MilliporeSigma (Sigma-Aldrich)
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⊗	N-(1-Naphthyl)ethylenediamine dihydrochloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #222488
⊗	Sulfanilamide Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9251
⊗	Phosphoric acid Meyer Catalog #7664-38-2
⊗	Sodium nitrate Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5506

Biochemical Measurements

- 1
 - a) 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.
 - b) Whole blood was centrifugated to separate RBC and serum samples

IMJ 8 Mass / % volume

Expected result

We extract proteins from blood

⊗ Perchloric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #244252**

Safety information

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

🧊 4 °C on ice

- 2 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 **Bio Basic Inc. Catalog #SB0617.SIZE.500G**

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

Glucose determination

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

Equipment

		NAME
Biotek instruments		BRAND
ELx800		SKU
USA		SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

Aspartate aminotransferase (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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

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.

Equipment

	NAME
Biotek instruments	BRAND
EL x 800	SKU
USA	SPECIFICATIONS

Malondialdehyde (MDA) determination (Hernandez-Munoz e..

- 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 8% 2-thiobarbituric acid

⊗ Potassium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767**

⊗ 2-Thiobarbituric acid **Merck MilliporeSigma (Sigma-Aldrich)**

⊗ Perchloric acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #244252**

⊗ Acetic Acid, Glacial **Meyer Catalog #0040**

⊗ Potassium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1767**

⊗ 2-Thiobarbituric acid **Merck MilliporeSigma (Sigma-Aldrich)**

[M] 6 Mass / % volume 6% perchloric acid w/v

[M] 20 % volume 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 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 mix butanol-pyridin (15:1, v/v)

Equipment

	NAME
Beckman Coulter	BRAND
DU 640	SKU
Spectrophotometer	SPECIFICATIONS

1,1,3,3-Tetraethoxypropane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #9889**

Arginine determination (Colombo, JP and Konarska L.; Argin..

- 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 (MnCl₂) at 55 °C.

⌚ 00:20:00 20 minutes incubation

🌡 55 °C for incubation

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

⊗ Manganese (II) chloride tetrahydrate **Merck MilliporeSigma (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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #222321**

🧴 10 µg 10 µg of cytosolic protein.

⊗ Acetic Acid, Glacial **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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876**

⊗ Acetic Acid, Glacial **Meyer Catalog #0040**

⊗ Phosphoric acid **Meyer Catalog #7664-38-2**

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

⌚ 00:45:00

Arginase determination (Colombo, JP and Konarska L.; Argi...

- 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 **Merck MilliporeSigma (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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #223484**

🌡 37 °C incubation temperature

⊗ Acetic Acid, Glacial **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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876**

⊗ Acetic Acid, Glacial **Meyer Catalog #0040**

⊗ Phosphoric acid **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 v/v acetic acid:6 M phosphoric acid

Equipment	
	NAME
Beckman Coulter	BRAND
DU 640	SKU
spectrophotometer	SPECIFICATIONS

Hb determination (Colombo, JP and Konarska L.; Arginase i...

- 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 centrifuged 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 absorption coefficient $E=44 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.
 Molecular weight of Hb is 64 458.

⊗ Potassium ferricyanide (III) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #702587**

⊗ Potassium cyanide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #207810**

⊗ Monobasic potassium phosphate **Merck MilliporeSigma (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

Equipment	
	NAME
Beckman Coulter	BRAND
DU 640	SKU
Spectrophptometer	SPECIFICATIONS

Protein determination (Lowry OH et al. (1951); Protein mea..

- 22 We prepared the following reagents:
- Reagent A, 2 per cent NaCO_3 in 0.10 N NaOH
- Reagent B, 0.5 per cent $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ 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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #223484**

⊗ Copper (II) sulfate pentahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #209198**

⊗ Potassium sodium tartrate tetrahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #217255**

⊗ Folin & Ciocalteus phenol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F9253**

⊗ Bovine Serum Albumin (BSA) **Merck MilliporeSigma (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 μl 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 μl 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

Equipment	
	NAME
Beckman	BRAND
DU 640	SKU
Spectrophotometer	SPECIFICATIONS

Ammonia determination (Bergmeyer HU, Beutler HO. Ammo.

- 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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A2754**

⊗ alpha-Ketoglutaric acid sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #K1875**

⊗ Beta-nicotinamide adenine dinucleotide, reduced disodium salt hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N8129**

⊗ Triethanolamine **Merck MilliporeSigma (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) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10197734001**

⊗ Triethanolamine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #90279**

Equipment

	NAME
Beckman Coulter	BRAND
DU 640	SKU
spectrophotometer	SPECIFICATIONS

Urea determination (Kerscher L, Ziegenhorn J. Urea, in: Ber..

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 after 10 min read (339 nm) A(0)

⌚ 00:10:00 mix thoroughly

⊗ Tris base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T1503**

⊗ Succinic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #398055**

⊗ alpha-Ketoglutaric acid sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #K1875**

⊗ Adenosine 5-diphosphate sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A2754**

⊗ Glutamate dehydrogenase (Gldh) **Merck MilliporeSigma (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) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #U7752**

🔗 Ammonium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #254134**

Equipment

	NAME
Beckman Coulter	BRAND
DU 640	SKU
spectrophotometer	SPECIFICATIONS

Ornithine determination (A spectrophotometric Method for ..

- 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 **Meyer Catalog #0040**

🔗 Ninhydrin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N4876**

🔗 Phosphoric acid **Meyer Catalog #7664-38-2**

🔗 Ethanol absolute **JT Baker Catalog #13225483**

🕒 00:10:00 boiling water bath

🔗 L-Ornithine monohydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #02375**

Equipment	
	NAME
Beckman Coulter	BRAND
DU640	SKU
spectrophotometer	SPECIFICATIONS

Citrulline determination (Ceriotti G. Ornithine Carbamoyltra..

- 30** The citrulline is formed from serum ornithine and carbamyl phosphate by ornithine carbamoyl transferase. This method is based on the 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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #258105**

⊗ Iron (III) sulfate hydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #307718**

⊗ 2,3-Butanedione monoxime **Merck MilliporeSigma (Sigma-Aldrich) Catalog #31550**

⊗ Phenazone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P0800000**

⊗ Acetic Acid, Glacial **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

Equipment	
	NAME
Beckman Coulter	BRAND
DU 640	SKU
spectrophotometer	SPECIFICATIONS

Nitrites determination (Green, L.C. et al. (1982). Analysis o..

- 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 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #222488**

⊗ Sulfanilamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9251**

⊗ Phosphoric acid **Meyer Catalog #7664-38-2**

⊗ Sodium nitrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5506**

Equipment	
	NAME
Beckman Coulter	BRAND
DU 640	SKU
spectrophotometer	SPECIFICATIONS