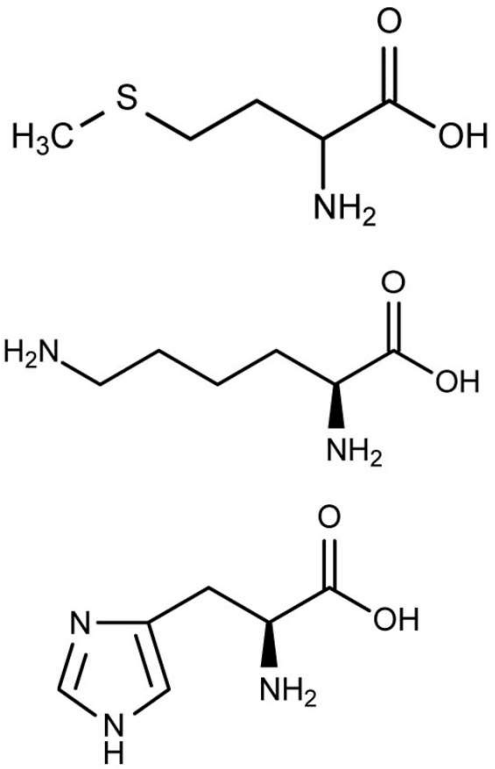
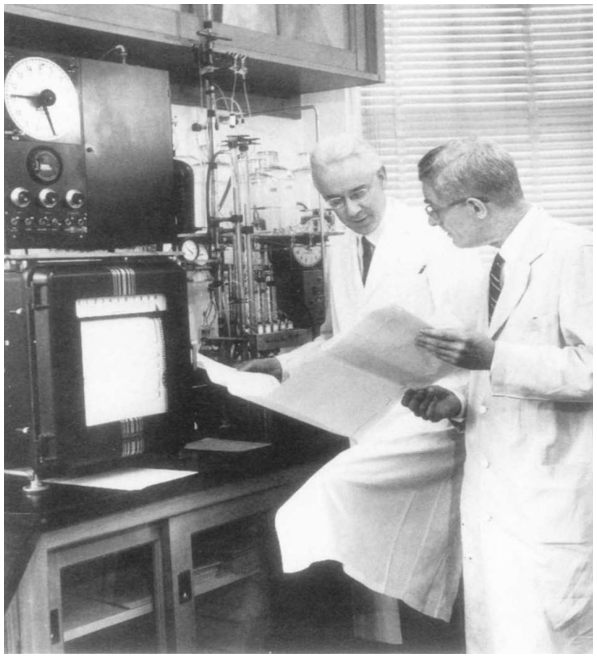
Protein Analysis Methodology





ASDA NANP Symposium

Glen Broderick

USDA-ARS (retired)

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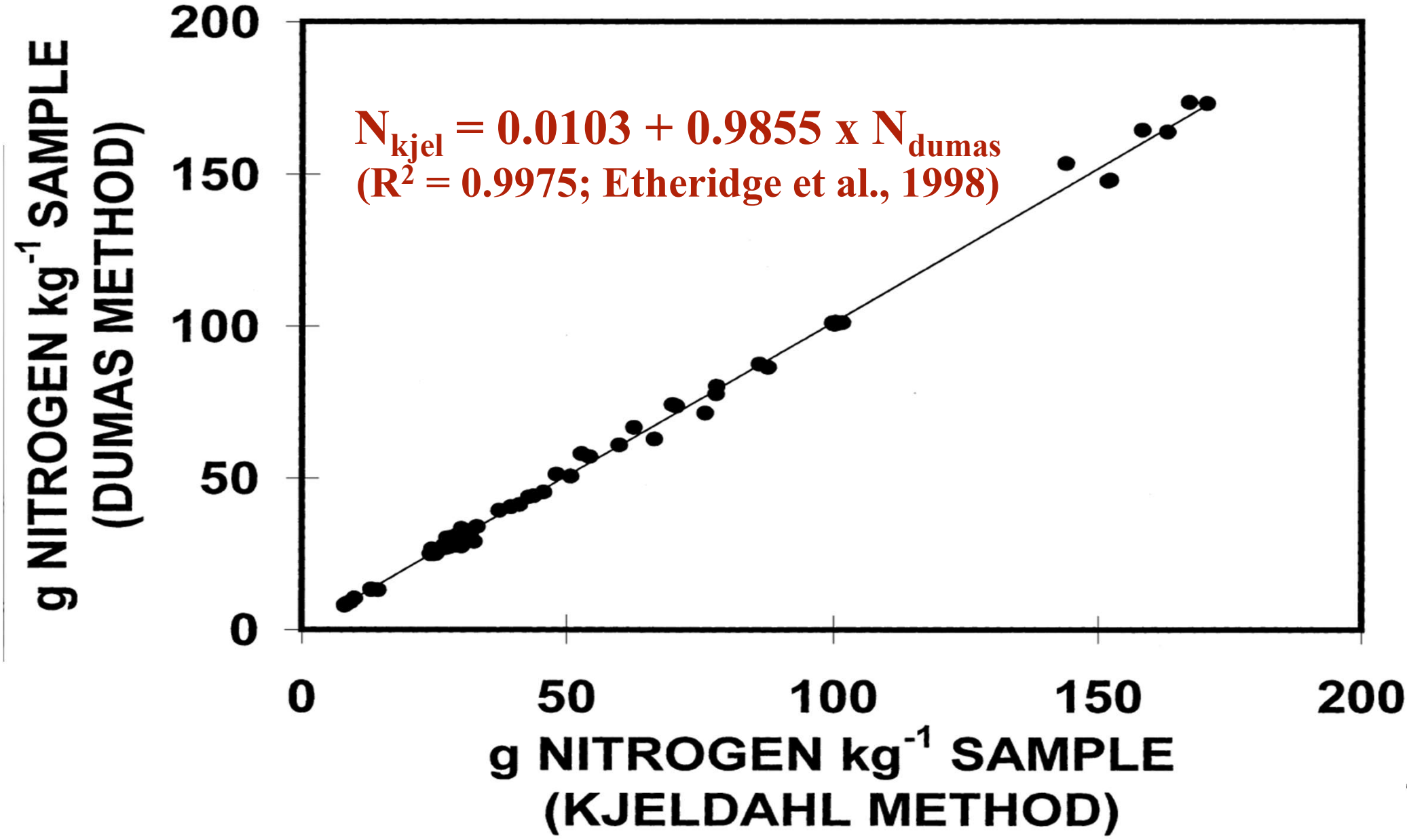
Protein Analysis Methodology

1. Elemental N Analysis Can Replace Kjeldahl
2. Quantifying RDP & RUP
    a. In Situ Methods (Comments; Problems)
    b. New Methodology Needed
    c. Intestinal Digestion of RUP
3. Quantifying Microbial Protein Formation
4. Amino Acid Analysis (AAA)
    a. Losses During Protein Hydrolysis
    b. Separation & Detection Methods
5. Milk Urea

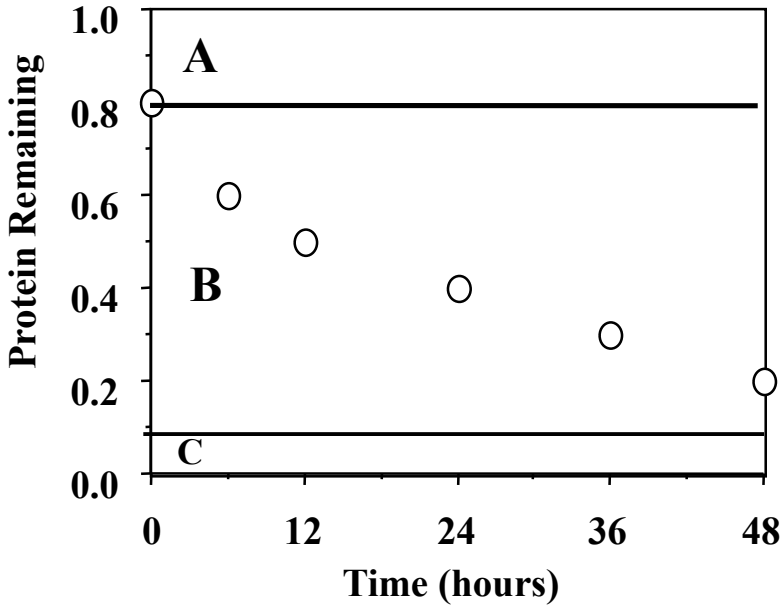
Kjeldahl vs. Elemental N (Dumas Assay)

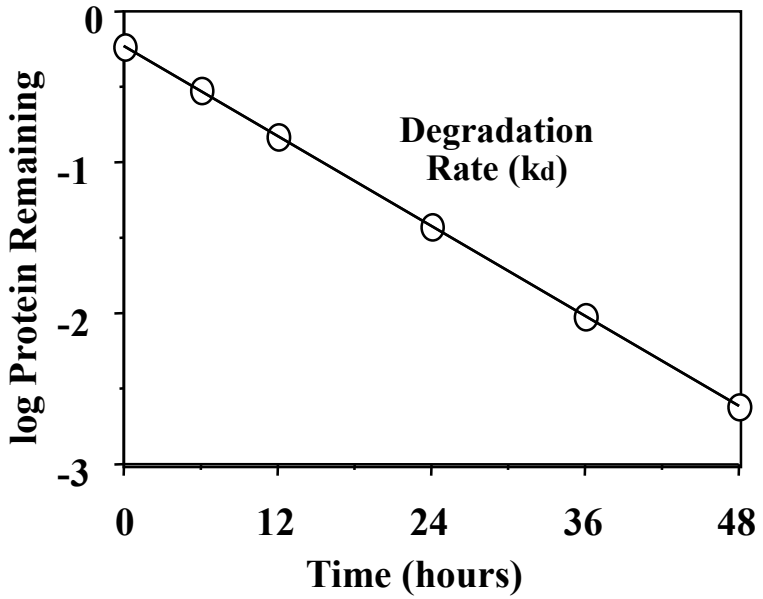
Elemental N (Dumas Assay) vs. Kjeldahl



In Situ Incubations in the Rumen

Data Analysis for First-Order Models





$$\text{Degradation} = A + B\left(\frac{kd}{kp + kd}\right)$$

In Situ Protein Degradation

(Huhtanen & Ahvenjarvi, 2022)

1. **NASEM (2021) Still Using In Situ Because of Large Literature Base on Methodology & Data**
2. **Soluble CP Leak Out (Assumed Escape = 6.4%)**
3. **Microbial Contamination Inside Bag (Especially Low CP Feeds)**
4. **Single kp-Values for Concentrates & Forages**
5. **No Accounting for Passage & Degradation Lags**
6. **What about Fraction C, Including ADIN (Useful or Indigestible?)**

NRC-2001 vs. NASEM-2021 kp

1. **Lowered kp (Reduces RUP):**
   - a. **NRC-2001:** **Wet Forages = 0.055/h**
     (DMI = 4.0% BW) **Concentrates = 0.067/h**
   - b. **NASEM-2021:** **Forages = 0.053/h**
     (Seo et al., 2006) **Concentrates = 0.049/h**
2. **RUP Estimates:**
   - a. **NRC-2001 (kd):** **Solvent SBM (.075) = 43% RUP**
     (DMI = 4.0% BW) **Canola Meal (.104) = 36% RUP**
   - b. **NASEM-2021 (kd):** **Solvent SBM (.090) = 33% RUP**
     (A = 6.4% escape) **Canola Meal (.105) = 30% RUP**

Rumen Passage Rates Vary with Phase & Especially Animal

Source	Phase (Marker)	Mean (/h)	Range
Broderick (1985)	Liquid (Cr-EDTA)	0.17	0.12-0.22*
Reynal (2003)	Liquid (Co-EDTA)	0.13	0.07-0.26
Reynal (2003)	Small particles (Yb)	0.14	0.08-0.23
Brito (2007)	Solids (Prot.-mordant)	0.05	0.03-0.07

*Significant Animal Effect ($P < 0.05$)

Rumen Passage Rates Vary with Phase & Especially Animal

Source	Phase (Marker)	Mean (/h)	Range
Broderick (1985)	Liquid (Cr-EDTA)	0.17	0.12-0.22*

Liquid Rates (Soluble NAN) 3X > Solids (Insoluble NAN)

Wide Animal Variation

Brito (2007)	Solids (Prot.-mordant)	0.05	0.03-0.07

*Sgnificant Animal Effect ($P < 0.05$)

NRC-2001 vs. NASEM-2021 kp & RUP

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True Proteins Contribute to Microbial Protein & Different RUP (Brito et al., 2007)

Item	Diets				*Prob.*
	Urea	Soybean Meal	Cottonseed Meal	Canola Meal	
RUP, kg/d	0.54^{c}	0.99^{b}	1.35^{a}	1.15^{ab}	<0.01
Microbial protein, kg/d	2.34^{b}	2.71^{a}	2.71^{a}	2.78^{a}	0.04
Milk protein yield, kg/d	0.92^{c}	1.23^{ab}	1.18^{b}	1.27^{a}	<0.01

Omasal Flows

Diets = Alfalfa & Corn Silages + High Moisture Corn; 16.5% CP

$^{a\text{-}c}(P < 0.05)$

In Situ Protein Degradation

(Huhtanen & Ahvenjarvi, 2022)

1. NASEM (2021) Continues Using In Situ Because of

We Should be Looking at

Alternative Methods for RDP/RUP

1. Micr. Prot. Stimulated by RDP

2. RUP Varies in Value

5. No Accounting for Passage Lag
6. No Accounting for Degradation Lag
7. What about Fraction C, Including ADIN (Useful or Indigestible?)

Quantifying In Vitro Degradation Rate from End-Product Appearance

1. **Rumen Inoculum with Inhibitors of Microbial Growth ("MMIIV" Method)**
   a. Appearance of TCA Soluble-N
   b. Appearance of NH_3, AA & Peptides
2. **Ruminal Inoculum Using:**
   a. Gas Production to Quantify Microbial Growth ("Menke-Raab")
   b. Marker to Account for Microbial Growth (^{15}N)
3. **Utilizable CP (RUP + Microbial CP)**
4. **Use of Proteolytic Enzymes**

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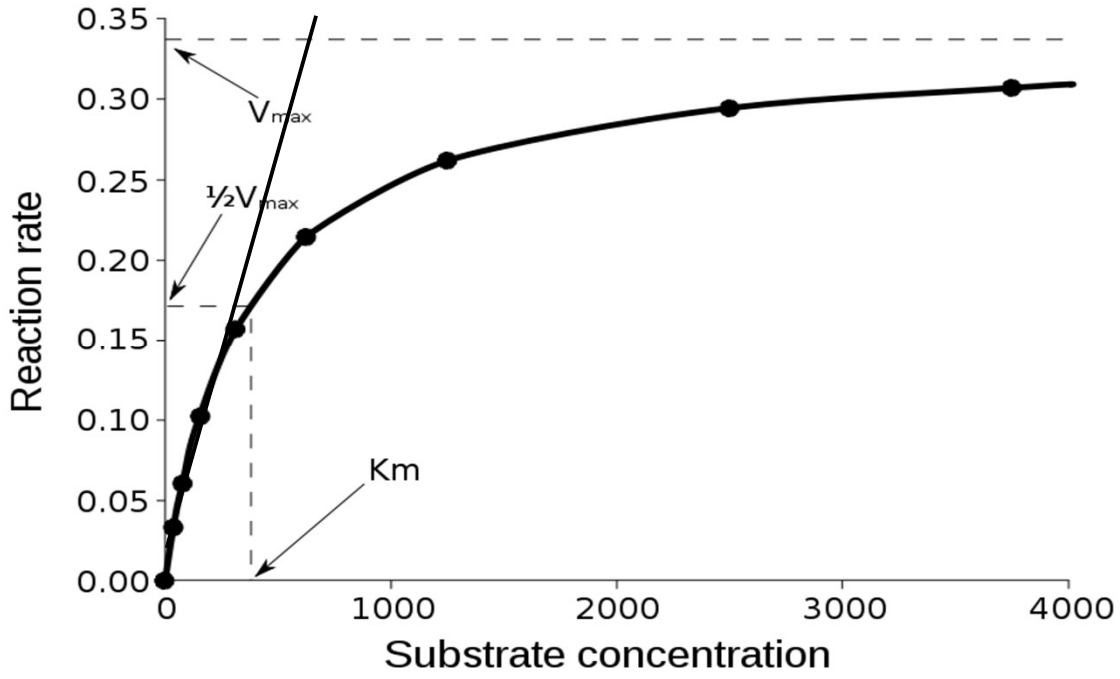


Michaelis-Menten Inhibitor Invitro (MMIIV) Method (Colombini et al., 2011).

Degradation rate (Theta) estimated as the tangent through the origin of the velocity vs. substrate concentration ([S]) curve:

$$\text{Theta} = V_{max}/K_m$$

$$\text{Adjusted kd} = \text{Theta} - \text{Degrad}\ (t = 0)$$

Mean Responses (Leu = 1.0) of AA & Peptides to OPA-Absorb. or Fluor.

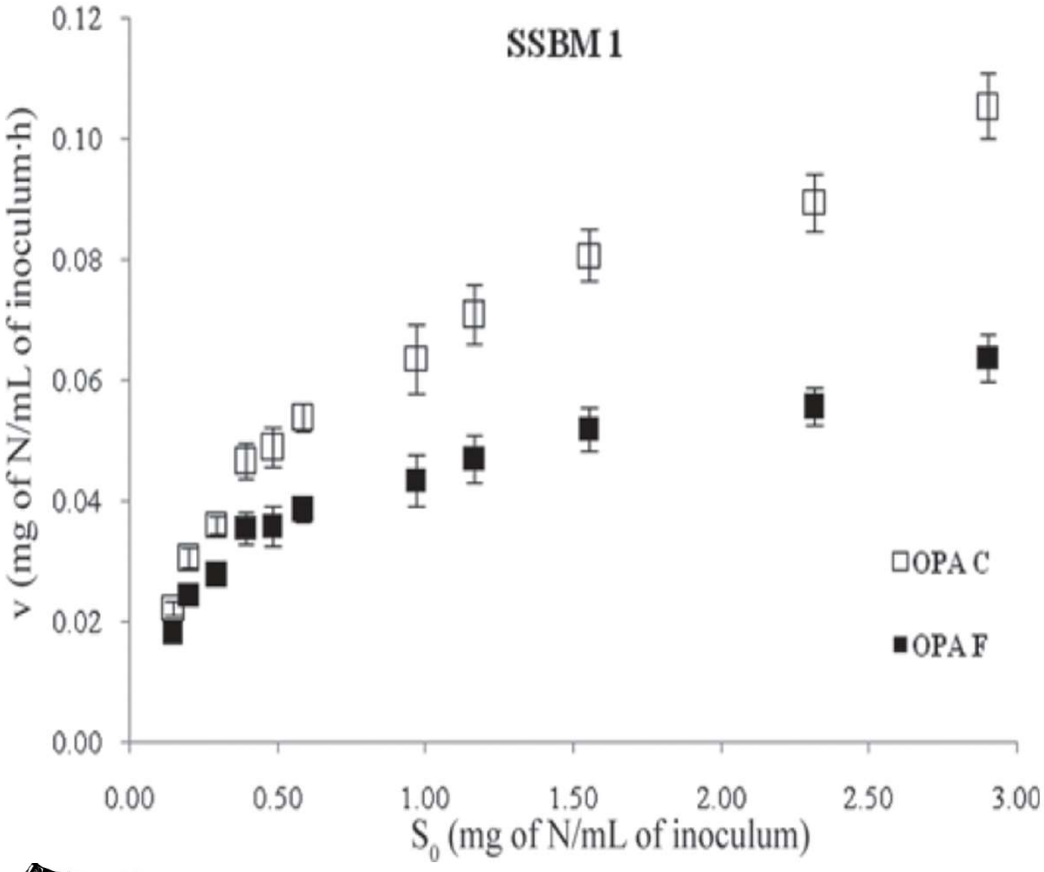
Source	OPA-Absorb.	OPA-Fluor.
20 Protein AA	0.95	0.81
19 Protein AA (w/o Pro)	1.00	0.85
12 Dipeptides	1.04	0.16
14 Tri- & Oligopeptides	1.02	0.11

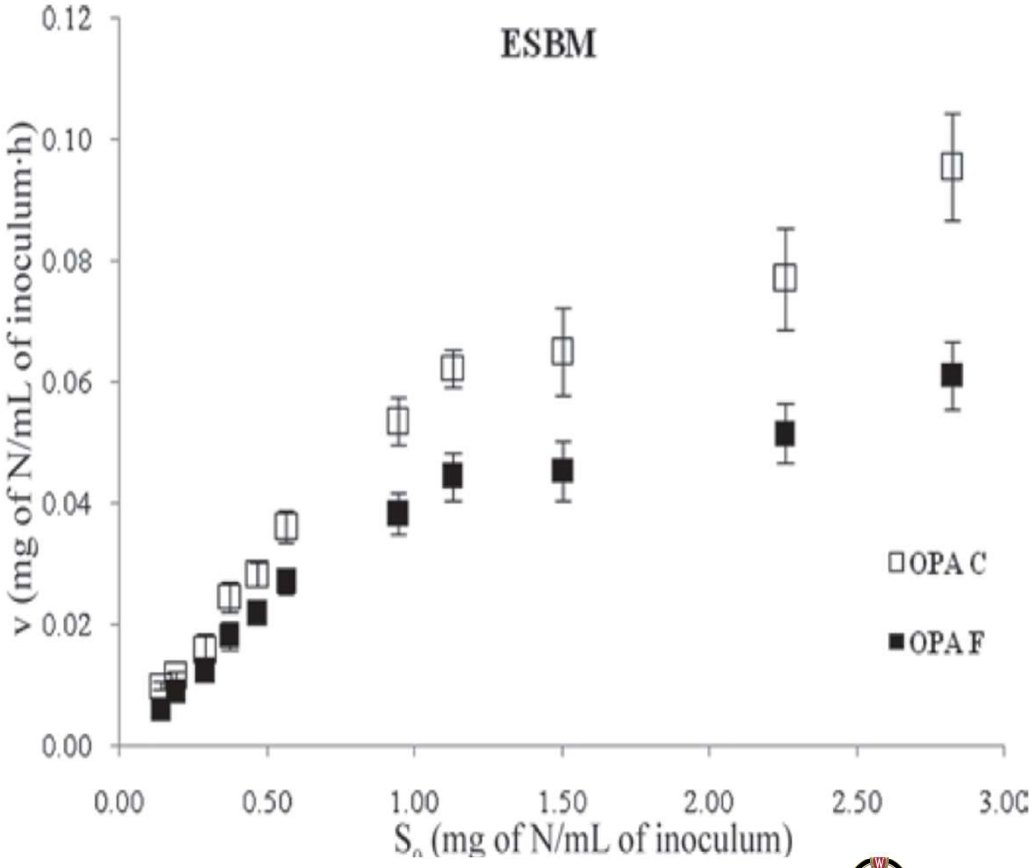
OPA = *O*-Phthalaldehyde

SBM Degradation Estimated by Net Release:

NH_3 + TAA (OPAF, ■)

NH_3 + TAA + Peptides (OPAC, □)





(Colombini et al., 2011)

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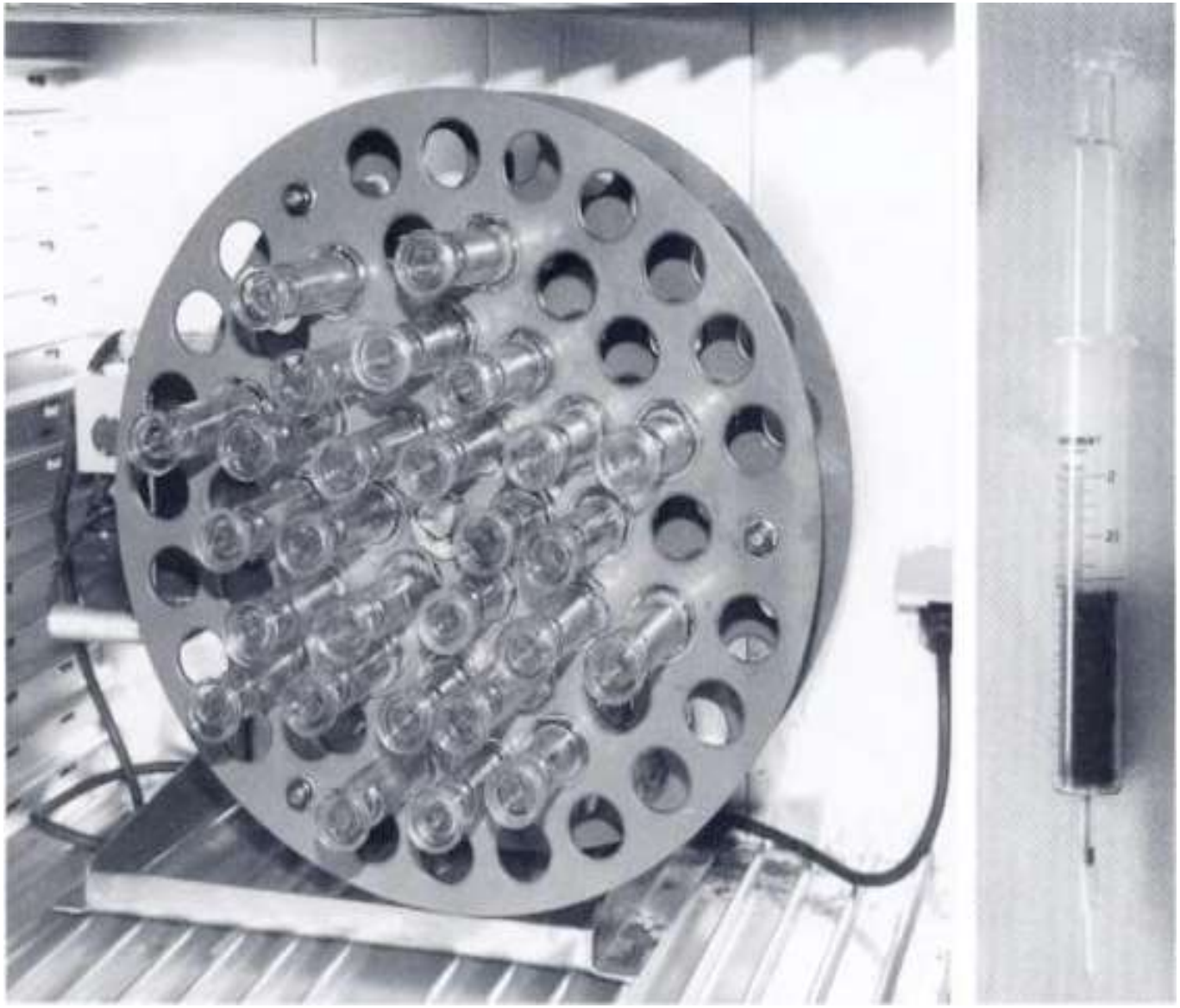
Menke-Raab Gas Production Method



Hohenheimer Gas Production System (Menke et al., 1979)

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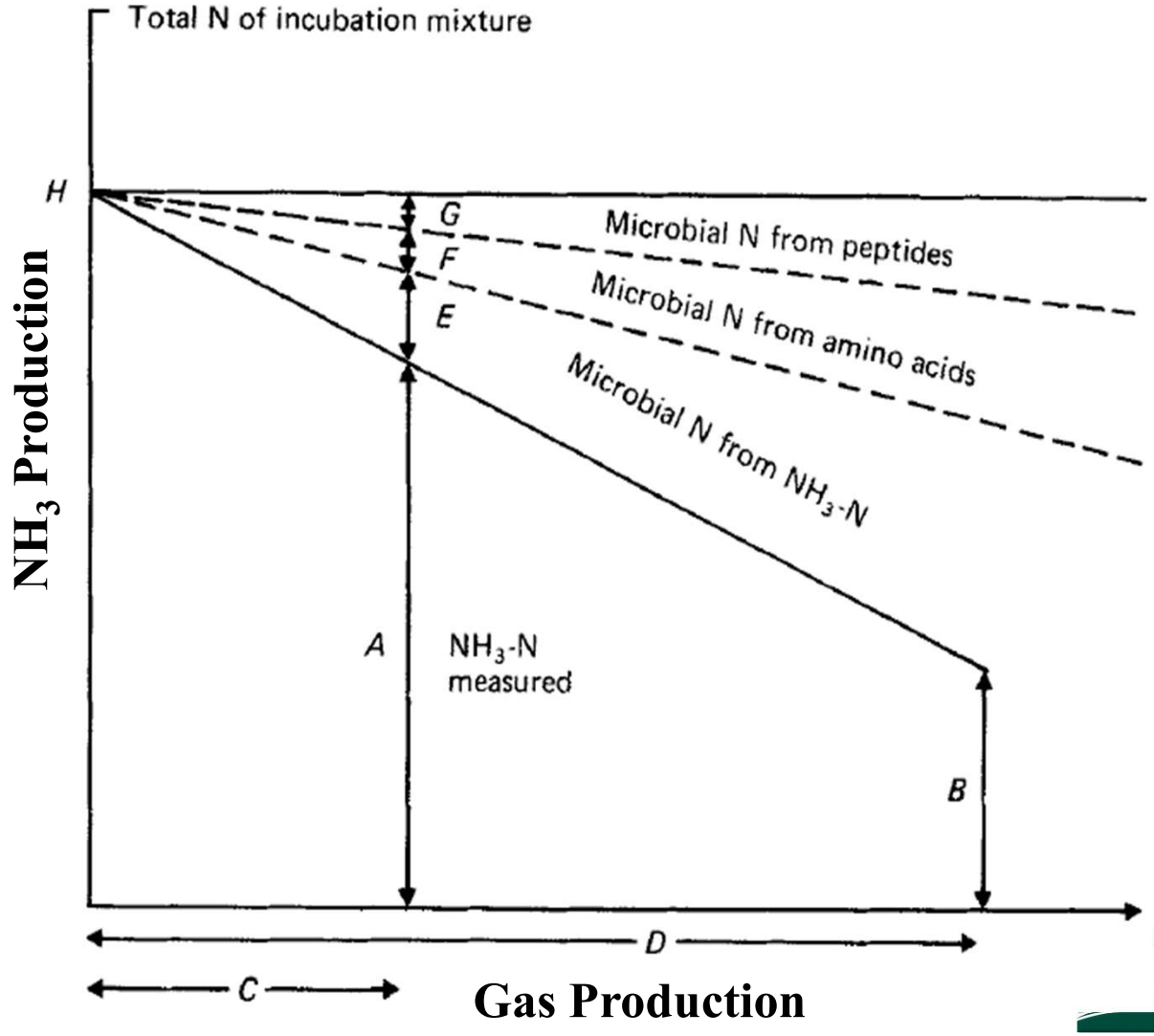
Schematic Representation of Protein Degradation & Protein Synthesis in Vitro

(Raab et al., 1983)

A = NH_3 From Feed Alone
B = NH_3 from Feed + Starch
C = Gas Production from Feed Alone
D = Gas Production from Feed + Starch
E, F, G = Microbial N from NH_3, AA, Peptides from Feed Alone
H = NH_3 Expected at 0-Gas Production



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Estimation of Degradation Rates from Gas Production Data (24 h data; Raab et al., 1983)

Protein	Intercept	Net N	IVDN	kd
	----(mg NH_3-N)----			/h
Casein	8.80	4.12	0.990	0.194
Soybean meal	8.61	3.93	0.945	0.121
Rapeseed meal	8.19	3.51	0.844	0.077

r = -0.993-0.998
Added N = 4.16 mg
Blank = 4.68 mg NH_3-N

Protein Degradation Rates using Proteases from Mixed Rumen Microbes or Strep. Griseus

(Mahadevan et al., 1987)

Protein	Protease Source	
	Rumen microbes	S. griseus
	-----(mg protein degraded to AA/h)-----	
Corn Gluten Meal	0.37	0.35
Fish Meal	0.62	1.65
Soybean Meal	1.06	0.70

Calsamiglia & Stern (1995) 3-Step In Vitro Method

1. Rumen In Situ Incubation of Protein (16-h)
   a. 1/0.06 ≅ 16 h (not = Escape at kp = 0.06/h)
   b. kp & kd both = 0.06/h, t = 11.6 h
   c. Probably OK for Intest. Digestibility
2. Protein Incubated with Pepsin (0.1 N HCl; 1-h)
3. Protein Incubated with Mixed Pancreatic Enzymes (pH 7.6; 24-h)
4. Generally Lower Digestibilities than Mobile Bag

Ross et al. Modification of Calsimiglia-Stern
(Gutierrez-Botero et al., 2022)

1. In Situ Bags Replaced by Rumen In Vitros in Flasks
2. Set 1—Undegraded CP Trapped:
   a. Micro-Pore Filters or Freeze-Drying ("Soluble" Feeds)
   b. Blank Corrected for Microbial CP (NDF Flasks)
3. Set 2—Rumen In Vitro Run, Followed by
   a. 1-h Treatment w/ Pepsin (2 N HCl), then
   b. 24-h Treatment w/ Trypsin, Chymo., Amylase & Lipase
   c. Blank Corrected for Microbial CP (NDF Flasks)
4. Computations:
   a. Set 1 = RUP; Set 2 = Total Undigested CP
   b. Set 1 – Set 2 = Intestinally Digested RUP (AA Analysis)

Microbial Protein Markers: $^{15}NH_3$ vs. Purines

(Reynal et al., 2005)

	Dietary RDP, % of DM				Linear
Microbial Eff.	13.2	12.3	11.7	10.6	Prob.
$^{15}NH_3$ (g NAN/kg omtdr)	32.3[a]	30.1[b]	28.1[c]	28.0[c]	<0.01
Total Purines (g NAN/kg omtdr)	28.4	27.1	26.4	26.8	0.17

Omasal Flows

[a,b,c](RDP affect P < 0.01)

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Microbial Protein Markers: $^{15}NH_3$ vs. Purines

(Reynal et al., 2005)

	Dietary RDP, % of DM				Linear
Microbial Eff.	13.2	12.3	11.7	10.6	Prob.
$^{15}NH_3$ (g NAN	$^{15}NH_3$ is Best Microbial Marker; Total Purines More Variable as Marker				<0.01
Total Purines (g NAN/kg omtdr)	28.4	27.1	26.4	26.8	0.17

Omasal Flows

[a,b,c](RDP affect P < 0.01)

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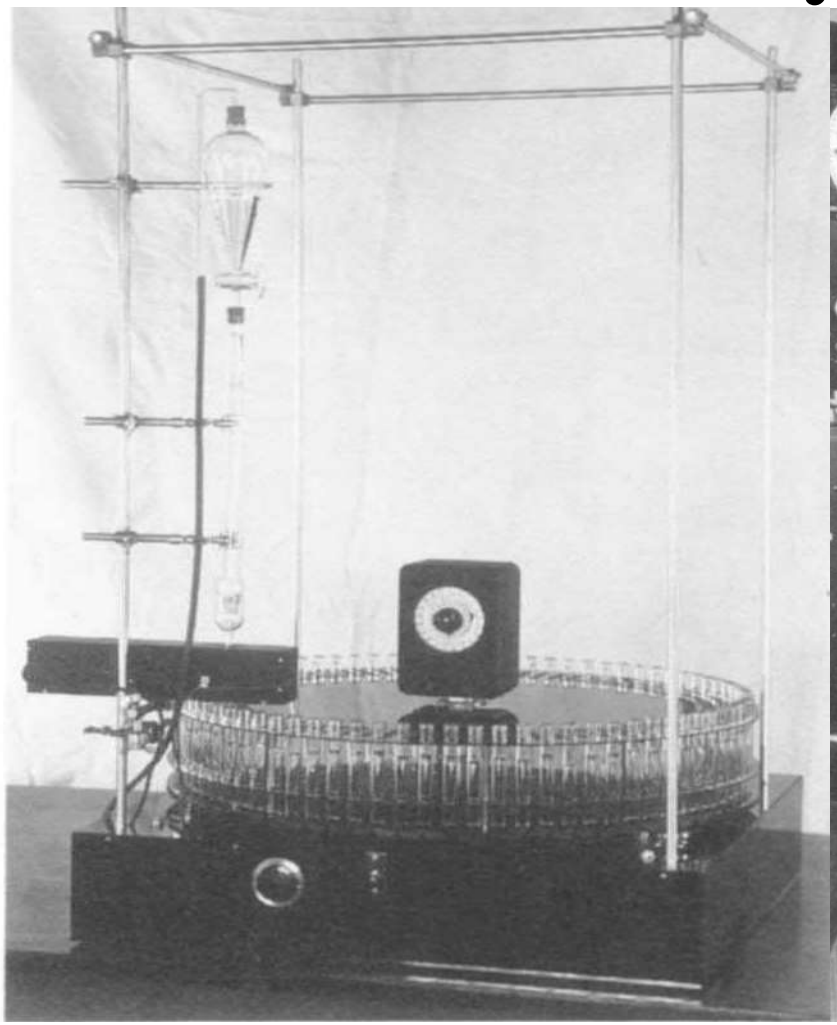
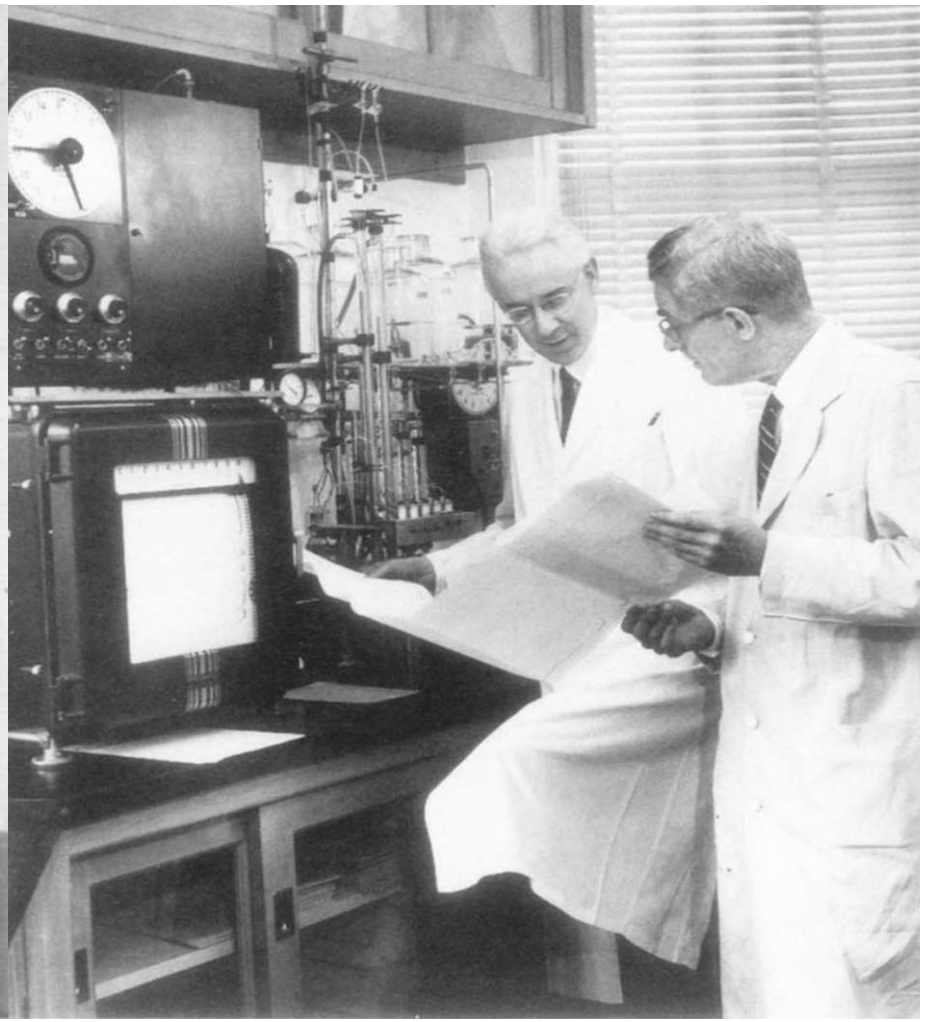
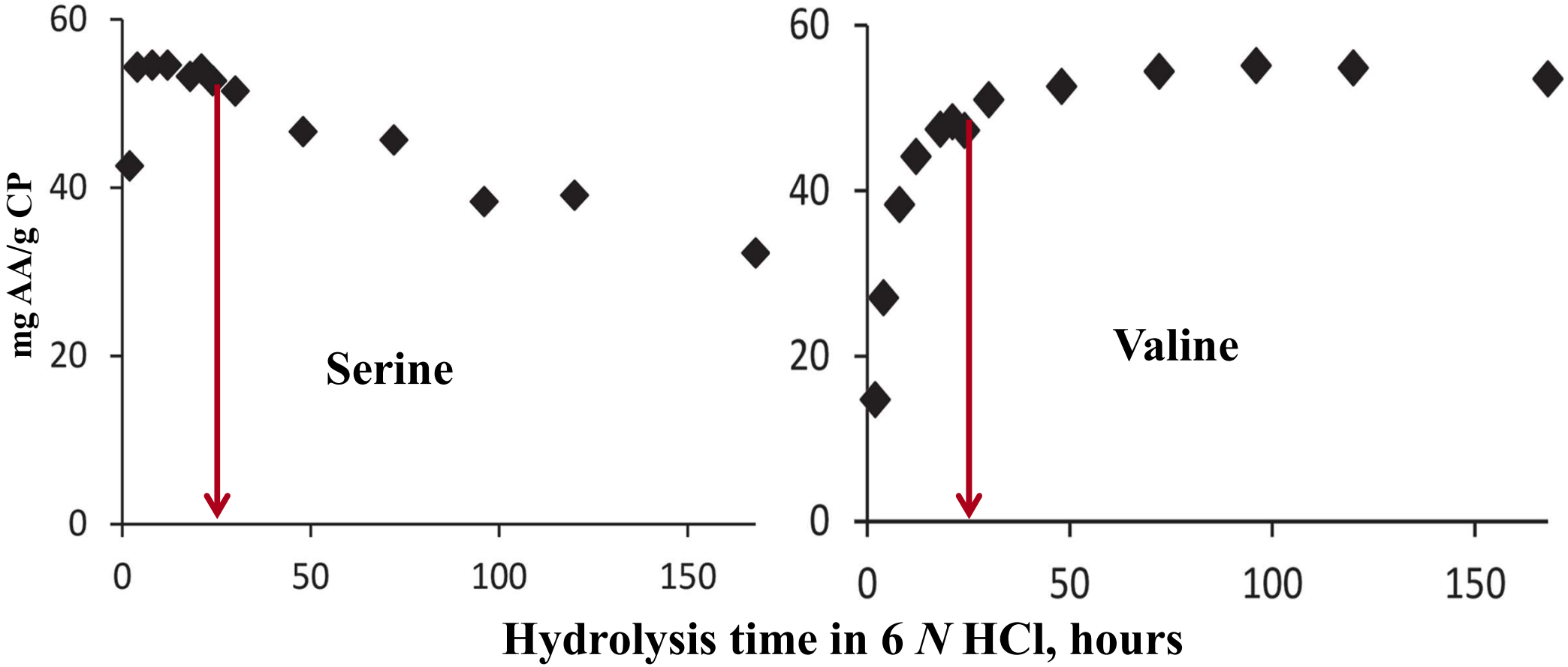
Amino Acid Analysis—Moore & Stein





Time-Course of AA Release During HCl Hydrolysis (Lapierre et al., 2019)

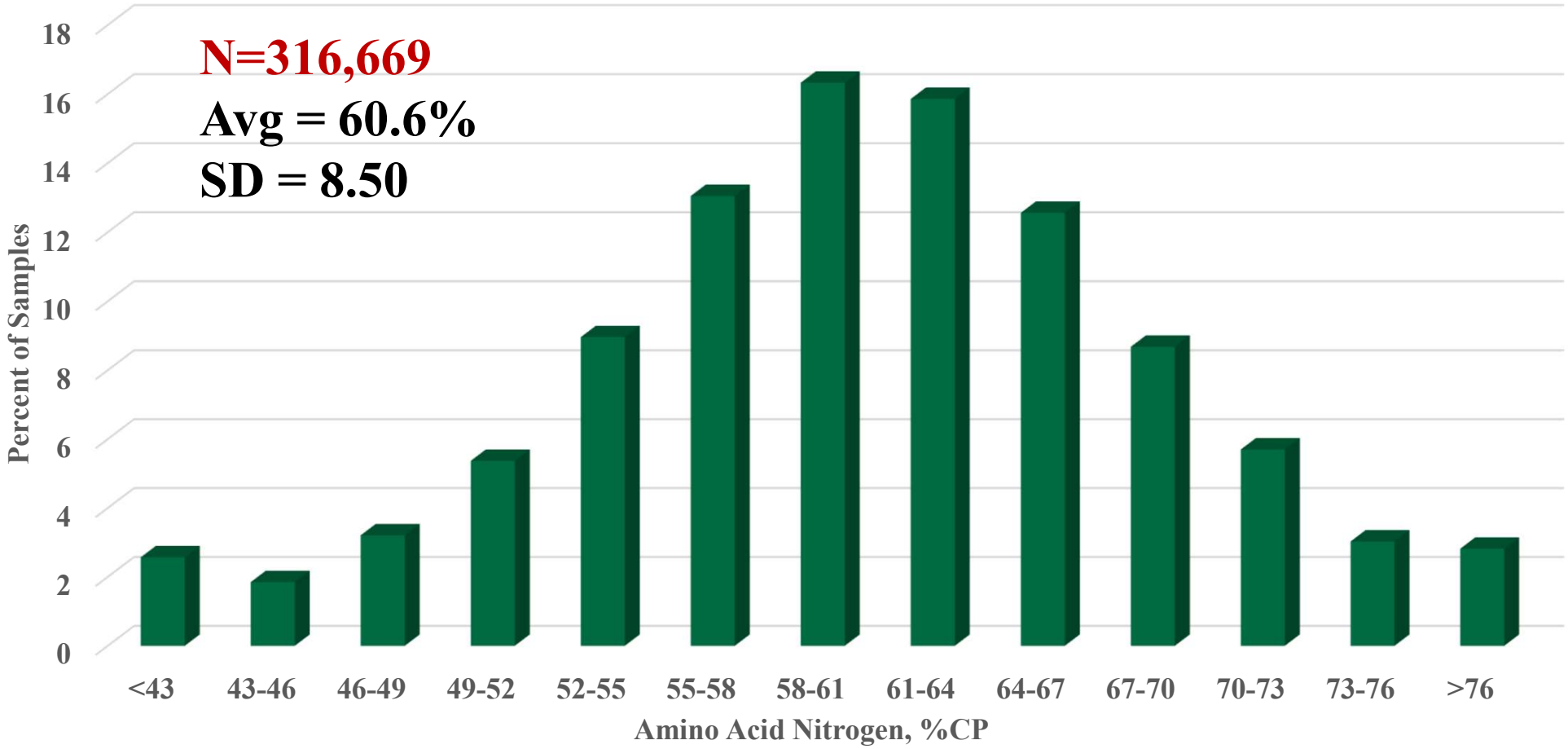


Laboratory AA Analyses

Source	Item	Comments
AAA with ^{15}N or ^{13}C Std AA	Calder et al., RCMS 13: 2080, 1999	Greater Accuracy & Precision
AAA by NIRS (Evonik)	Developed Calibrations (Fontaine et al., JAFC Vol. 49-52, 2001-06)	Various Feeds & Foods; Collaborative Studies; Calibration Available
Univ. of Missouri Labs	Chromatographic AAA	Relatively Expensive
Dairyland Lab	Both Chrom. & NIRS	All AA or NASEM AA
Cumberland Valley	Both Chrom. & NIRS	Large NIRS AA Database
Rock River Lab	NIRS Total AA; Table Feed Compostions	Satisfied w/ results

Distribution of NIRS AA-N (% Total CP) in Haylage

(Ward; CVAS; 2022-2025)



Jugular Infusions of Met+Lys+His (MKH) & Ile+Leu (IL) on Production (Yoder et al., 2020)

	Treatment				Prob.[3]	
Trait	Cntrl[1]	MKH[2]	IL[2]	MKH+IL	MKH	IL
ECM, kg/d	47.2	48.3	49.1	50.1	0.14	<0.01
Milk prot., kg/d	1.46	1.52	1.50	1.60	<0.01	<0.01
Milk fat, kg/d	1.68	1.72	1.74	1.74	0.60	0.22
N-eff., %	38.1	*38.1*	38.3	39.6	*0.09*	0.19

[1]Basal = 1.69 Mcal NE_L/kg DM; 15% CP; RDP Adequate; Met supply/req = 72%
[2]MKH = Limiting EAA Effect; IL = Anabolic Signaling ("mTOR") Effect
[3]MKH x IL Interaction Prob. = 0.18 for N-eff.

Is MidIR-Absorbance Reliable for MUN?

1. **Early $MidIR_{abs}$ Gave Variable MUN Results**
   a. **GAB (2003)--$MidIR_{abs}$ Accurate [MUN] (Period-Model)**
   b. **Recent $MidIR_{abs}$ [MUN] Calibrations Improved (Dave Barbano)**
2. **DHI MUN Data Not Timely to ID Diet Mix-ups**
3. **MUN Assay in the Parlor? (BouMatic MilkGenius)**

Summary

1. Measure Total N w/ Elemental Analysis (not Kjeldahl)
2. Quantifying RDP & RUP
   a. In Situ Methods Still "Useful"; New Methods Needed
   b. Intestinal RUP Digestion of RUP by 3-Step or Ross Assay
3. Measuring Microbial Protein in the Rumen
   a. Labeling MP w/ $^{15}NH_3$ Superior to Purines
   b. Urinary Purine Derivatives Detect Trt Differences
4. Use Lapierre Correction Factors to Account for AA Hydrolysis Losses
5. Knowing EAA Supply Essential to Optimizing Milk
6. MidIR-Absorbance Milk Urea Values are Satisfactory

Questions
&
Comments

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