PROTEIN ASSAY BY MICROBIURET: STANDARDIZATION

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From DBF's Hopkins Notebooks, **III**, p. 102 & **VI**, p. 75.
http://biology.clc.uc.edu/fankhauser/Labs/Cell_Biology/Microbiuret/Microbiuret.htm

CAUTION: Wear safety goggles and handle the microbiuret reagent with care since it is a caustic solution of ~30% NaOH (lye). Any hint of slipperiness on your fingers should be rinsed off with a solution of **5% boric acid** (in squirt bottles) followed by thorough hand washing.

Microbiuret reagent is an alkaline solution of copper ions which complex with the peptide bonds in protein to produce a blue-purple color (absorption max = 310 nm)[1]. Following Beer's Law, the color intensity should be proportional to protein concentration, and the amount of protein in a sample can be assayed in unknown solutions. The first step is to construct a "standard curve" by assaying known amounts of protein and deriving a conversion factor to convert from A_{325} to mg protein. Then we will assay the concentration of protein in a variety of unknowns.

EQUIPMENT AND SUPPLIES, FOR A TABLE OF TWO (perform experiment in pairs):

	Microbiuret reagent[2]		30-40 mL dH_2O in 125 mL flask
	Eppendorf Repipeter (front of room)		vortex
	Squirt bottles: 5% boric acid to neutralize microbiuret spills		safety glasses
3	test tube racks: 2 for 13x100 mm, 1 for 16x150 tubes		spectrophotometer, warmed up
12	13 x 100 mm test tubes		two cuvettes in test tube rack
2	16x150 test tubes		Kimwipes, paper towel
1	1 mL displacement pipet (for dH_2O and protein solution)		
1	8 mL of mg/mL protein standard[3] in 13x100 test tube		

STANDARDIZATION OF MICROBIURET REAGENT:

1) Write out an experiment table in your book, then set up 13x100 mm tubes for the standardization, then add in sequence 1) water (use 1 mL pipet twice), 2) protein and 3) microbiuret reagent (from repipet):

tube	mL water	mL 1 mg/mL	mL microbiuret	mg protein	A_{325} _____
B	2.0	0.0	1.0		
1	1.9	0.1	1.0		
2	1.8	0.2	1.0		
3	1.6	0.4	1.0		
4	1.2	0.8	1.0		
5	0.0	2.0	1.0		

2) Calculate the mg protein in each tube and enter in your table. (1 g = 10^3 mg = 10^6μg).
3) Vortex to mix well, let sit 15 min,, and
4) Read A_{325} of each tube in a spectrophotometer, using tube B as the blank. (Read at 310 nm if you have a UV capabilities.)
5) Plot standardization curve (protein vs A_{325}), determine the inverse of the slope for the conversion factor to convert optical density (OD) units to mg protein. (*If* the slope of the line were linear, A_{325} of 1 mg/tube ≈1.25 mg/OD unit).

Wash work areas well when finished to clean up any spilled caustic materials.

[1] Our Spectronic 20s cannot measure in the UV range, but only measure absorbency down to 325 nm.

[2] **MICROBIURET REAGENT:** (Safety glasses should be worn during this experiment since this solution is close to a 30% solution of NaOH. *Handle with extreme caution.*)

Solution A:		Solution B:	
40 g	NaOH (caution, caustic)	400 mg	$CuSO_4$
100 mL	dH_2O to dissolve NaOH with caution	40 mL	water, agitate to dissolve

1) *Q.s.* To 150 mL with dH_2O
2) Add solution B *slowly* to solution A with stirring.
Store in labeled bottle marked **CAUTION**: *caustic.*

[3] **STANDARD PROTEIN, mg/mL:** Prepare 1 mg/mL solution of standard protein (bovine serum albumin [BSA], or egg albumin) by adding 100.0 mg of powder to 80 mL dH_2O, thoroughly dissolve with stirring, avoiding foaming which denatures protein. If possible, let sit at 4°C for a week to completely dissolve. *Q.s.* to 100.0 mL. Store at 4°C. (Need ~5 mL/student).

SAMPLE LAYOUT OF AN EXPERIMENT: PROTEIN CONCENTRATION IN UNKNOWNS BY MICROBIURET

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1. **PLAN YOUR EXPERIMENT, WRITE OUT YOUR EXPERIMENT TABLE:**
   Calculate the dilutions of unknowns needed to bring their protein concentrations down to approximately 1-5 mg/mL. Plan two tubes per each diluted sample, one with 0.1 mL, the other with 1.0 mL. Calculate and record the amount of water required for each tube to *q.s.* to 2.0 mL. **Create a table** similar to one in the standardization with these nine columns:

tube #	specimen	dilution factor	mL H_2O	sample aliquot	mL microbiuret	A_{325}	mg protein per tube	mg protein in 100 g sample

   Include a blank, as in the standardization procedure, and **standardization tubes** with 0.5 and 1.0 mg standard protein each.
2. **PREPARE SAMPLE: DILUTE, SUSPEND OR DISSOLVE:**
   The final concentration of protein in the diluted samples should be between 1 to 5 mg/mL.
   Solids: For a 1% suspension: weigh out 300-500 mg. Grind very fine in mortar and pestle. Add a few drops dH_2O, grind to paste, add few more drops, make slurry, wash grindings into graduated cylinder, q.s. to 100x weight with dH_2O (i.e., 30 mL for 300 mg solid).
   Liquids: For dilute biological fluids like salivaor urine, use 50 uL and 200 uL undiluted. Collect saliva in 10 mL beaker. For concentrated fluids (egg white or yolk, blood, milk, etc) make a 1:50 dilution: add 0.020 mL to 0.980 mL dH_2O. Vortex thoroughly after the diluting.
3. **SET UP TUBES, ADD dH_2O TO TUBES AS IN YOUR TABLE:**
   Set up the appropriate number of labeled, clean 13 x 100 mm test tubes in a rack (2 tubes/sample). Add the dH_2O first *then* the protein sample, *finally* the microbiuret reagent.
4. **ADD PROTEIN ALIQUOTS TO THE SET OF TUBES:**
   Carefully following your protocol table, add the prescribed amounts of standard protein to the standardization tubes, and 0.1 and 1.0 mL aliquots of diluted specimens to their tubes. Samples should always be added just below the surface of the water.
5. **ADD 1 mL OF MICROBIURET TO EACH TUBE:** Make a visual check to see that all tubes appear to have a identical final volume of 2 mL in them (water + sample). *Then* add 1.0 mL of microbiuret reagent by Eppendorf Repipeter , mix well by vortex, let sit for 15 min.
6. **READ ABSORBENCY AT 325 nm:** Use the B tube (containing no protein) as the blank, determine the A_{325} of each tube in succession. You may not need to wash the cuvette between samples, but drain it thoroughly, touching off the last drop from the cuvette on a paper towel prior to adding the next specimen to minimize cross contamination. (Our Spect 20s may not blank out at 325. Increase the wavelength until you *can* blank it out. Note wavelength used.
7. **CALCULATE THE PERCENT PROTEIN IN THE ORIGINAL SPECIMEN**:
   Calculate how much protein is in each tube using the conversion factor from the previous lab (A_{325} of the specimen x conversion factor (C.F.). Do the two standard tubes agree with the standardization? Calculate the concentration in the original sample:

mg/mL in sample = (A_{325}) x (C.F. mg/A_{325}) x (1/ mL aliquot used) x (dilution factor) x (suspension factor)

examples:	A_{325}	conversion factor	aliquot fact	Dil.F.	Sus.Fac.	
%protein in saliva =	0.052 x	(2.80 mg protein/A_{325}) x	(1/0.3 mL) x	5 x	1	=2.43mg/ml = 0.24%
%protein in dog food =	0.144 x	(2.80 mg protein/A_{325}) x	(1/0.1 mL) x	1 x	100	= 400mg/g = 40%

Prepare a table of data of the results from the entire class.

Set up tables with prepared suspensions/dilutions of dog food, cat food, milk, etc.
Place pipeters with 10. And 0.1 volumes at each table, rotate around with tubes having appropriate volume of water in them.

Used for distribution of MB.

MICROBIURET DATA TABLE

Date: Name:

tube	specimen	dil. fact.	mL H2O	mL samp aliquot	mL MB	$\underline{A_{325}}$	mg prot/ tube	mg prot/ 100 g samp
B	dH2O	--	2.00	--	1.0			
1	mg/mL BSA	1	1.9	0.1	1.0			
2	mg/mL BSA	1	1.8	0.2	1.0			
3	mg/mL BSA	1	1.6	0.4	1.0			
4	mg/mL BSA	1	1.2	0.8	1.0			
5	mg/mL BSA	1	--	2.0	1.0			
6								
7	saliva	1		0.050	1.0			
8	saliva	1		0.200	1.0			
9	milk	50		0.050	1.0			
10	milk	50		0.200	1.0			

Dilute the milk 1 to 50: 0.980 mL dH2O + 0.020 milk