PROTEIN IODINATION TO KNOWN SPECIFIC ACTIVITY USING A SPARINGLY SOLUBLE CHLOROAMIDE, 1,3,4,6-TETRACHLORO-3A,6A-DIPHENYLGLYCOLURIL (IODO-GEN, AVAILABLE FROM PIERCE)

Principle: $^{125}I^-$ is oxidized to $^{125}I^+$, which covalently labels protein tyrosines through electrophilic substitutions. By using tubes coated with a small amount of sparingly soluble yet highly efficient chloroamide, Iodo-gen, instead of soluble chloroamine-T, damage to proteins is minimized (Fraker and Speck, BBRC 80:849-857, 1978). Using quantitative methods, the specific activity of iodinated antibodies may be calculated and used to determine the number of antigen sites/cell with which an antibody reacts. For more detail on iodination see Bolton, Amersham Review 19, and for site number quantitation with ^{125}I- $F(ab')_2$ fragments see Jensenius and Williams, Eur. J. Immunol. 4:91-97, 1974.

Reagents:
- Protein to be iodinated, 0.1 mg/ml in borate saline
- Iodogen (Pierce), 0.1 mg/ml in methylene chloride
- pH 8.2 borate saline: 0.01 M sodium borate, pH 8.2, 0.14 M NaCl
- Tyrosine, 0.4 mg/ml in borate-saline
- Tris-saline-azide dialysis buffer (TSA)
- 1% bovine serum albumin (BSA) + 1 mg/ml KI or NaI in TSA

Procedure: Dissolve Iodogen in methylene chloride, 0.1 mg/ml. Coat 12 x 75 mm glass, disposable, annealed tubes with 20 µl of solution by vortexing when rotating tubes in a 37° bath in the hood. Coated tubes may be stored in a desiccator over drierite protected from light for at least 2 weeks.

In a 1.5 ml Sarstedt conical tube mix 100 µl 0.1 mg/ml protein and 200 µCi ^{125}I (2 µl of 100 µCi/µl ^{125}I brought to 0.04 M in Na phosphate, pH 7.5 and neutralized). Assuming 100% incorporation, 2 atoms of ^{125}I will be incorporated/150,000 MW IgG.

Immediately mix and transfer 95 µl to a pre-cooled Iodogen-coated tube on ice. Cover with parafilm and allow reaction to proceed for 5 min. on ice with gentle hand rotation of the tube. Terminate the reaction by transferring 85 µl to a tube containing 20 µl of 0.4 mg/ml tyrosine in borate-saline.* After 1 min, add 400 µl 1% BSA + 1 mg/ml KI. Remove 10 µl and dilute into 990 µl borate-saline and later count 10 µl of this 1/100 diluted material to determine total radioactivity. Transfer the remaining 500 µl in the tube into a dialysis sack and ensure quantitative recovery by rinsing the tube and transfer pipette with 500 µl 1% BSA-1 mg/ml KI and adding this to the dialysis sack. Dialyze each protein separately vs exactly 1,000 ml of tris-saline-azide in a covered vessel protected from light overnight. Remove a 1 ml aliquot for ^{125}I counting. Quantitatively transfer dialysis sack contents to a tube. Measure the volume and count 10 µl of 1/100 diluted material. Also count the empty sack. Dilute ^{125}I protein appropriately in 10% BSA and store at -80°.

Calculating specific activity: Assume quantitative recoveries of protein at all steps except dialysis. Assume that all counts which do not dialyze out are due to protein. This follows the finding of Bolton (Amersham Review 19) that free ^{125}I is quantitatively recovered in gel filtration, while

^{125}I-protein is not. Before counting, dilute all samples to 1 ml and use the same plastic (less γ absorption than glass) tubes for all. Volume and containers affect count rate.

*No metabisulfite is added, thus avoiding S-S reduction if $F(ab')_2$ fragments are being labeled.

CALCULATIONS:

recovery of protein from dialysis sacks =

$$\frac{^{125}\text{I in protein after dialysis}}{^{125}\text{I total input} - {}^{125}\text{I dialyzed out}}$$

protein concentration after dialysis or after dilution for storage =

$$\frac{\text{input } \mu g}{\text{final vol}} \times \frac{85\ \mu l}{100\ \mu l + \text{vol } ^{125}\text{I}} \times \frac{495\ \mu l}{505\ \mu l} \times \text{recovery}$$

Remove a standard volume of the samples, e.g., 10 µl, and place in the same type of tube as samples will be counted in the saturation binding assay. This allows determination of the cpm/µg.

For binding assay, calculate:

molecules/cpm =

$$\frac{\mu g \text{ prot}}{\text{cpm}} \times \frac{6 \times 10^{23} \text{ molecules}}{\text{mole}} \times \frac{\text{mole}}{150{,}000 \text{ g (IgG)} \text{ or } 105{,}000 \text{ g } F(ab')_2} \times 10^{-6} \text{ g}/\mu g$$

In each new assay, recount this standard sample to correct for differences in machine efficiency and decay.

In a saturating binding assay, molecules/cell =cpm/cell x molecules/cpm.

For each ^{125}I-protein preparation calculate 1) dpm/µg using the ^{129}I standard and assuming the same efficiency for ^{125}I and ^{125}I, and 2) the molar ratio of

^{125}I: protein =

$$\text{dpm}/\mu g \times \frac{\text{mCi}}{2.2 \times 10^{9} \text{ dpm}} \times \frac{\text{n mol } ^{125}\text{I}}{1.5 \text{ mCi (carrier-free)}} \times \frac{150\ \mu g/\text{NMol (IgG)}}{\text{or } 105\ \mu g/\text{NMol } (F(ab')_2)}$$

IODINATION OF ANTIBODIES ON IMMUNOADSORBENT COLUMNS

<u>Principle</u>. Antibodies are iodinated while bound to antigen-coupled Sepharose and then acid eluted. The advantages are that no prior purification of the antibody is required, and that complementarity determining tyrosines are protected from iodination. Ref: Miles and Hales, Biochem. J. <u>108</u>:611 (1968) and Herzenberg and Herzenberg, in Handbook of Experimental Immunology, 3rd Edit., p. 12.21 (1978). For monoclonal antibodies which have already been purified, iodination in solution gives a much higher yield of ^{125}I-antibody, which is equally active.

Reagents:		
	1-5 mCi ^{125}I, carrier free, high conc. (NEZ-033H)	
	antigen-Sepharose	
	Sephadex G-25	
	$F(ab')_2$ or IgG antibody	
	0.8% NaCl, 0.05 M Na Phosphate pH 7.5 (PBS)	50 ml
	0.2 M Na phosphate pH 7.5	1 ml
	0.1 M glycine HCl pH 2.5	2 ml
	0.1 M glycine HCl pH 2.5, 1% BSA	2 ml
	2 mg/ml chloroamine T, dissolved just before use in PBS	1 ml
	0.1 N HCl	1 ml
	1 M Tris-HCl pH 8.4	1 ml

<u>Procedure</u>

1. Prepare a mini-column from Sarstedt 701 tubes (0.4 ml), pack with glass wool, and add a 50 µl bed of Sephadex G-25 and then a 10 µl bed of 2 mg coupled antigen/ml Sepharose for 1-2.5 mCi ^{125}I or 20 µl for 3-5 mCi ^{125}I.

2. Pre-elute with 1 ml glycine-HCl pH 2.5 and wash with 2 ml PBS.

3. Slowly pass through sufficient antibody to saturate the column ~ equivalent of 0.2 ml of serum of mouse Ig-absorbed rabbit anti-rat IgG, or ~ 1 mg $F(ab')_2$ per 20 µl bed.

4. Wash with 4 ml PBS and pinch off effluent.

5. Prepare ^{125}I: add 10 µl 0.2 M Na phosphate pH 7.5, then sufficient 0.1 N HCl to neutralize the NaOH in the ^{125}I.

6. Add 2 mg/ml Chloroamine-T to the ^{125}I, (5 µl for 10 µl bed of Sepharose and 10 µl for a 20 µl bed), mix, and immediately add to the column and suspend the Sepharose layer by mixing for 1 min.

7. Wash with 2 ml PBS (collect as 'flow-through').

8. Elute with 2 ml 0.1 M glycine-HCl pH 2.5 + 1% BSA into a tube containing 1 ml of 1 M Tris-HCl, pH 8.4 ('eluate').

9. Determine with a geiger counter the relative proportion of counts in the 'flow-through', 'eluate', and remaining on the column. About 30%

incorporation into the eluate is normally achieved.

10. Dialyze O/N vs. tris-saline-azide, dilute to 10,000-20,000 cpm/μl with 10% BSA in Hanks HEPES and store at -80° in 2 ml aliquots.

T. Springer, January 1984

IODINATION OF CELLS WITH GLUCOSE OXIDASE-COUPLED LACTOPEROXIDASE

Ref: Hubbard and Cohn 1972 J. Cell Biol. 55, 390; Trowbridge and Hyman 1975 Cell 6:279.

Lactoperoxidase catalyzes iodination using H_2O_2 as oxidant; glucose oxidase supplies H_2O_2 by the reaction glucose + H_2O + O_2 $\rightarrow$ H_2O_2 + gluconic acid.

Reagents
^{125}I : NEZ-033A, neutral pH, 100mCi/ml.
Hank's-HEPES-glucose: Hank's balanced salt solution (without phenol red) + 10mM HEPES pH 7.2 + an additional 0.2% glucose (Hank's contains 0.1% glucose, this brings to 0.3%). Be sure to exclude phenol red, since it would be iodinated. Exclude azide since it inhibits lactoperoxidase.
Lactoperoxidase, crystalline suspension in $(NH_4)_2SO_4$, ~1,000 units/ml, Boehringer Mannheim 107 174. Resuspend crystalline suspension, remove a 10µl aliquot, microfuge, aspirate supernatant, and dissolve in Hank's-HEPES to bring to 62.5 units/ml.
Glucose oxidase, solution in 0.1M acetate pH 4 containing thimerosal, ~1,000 units/ml, Sigma Type 5, G6500. Remove a 5µl aliquot and dilute to 12.5 units/ml in saline with no glucose, e.g. .14M NaCl.
12 ml conical polypropylene tubes (Sarstedt 57.527). 1.5 ml microfuge tubes, ultracentrifuge tubes, small diameter dialysis tubing.

Procedure.

Wash cells 3x in Hanks-HEPES-glucose. Cells are washed and held at 4^0 until the iodination. Filter through nytex to remove aggregated, dead cells. Resuspend to 2 x 10^7/ml. Viability should be >95%. Place in 12 ml polypropylene tube. The iodination should be done immediately after cells are washed, as they deteriorate in protein-free solutions. Enzymes should be stored on ice and used not longer than 8 hours after dilution.

Warm the cells to room temperature and per 1 ml add
1) 0.625 units lactoperoxidase (10µl)
2) 0.125 unit glucose oxidase (10µl)
3) immediately, 1 to 5 mCi of ^{125}I (10 to 50 µl).

Allow to react 15 min at room temp with resuspension every 5 min. Dilute to 10 ml with Hank's-HEPES-glucose. Remove 5 µl aliquot for counting input counts. Pellet cells. Wash 1x.

Lyse cells at 10^7/ml in: 0.01M tris-HCl, pH 8, 0.14M NaCl, 1% Triton X-100, 1% hemoglobin, 1mM iodoacetamide, 1mM PMSF (add PMSF fresh from a 50 mM solution in absolute ethanol just before addition to cells; its $t_{1/2}$ in aqueous solution is 8 min.). Incubate 10 min at 4^0C. Microfuge x 5 min. and save supernatant. After preclearing, ultracentrifuge at 100,000 g x 1 h or 200,000 g x 1/2 h. Dialyze versus 0.01M tris-HCl pH 8.0, 0.14M NaCl, 0.05% NaN_3. Count a 5µl aliquot and determine % incorporation into detergent-solubilized, non-dialyzable material, which sould be 1-2%.