BioAMS Tutorial
Introduction

The first papers on Accelerator Mass Spectrometry (AMS) using modern particle accelerators were published in Science in 1977. These papers were from two groups working on AMS, one from Simon Fraser University (Nelson D.E., Korteling R.G., Stott W.R. “Carbon-14: Direct Detection at Natural Concentrations” Science, 1977, 198(4316), p.507) and the other at the University of Rochester (Bennett C.L., Beukens R.P., Clover M.R., Gove H.E., Liebert R.B., Litherland A.E., Purser K.H., Sondheim W.E. “Radiocarbon Dating Using Electrostatic Accelerators: Negative Ions Provide the Key” Science, 1977, 198(4316), p.508.) After these reports, many isotopes were investigated and developed for AMS analysis. In all instances, the use of AMS has shown unparalleled sensitivity in radioisotope detection. These investigations led to utilizing AMS for radiocarbon-dating archaeological artifacts. Some of the processes developed for this technique are now used in biological investigations utilizing AMS.

Radiocarbon and other radioisotopes have been in use as biological tracers for many years but the investigations were limited by the detection techniques then available. The proven sensitivity of AMS led to interest in doing these types of experiments using AMS for the detection of the label. This usage is what we refer to as BioAMS. The first BioAMS paper was published in 1990 by Turteltaub et.al. (K. W. Turteltaub, J. S. Felton, B. L. Gledhill, J. S. Vogel, J. R. Southon, M. W. Caffee, R. C. Finkel, D. E. Nelson, I.D. Proctor, and J. C. Davis (1990). “Accelerator mass spectrometry in biomedical dosimetry: Relationship between low-level exposure and covalent binding of heterocyclicamine carcinogens to DNA (14C detection of 2-amino-3,8 dimethylimidazo[4,5-f]quinoxaline)” Proc. Natl. Acad. Sci. USA 87 (July 1990, Medical Sciences): 5288-5292).
This tutorial is presented using slides from a presentation followed by a discussion of the material presented on the previous slide. The discussion slides have the same titles as the presentation slide to which they refer.

This tutorial also emphasizes radiocarbon ($^{14}$C) since it has been the predominant isotope used as a tracer in BioAMS. However, BioAMS studies have been performed using other isotopes, including tritium ($^{3}$H) and $^{41}$Ca. All isotopes used in BioAMS have considerations analogous to those expressed for radiocarbon.
AMS quantitates by directly counting rare atoms.

Isotope’s half-life determines efficiency of decay counting.

Ionization and ion transport determine efficiency of AMS.

1 dpm = 7 fmol $^{14}$C

Requires large doses of tracer.

Attomole sensitivity for mg-sized samples

Enables low dose/low exposure tracer studies.
AMS quantitates by directly counting rare atoms

The isotope detection method of BioAMS differs from that used by other isotope detection systems. The usual method used in such techniques as Liquid Scintillation Counting (LSC) is to detect each decay event in a radioactive sample and relate that to the amount of radioactive isotope in the given sample. The accuracy of measuring radioisotope amounts using this method is limited by the time necessary for accumulating enough decay events to achieve a statistically relevant outcome. Therefore the half-life of the isotope in question becomes relevant in determining the efficiency of this type of isotopic measurement. Also, since 1 dpm is equivalent to 7 fmol $^{14}$C, using this method for radiocarbon tracer experiments necessitates that a relatively large dose of chemical with the tracer be administered to the system. These large radiological and chemical doses might have adverse effects and perturb the biology of the system under study.

BioAMS uses a completely different detection system. The BioAMS spectrometer counts individual rare isotope atoms using an ion beam. This enumeration is irrespective of decay events. So the only limit on the efficiency of this measurement technique is the ability to form an ion beam and transport this beam through the detection system. The number of decays happening in the measurement time is irrelevant, as long as the radioactive half-life is sufficiently long compared to the time frame of the experiment. Therefore, BioAMS gives us sensitivity down to attomole levels in milligram-sized samples, which enables the performance of very low dose and low exposure tracer studies. The dosage levels used in BioAMS are so low that the adverse effects of radiological dosing are negligible and only the effects of the chemical activity of the agent are detected with the tracer. This can even allow for the possibility of studies to be performed with human volunteers.
AMS meets criteria for validation of a bioanalytical method

- **SELECTIVITY**: specific to the measured parameter. Molecular destruction with isotope selection
- **STABILITY**: invariant response after sample definition. AMS does not rely on biochemical specificity
- **RECOVERY**: aliquots are representative. As long as measured sample is homogenized
- **LIMIT OF QUANTITATION**: sensitivity limit in normal use. LLOQ: 3 amol $^{14}$C (LOD: 1 amol $^{14}$C); ULOQ: ~100 fmol $^{14}$C
- **RANGE & LINEARITY**: proportionality without saturation. 5 orders in magnitude dynamic range
- **ACCURACY**: reproducibility of nominal standards. AMS tuning procedures and linear normalization
- **PRECISION**: reproducibility of independent repetitions. Precision of AMS driven by counting statistics
AMS meets criteria for validation of a bioanalytical method

Validation is necessary for any novel bioanalytical methodology. This validation is usually reflective of the following parameters: selectivity, stability, recovery, limit of quantitation, range & linearity, accuracy and precision. BioAMS exhibits desirable performance in all of these parameters. Its selectivity is specific to the isotope through momentum and energy selection with the destruction of interfering molecular isobars. Its stability is demonstrated by the exhibition of an invariant measurement response after the sample is defined and prepared. There are no biochemical interferences or degradations to the sample once it has been defined. In recovery all aliquots of a sample are representative since the measured sample goes through a homogenization step in its preparation. The limit of quantitation in normal use has a lower limit of 3 amol $^{14}$C with a limit of detection of 1 amol $^{14}$C, while its upper limit is approximately 100 fmol $^{14}$C limited only by the parameters of the detection system. This gives the researcher the possibility to perform experiments with a dynamic range of 5 orders in magnitude with linear responses throughout the entire range. The accuracy of AMS is proven through the use of measuring nominal standards as referents, exacting tuning procedures, and linear normalization of measurement outcomes against the standard referents. The precision is demonstrated by the reproducibility of the independent repetitions made during the measurement cycle and is driven by the counting statistics of the detection system.
AMS minimizes radiation risk

Radiation doses can be expressed in hours of plane flight.

Human Subjects Review Committee (IRB) focus on chemical (not radioactive) risk

Typically no samples are radioactive

Low dose leads to a reduction in radiological waste disposal fees:

10CFR20.2005(a)(2): “A licensee may dispose of the following licensed material as if it were not radioactive: 0.05 microcurie or less of \(^3\)H or \(^{14}\)C per gram of animal tissue, averaged over the weight of the entire animal.”
AMS minimizes radiation risk

Using radiological isotopes in BioAMS experiments is not significant in effecting biological changes to study systems due to the radiation dose. This graph demonstrates that concept by comparing radiological exposures from normal activities to the radiological risks in the experimental exposures in a typical AMS experiment. The radiological risks are compared by determining the radiation dose as a function of the $^{14}$C-labeled compound’s biological half-life. Therefore the radiological dose from a 100 nCi exposure of drugs, toxins, or nutrients is much less than the radiation dose received from a 1 hour plane flight or the dose due to the natural abundance of $^{14}$C in a human’s body (1.3 mrem/yr). This is due to the low biological mean life of these agents as compared to the whole body doses received from the “normal” activities and natural $^{14}$C abundance.

In many instances this allows an IRB to focus on chemical risks, not radioactive risks when examining new experimental protocols using BioAMS. The sample materials used in such studies are typically not classified as radioactive. Therefore any wastes produced are not radioactive per the definitions for radiological wastes. This leads to lower costs associated with performing radiological tracing experiments as there would be no radiological waste disposal fees associated with the samples.
Only AMS offers proven ppq $\left(1 \times 10^{15}\right)$ quantitation.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Concentration</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol</td>
<td>$10^{-3}$</td>
<td>NMR, Isotope Ratio-MS</td>
</tr>
<tr>
<td>nmol</td>
<td>$10^{-6}$</td>
<td>HPLC-UV/VIS</td>
</tr>
<tr>
<td>pmol</td>
<td>$10^{-9}$</td>
<td>LSC, LC-MS, ECD</td>
</tr>
<tr>
<td>fmol</td>
<td>$10^{-12}$</td>
<td>ICPMS, Antibody</td>
</tr>
<tr>
<td>amol</td>
<td>$10^{-15}$</td>
<td>AMS</td>
</tr>
</tbody>
</table>
Only AMS offers proven ppq \((1:10^{15})\) quantitation

The quantitation limits of BioAMS lower the sensitivity of measurements made with the technique down to parts per quadrillion \((1:10^{15})\). As compared to the detection limits for other measurement techniques commonly used in biology, only AMS proves to be sensitive down to this (attomole) concentration level. This makes AMS the most sensitive analytical technique in the biologist’s arsenal. It becomes the tool that studies need for achieving extremely accurate and precise results, enabling the performance of low-dose radioactive tracer experiments, as the radiological effects from higher doses would be of concern biologically and financially.
AMS analysis is compatible with many standard biochemical techniques

Sample Definition

Direct sampling of tissues - Mass balance/distribution (homogenization)

HPLC/Chromatographic separations - Small molecule/metabolites
   (peak identification/purity)

Biomolecule extraction - DNA, RNA, protein binding (solvents/reagents)

Electrophoresis - Protein quantification (matrix)

FACS - Cell types (solvent/reagents)

Sample Preparation

Routine preparation for analysis requires milligram-sized samples

Elemental carbon analysis sometimes required for carbon content

Homogenization is key : easy for fluids; tissues must be “liquidified”

Extraneous sources of carbon must be controlled : non-volatile solvents and reagents can add carbon
AMS analysis is compatible with many standard biochemical techniques

Whenever a novel bioanalytical system is developed, its applicability can be limited by the biochemical technique it was designed to evaluate. BioAMS has been shown to be compatible with many techniques. These techniques include methods for determining mass balance or distribution of a biomolecule, detecting small molecules or metabolites, extracting DNA, RNA or proteins, quantifying proteins, and sorting cells. As with other systems, while BioAMS is compatible with these techniques, there are concerns in sample definition and preparation that need to be addressed to enable the optimum results. Sample definition issues to be aware of when performing these techniques include homogenization when directly sampling tissues, accurate peak identification and purity when performing HPLC or other chromatographic separations, solvents and reagents for biomolecule extraction or cell sorting, and the matrix used for electrophoresis separation. When preparing the sample, the issues of concern include size, carbon content, sample homogeneity, and control of extraneous sources of carbon. These concerns are easily addressed by ensuring proper sample sizing (micrograms to milligrams for routine analysis), performing elemental analysis to determine carbon content, liquifying tissues to ensure homogenization, and realizing that non-volatile reagents or solvents could add carbon to the sample.
AMS quantifies long-lived isotopes by mass spectrometry

Absolute Quantitation

\[
R_{\text{measured}} = \frac{\left(\frac{\text{counts }^{14}\text{C}}{\text{nCoul }^{13}\text{C}}\right)_{\text{sample}}}{\left(\frac{\text{counts }^{14}\text{C}}{\text{nCoul }^{13}\text{C}}\right)_{\text{standard}}} = \left(\frac{^{14}\text{C}}{^{13}\text{C}}\right)_{\text{sample}}
\]
AMS quantifies long-lived isotopes by mass spectrometry

The AMS spectrometer works by forming an ion beam in a cesium-sputter ion source and accelerating that beam through two magnetic fields that select for the masses of interest, and then measures the number of ions that are in those masses of interest. For example, during radiocarbon analysis, the ion source produces a beam of both elemental and molecular ion particles that are first passed through a magnetic field to bend the beam 90° and only ions of mass 13 or 14 are selected for injection into the particle accelerator. Ions of mass 12 are quantified in an off-axis Faraday cup. This injection process first injects mass 14 ions and then the “bouncer” changes the energy of the ion beam to inject mass 13 ions. This process is repeated many times per second. The tandem accelerator accelerates the ions to high velocity (~1% the speed of light) before collisions with a diffuse gas or thin foil eliminates any molecular ions from the ion beam and strips off one or more electrons in a molecular ion dissociator. The fragment of the initial ion beam that exits the accelerator is then passed through another magnetic field to separate the ion of interest from the ion beam. The mass 13 ions are quantified in a Faraday cup and, after final energy analysis, the mass 14 ions are detected at the end of the beam path. This process demonstrates the “bag of tricks” used in performing AMS: isotopes are selected by dispersing the ion beam according to mass in a magnetic field, the destruction of any interfering molecular isobars, and the highly efficient atom counting done by the $^{14}$C detector at the end of the beam path. Absolute quantification is performed by taking the output of the spectrometer, which is counts of $^{14}$C per nanocoulombs of $^{13}$C in the sample, normalizing that measurement versus a similar measurement of a standard material, and expressing that measured ratio as the amount of $^{14}$C per amount of carbon in the sample.
Historically, all samples were converted to graphite for AMS analysis.

Combustion (under excess O\textsubscript{2} and heat)
Sample $\rightarrow$ CO\textsubscript{2} + N\textsubscript{2} + H\textsubscript{2}O + SO\textsubscript{2} + ........

Reduction (with metal catalyst and heat)
Zn + H\textsubscript{2}O $\rightarrow$ ZnO + H\textsubscript{2}
CO\textsubscript{2} + H\textsubscript{2} $\xrightarrow{\Delta_{Co}}$ CO + H\textsubscript{2}O and
CO + H\textsubscript{2} $\xrightarrow{\Delta_{Co}}$ C\textsubscript{gr} + H\textsubscript{2}O

Sample prep. process destroys chemical information.
Historically, all samples were converted to graphite for AMS analysis

Any sample material has to be converted into a form that is amenable to being used in an ion source to make an ion beam. Since, historically, the majority of BioAMS studies have been performed using $^{14}$C as the tracer isotope, the sample material has had to be converted into graphite. In the graphitization process the sample is first sealed in an evacuated quartz vial with added oxidant. This vial is then heated to 900ºC and the sample material is oxidized to produce a mixture of gaseous oxides. The CO$_2$ and H$_2$O in the gaseous mixture are then cryogenically separated out and trapped in another vial that contains zinc and a metal catalyst. This second vial is then heated to 500ºC and the reactions occurring in it produce graphite. This graphite is then pressed into the depression of an aluminum cathode which is placed in a wheel-shaped magazine for installation in the ion source.

This sample preparation process destroys all chemical information present in the sample material and only permits measurement of the isotopic ratio of the carbon that has been extracted from the material.
Currently, interfaces enable measurement of liquid samples and continuous HPLC eluent.
Currently, interfaces enable measurement of liquid samples and continuous HPLC eluent

The historical graphitization process is procedurally complex and time consuming, especially when handling HPLC eluents and samples with less than 0.5 mg carbon. The HPLC eluents had to be collected in fractions with each fraction separately graphitized and measured. These eluent fractions and any sample with less than 0.5 mg carbon had to have carbon added to them to achieve the sample size necessary to permit adequate graphite production for subsequent measurement.

In order to address this complexity, a moving wire interface has been developed enabling the neat measurement of liquid samples such as HPLC eluent streams and low carbon concentration solutions. The interface consists of a nickel wire pulled from a supply spool through a cleaning oven that removes manufacturing oils and oxidizes the nickel surface. The sample is then introduced onto the wire and the wire passes through a drying oven that removes solvent from the liquids that have been introduced onto the wire and stabilizes the sample’s position on the wire. The wire then carries the sample into a combustion oven where the sample is oxidized to CO$_2$ gas. The CO$_2$ gas is then injected into the ion source where the carbon ion beam is produced. The used wire is then collected on another spool for disposal.

The two charts on this slide show the results of measurement using the interface. The blue spikes are the counts of $^{14}$C and the red lines are the measurements of the $^{12}$C current. The chart on the left shows measurements of 10 drops of a solution containing 1ug of DNA per drop (290ng C per drop). These 10 measurements were done in less than 10 minutes. The equivalent measurement using the graphitization process would have taken over a day of sample preparation time. The chart on the right shows the results of a HPLC eluent stream that was measured using the interface. This measurement took 12 minutes compared to the 2-3 days it would take to prepare the eluent for measurement by graphitization and subsequent AMS measurement.
AMS introduces the unit “Modern”

“Modern” is a well-defined number:
- $1.18 \times 10^{-12} \ ^{14}\text{C}/\text{C}$
- 97.89 amol $^{14}\text{C}/\text{mg}$ C
- 6.11 femtoCi $^{14}\text{C}/\text{mg}$ C
- 13.56 mdpm $^{14}\text{C}/\text{mg}$ C

- Contemporary terrestrial biological material is ~1.05 Modern
AMS introduces the unit “Modern”

Because of a historical connection to archaeological radiocarbon dating, BioAMS introduces a unit called “Modern” to the biologist. This unit is a well-defined number expressed as a ratio of $^{14}$C to total carbon in a sample. In different units, modern is $1.18 \times 10^{-12}$ $^{14}$C/C, 97.89 amol$^{14}$C/mg C, 6.11 femtoCi $^{14}$C/mg C, or 13.56 dpm/g C. The chart shows the BioAMS measurement range expressed in these various units. Due to the environmental introduction of radiocarbon in the 1960’s from above-ground nuclear tests and its subsequent elimination from the environment, contemporary terrestrial biological material measures ~1.05 Modern.
AMS provides a precise isotope ratio

“Science” is determined by the defined sample

Measured Ratio \( (R_{\text{meas}}) = \frac{\frac{^{14}\text{C}_{\text{tracer}}}{C_{\text{tracer}}} + \frac{^{14}\text{C}_{\text{tissue}}}{C_{\text{tissue}}} + \frac{^{14}\text{C}_{\text{carrier}}}{C_{\text{carrier}}}}{\text{pg/g}} \)
AMS provides a precise isotope ratio

The results of an AMS measurement are expressed as an isotope ratio of isotope of interest per common isotope for a given element. However since this isotope ratio is a dimensionless number, it can be converted to many useful units. For measurement of drugs or nutrients in tissue or fluids it can be converted to pg/g, for macromolecular binding studies it can be pmol/base, to determine a metabolic profile amol/min can be discovered and it can be expressed in fCi/g to determine specific activity.

The sole measured parameter of the defined sample is the isotope ratio. Any other sample parameter must be measured using other methods. This isotope ratio is the sum of the many carbon sources that are reflective of the individual components of a sample. The tracer, the tissue and the carbon carrier (if any was added) all contribute to the measured ratio. However, this ratio is provided without knowledge of any isotope source or molecular information in the sample. Therefore, the accuracy of the measurement must be ensured by strictly controlling these parameters. The measurement is also achieved without the use of any R&D in the measurement process, internal standards or standard curves.

AMS does not provide molecular identification, the only information provided is a ratio of rare to abundant isotope in a sample. All other desired information about a sample must be obtained using other methods.
Quantitation requires control of the carbon inventory

Measured Ratio ($R_{\text{meas}}$) = \frac{^{14}\text{C}_{\text{tracer}} + ^{14}\text{C}_{\text{tissue}} + ^{14}\text{C}_{\text{carrier}} + ^{14}\text{C}_{\text{unknown}}}{^{14}\text{C}_{\text{tracer}} + ^{14}\text{C}_{\text{tissue}} + ^{14}\text{C}_{\text{carrier}} + ^{14}\text{C}_{\text{unknown}}}

Tissue Dose

No Carrier; $^{14}\text{C}_{\text{tissue}} \gg ^{14}\text{C}_{\text{tracer}}$

HPLC Fraction

$^{14}\text{C}_{\text{carrier}} \gg ^{14}\text{C}_{\text{tracer}}, ^{14}\text{C}_{\text{tissue}}$

$^{14}\text{C}_{\text{tracer}} = ^{14}\text{C}_{\text{tissue}} \times (R_{\text{meas}} - R_{\text{tissue}})$

$^{14}\text{C}_{\text{tracer}} = ^{14}\text{C}_{\text{carrier}} \times (R_{\text{meas}} - R_{\text{carrier}})$
Quantitation requires control of the carbon inventory

Since the reported ratio is measured by summing all the carbon components contributing to the ratio, the measured ratio can be expressed as shown in the upper equation. This equation also expresses the existence of potentially unknown contributions to the measured ratio. In AMS, all sources of carbon in the defined sample must be known and accounted for. Carbon contributions can come from other sources than just the tracer, tissue or carrier. All of these contribution sources can also provide their own $^{14}$C contribution to the ratio. This contribution can come from solvent that have been insufficiently removed, plastics that have leached material in to the sample, or reagents that have been contaminated with $^{14}$C. Irrespective of the source of the carbon, it’s contribution will be reflected in the reported ratio.

This determination of the carbon inventory can be used to simplify the calculations in two general cases. The first is the “no carrier” example where the carbon contribution from the tissue is assumed to be much greater than the carbon contribution of the tracer. This is shown in the chart on the left. The carbon contribution from the tracer material is much less than the contribution from the tissue. Therefore, the denominator of the ratio can be reduced to just the carbon contribution from the tissue and the amount of $^{14}$C contributed from the tracer can be easily calculated by knowing the $^{14}$C/C ratio of un-dosed tissue. In a like manner, the chart on the right shows an example where the amount of carbonaceous material in the sample is so low that carrier needs to be added to it for graphitization. In this example, the carbon contribution is assumed to be predominantly from the carrier such that the contributions from the tracer and tissue components can be neglected in the calculation. In this case, the $^{14}$C contribution from the un-dosed tissue is also neglected as its total contribution is so low compared to the contribution from the tracer and the carrier. Again the calculation reduces to a simple equation where it is only necessary to know the ratio from the carrier, the measured ratio and the total carbon contribution from the carrier to determine the amount of $^{14}$C the tracer contributed to the result.
Case study highlights calculation of labeled compound in human tissue

**Tissue Dose**

- 59 nCi $^{14}$C-B12 oral dose
- Specific Activity: 58 mCi $^{14}$C/mmol B12

Useful conversion factors:
- 1 Modern = 97.89 amol $^{14}$C/mg C
- $1^{14}$C/C = 62.4 mCi $^{14}$C/mmol C

7-hour time point:
- $R_{\text{meas}} = 2.50$ Modern
- $R_{\text{tissue}} = 1.05$ Modern (measured from predose)
- Plasma: 0.042 mg C/mg plasma
  - $p_{\text{plasma}} = 1.02$ g/ml

\[ ^{14}\text{C}_{\text{tracer}} = (R_{\text{meas}} - R_{\text{tissue}}) \times C_{\text{tissue}} \]
\[ = (2.50 - 1.05) \times 0.042 \times 97.89 \]
\[ = 5.96 \text{ amol } ^{14}\text{C}/\text{mg plasma} \]

Concentration = $^{14}\text{C}_{\text{tracer}} \times (62.4/\text{Sp.Act.}) \times p_{\text{plasma}}$
\[ = 5.96 \times (62.4/58) \times 1.02 \]
\[ = 6.54 \text{ fmol B12/ml plasma} \]
The types of calculations that arise when performing AMS quantitation are demonstrated by this case study (C. Carkeet, et al. (2006). "Human vitamin B12 absorption measurement by accelerator mass spectrometry using specifically labeled 14C-cobalamin" Proc. Natl. Acad. Sci. USA 103(15), 5694-5699). The chart shows the results from the AMS measurements of the blood plasma from a time-course of dosing a human with a bolus of 14C-labeled vitamin B12. The results are expressed in fmol 14C-vitamin B12 per ml plasma. The calculations demonstrate the conversion from a measured ratio to a unit of biological relevance. An oral dose containing 59 nanoCuries of vitamin B12 with a specific activity of 58 mCi 14C/mmol was administered to a human volunteer. The absorption into the system was at a maximum at 7 hours and the calculation is to quantitate this maximum absorption. Using the definitions of Modern equating to 97.89 amol 14C/mg C, the observations that at the 7-hour time point the measured ratio was 2.50 Modern, the measured ratio of undosed tissue was 1.05 Modern, and the plasma had 0.042 mg carbon per mg, the amount of 14C tracer present at the 7-hour time point can be calculated by taking the difference between the measured ratio of the dosed tissue and the measured ratio of undosed tissue and multiplying by the percentage of carbon in the plasma and then converting from the Modern unit to give the amol 14C per mg plasma. This gives the result that at 7 hours there is 5.96 amol 14C per mg plasma. The concentration of vitamin B12 in the plasma is calculated by dividing by the fractional 14C labeling of the compound and multiplying the plasma density of 1.02 g/ml. The fractional 14C labeling of the vitamin B12 is determined by dividing the specific activity of the administered compound by the definition that 1 14C atom per molecule equates to 62.4 mCi 14C per mmol.

Using the proper definitions, constants and observations any BioAMS measurement can be converted from the 14C/C ratio that is the output of the measurement to any unit of interest for the biologist.