united nations
educational, scientific
and cultural
organization

international atomic
energy agency

the
abdus salam
international centre for theoretical physics

SMR 1550 - 9

WORKSHOP ON THE USE OF RECEPTOR BINDING ASSAY (RBA)

1 - 5 September 2003

Co-organized by the International Atomic Energy Agency (I.A.E.A.)

The Receptor Binding Assay for the Saxitoxins: Importance, Impediments and Solutions

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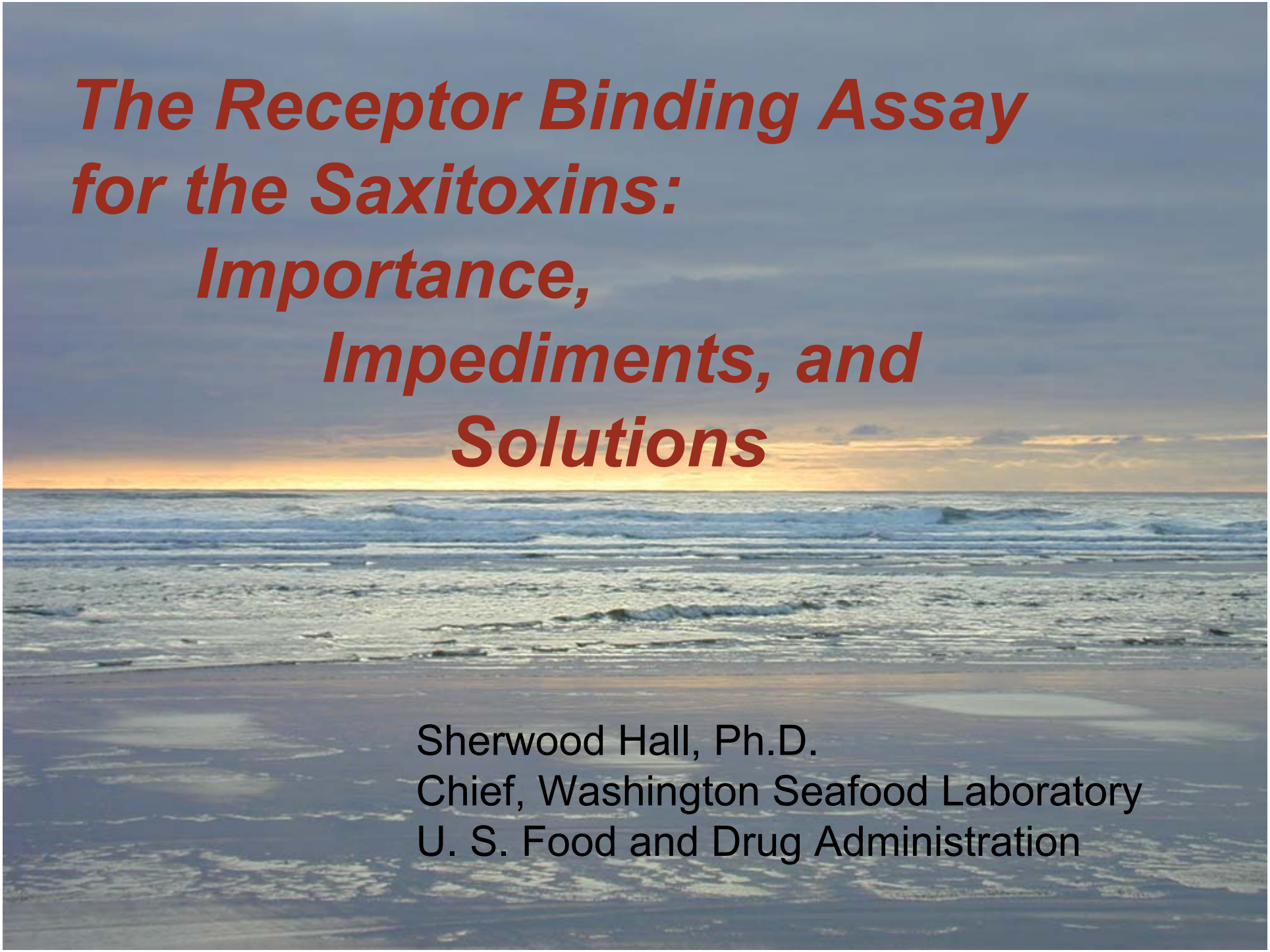


The Receptor Binding Assay for the Saxitoxins: Importance, Impediments, and Solutions

Sherwood Hall, Ph.D.
Chief, Washington Seafood Laboratory
U. S. Food and Drug Administration

S. Hall background:

Worked in marine science >30 years.
Worked with the saxitoxins since 1975.
Collaborations on receptor assay for STXs
started in 1983.
FDA since 1984,
concerned with seafood toxins in general,
involved with the development and
evaluation of management programs and
investigation of outbreaks
throughout the world

While we concluded long ago that the rba was the most promising option for replacement of the mouse bioassay for PSP, I will try to give a balanced view of the problem of seafood toxins and the various options for solution to help you make your own decisions about how the IAEA should proceed.

It must be kept in mind that neither toxicity monitoring nor the receptor binding assay will ‘solve’ the problem of marine biotoxins. But toxicity monitoring is an essential part of marine biotoxin management, and the receptor binding assay is the best way to monitor for the saxitoxins (STXs), the toxins that cause paralytic shellfish poisoning (PSP).

PSP is the most lethal of the many families of seafood toxins. This discussion will focus on the rba as applied to PSP.

The presentation, shortened to its essence:

Implementation of
the receptor binding assay
is essential, practical, and will have
significant benefits to human well-being.

1. PSP has severe negative impacts on human well-being.

2. Management programs are essential for minimizing these impacts.

3. Toxin detection is essential for management.

4. The rba is the best option to the mouse assay for the detection of PSP.

5. The availability of labeled saxitoxin is assured in the long term. The current lack of labelled saxitoxin is a short-term problem, which is being solved.

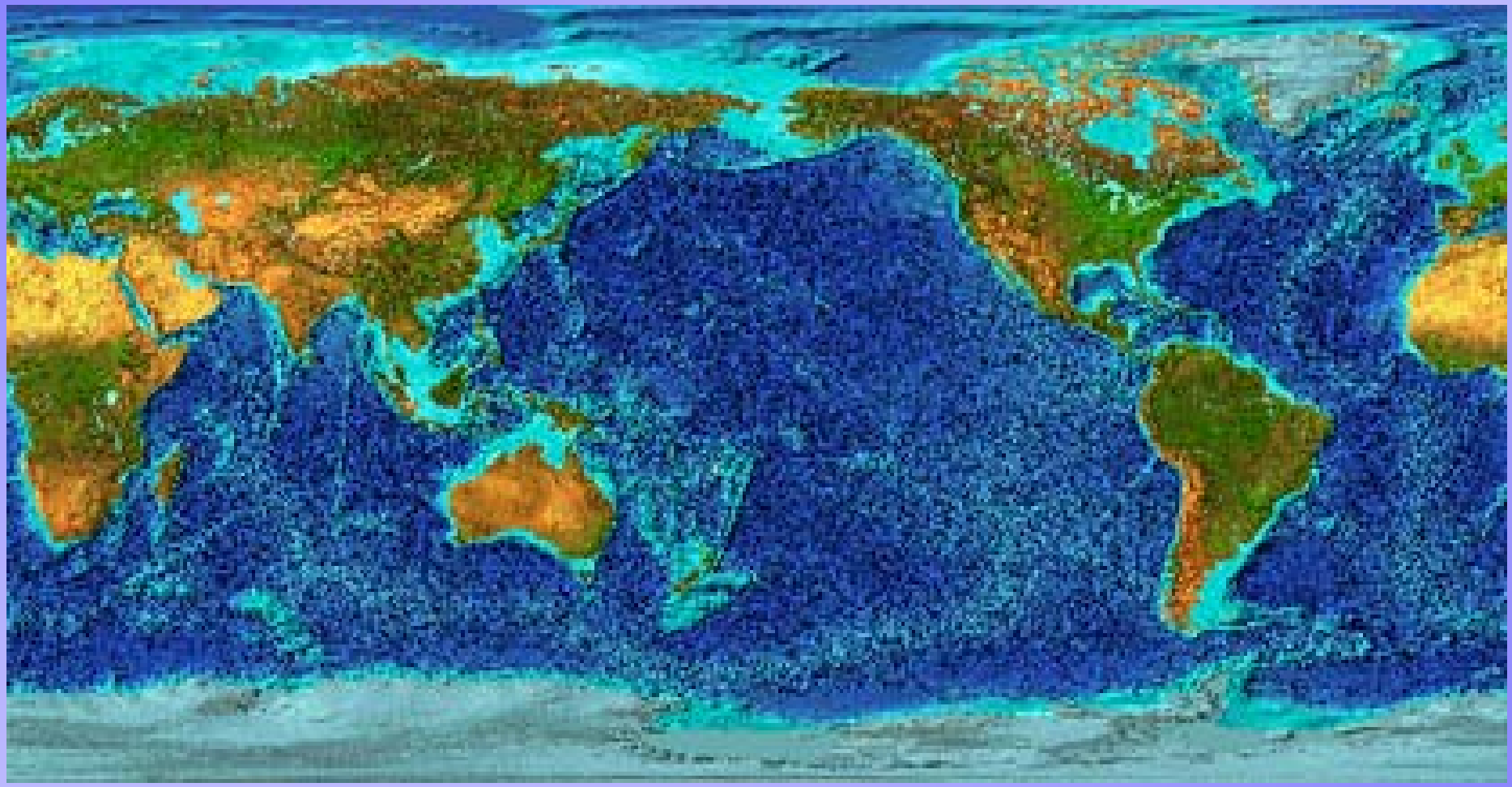
6. Implementation of the receptor binding assay is essential, practical, and will have significant benefits to human well-being.

1. PSP has severe negative impacts on human well-being

Alaska, 1799

Guatemala, 1987

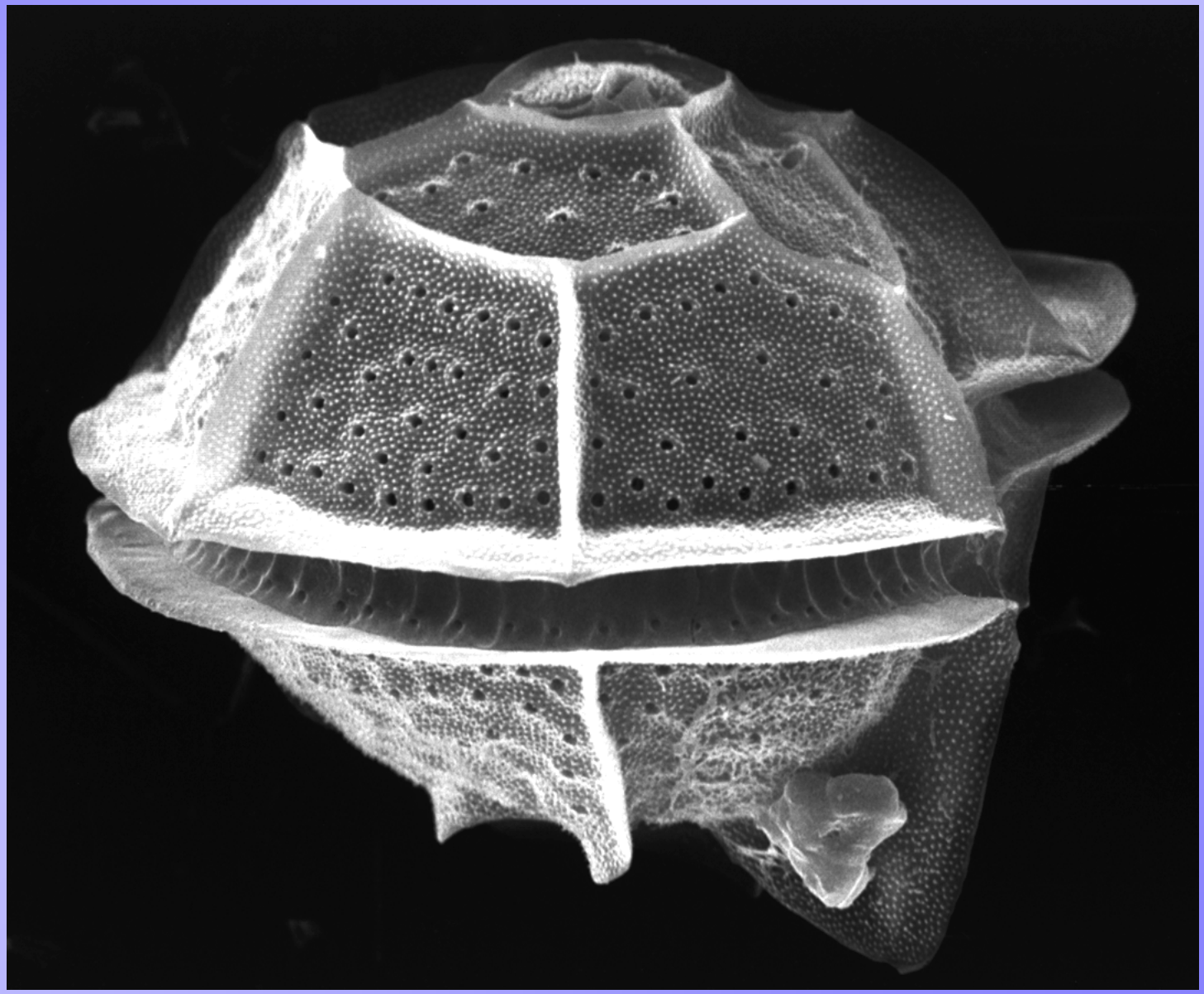

Peril Straits, Southeast Alaska




Champerico, on the Pacific coast of Guatemala


Amphichaena kindermanii

Pyrodinium bahamense



Guatemala, 1987:

26 dead

180 sick

In 3 days

In a region with no history of shellfish toxicity

Globally, the number of human fatalities from seafood toxins is not large compared to other causes of human suffering:

- infant diarrhea
- not wearing seat belts; smoking
- lightning strikes; bee stings

Very large economic and social impacts

- fear and uncertainty
- denial of a wholesome food source
- loss of market for all seafood
- deprivation of livelihood
- export/import relationships
- costs of management

Impacts often felt in the marketability of products not affected: The economic halo.

The costs of failing to manage biotoxins and prevent human illness are far greater than the cost of management.

Such problems are entirely preventable.

Effective management in the face of a severe outbreak can sustain market confidence and prosperity:

Canada- ASP

New Zealand- NSP

2. Management programs are essential for minimizing these impacts.

    Prevention?
    Prediction?
    Elimination?

In general, the problem is best managed by monitoring to identify affected product and ensure that it is not consumed

Seafood toxins are challenging to manage due to the characteristics of the plankton populations that produce the toxins

- patchy and ephemeral
- rapid increase
- diverse
  - families of toxins
  - toxins within families

3. Toxin detection is necessary for management.

Necessary, though not sufficient.

Monitoring strategies

plankton

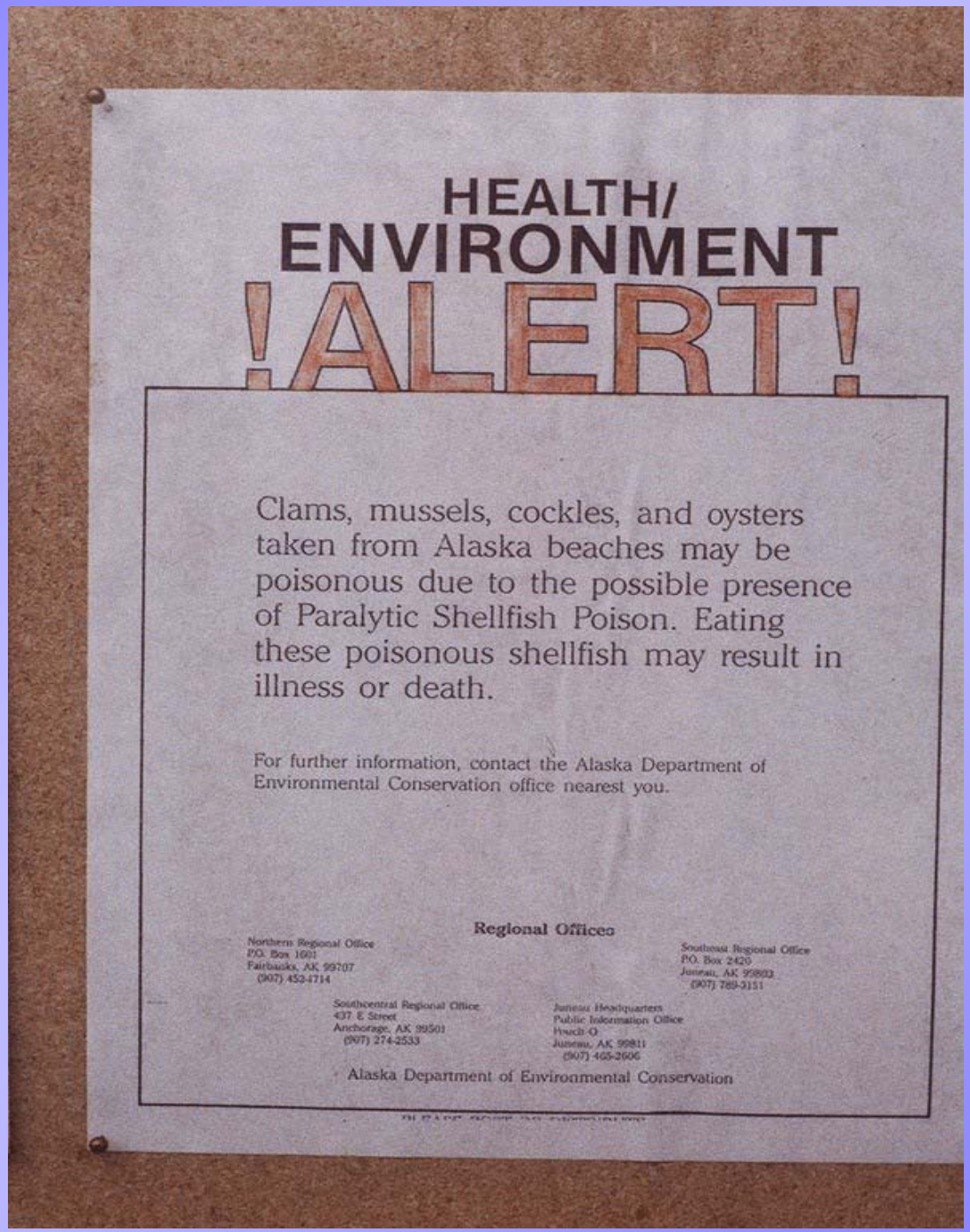
seafood toxicity

Monitoring programs are more efficient when they provide

high temporal and spatial resolution
- minimize unnecessary costs
- sustain confidence

timeliness
- due to the rapidity with which toxicity can increase

Both require intensive sampling.



HEALTH/ ENVIRONMENT !ALERT!

Clams, mussels, cockles, and oysters taken from Alaska beaches may be poisonous due to the possible presence of Paralytic Shellfish Poison. Eating these poisonous shellfish may result in illness or death.

For further information, contact the Alaska Department of Environmental Conservation office nearest you.

Regional Offices

Northern Regional Office
P.O. Box 1601
Fairbanks, AK 99707
(907) 452-1714

Southeast Regional Office
P.O. Box 2420
Juneau, AK 99803
(907) 789-3151

Southcentral Regional Office
437 E Street
Anchorage, AK 99501
(907) 274-2533

Juneau Headquarters
Public Information Office
Pouch O
Juneau, AK 99811
(907) 465-2606

Alaska Department of Environmental Conservation

CA mussel quarantine sign

Straits of Magellan

There are intrinsic limits to toxin testing

There are intrinsic limits to toxin testing:

1) Timing: No warning until
- toxins reach a detectable level
- a sample is taken, shucked, extracted
- detection method performed

(vs the rapidity with which toxicity can increase)

Frequency vs cost

2) Frequency vs cost:
Independent of the cost of toxin detection, the cost of sampling and sample prep limits the frequency (time, space) of toxin testing.

(vs the variability of toxicity in time and space)

Env obs can

Environmental observations:

CAN be conducted at a
high temporal and spatial frequency

CAN be conducted at relatively low cost, particularly through the use of volunteers

Environmental observations:

CANNOT replace toxin monitoring

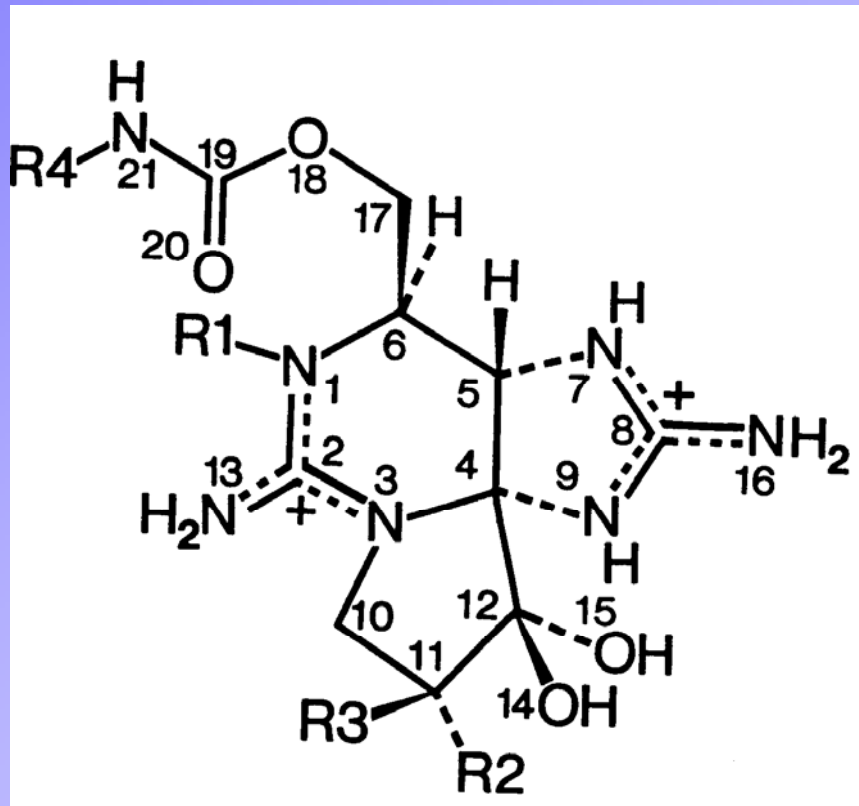
CAN focus toxin monitoring

- time
- location
- type of toxin

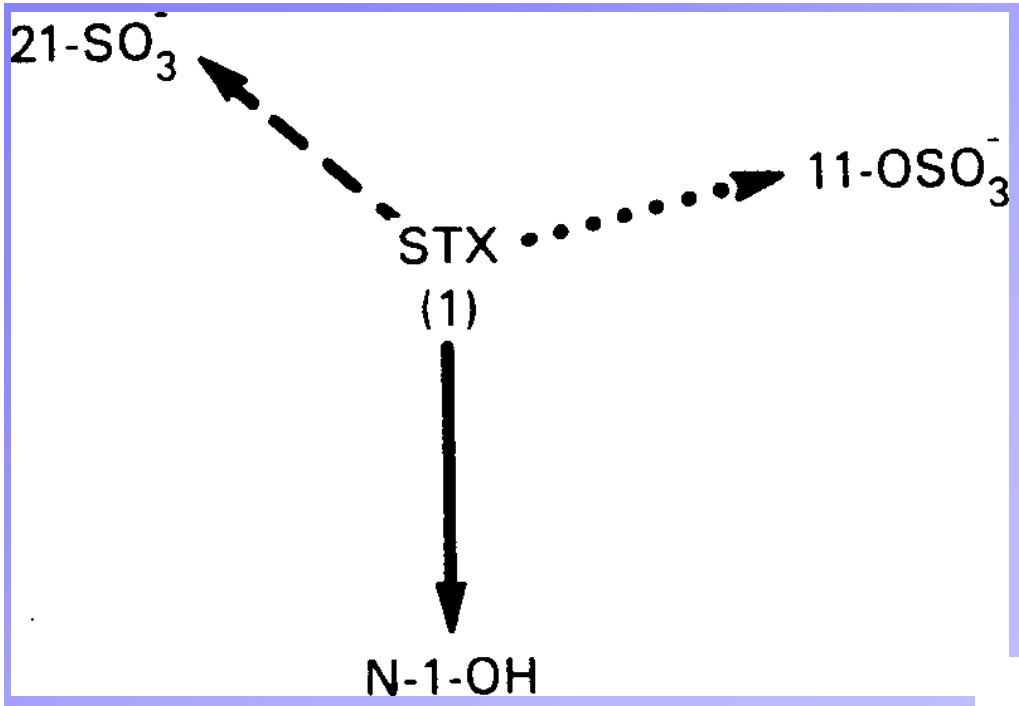
4 The rba is the best option for the detection of PSP

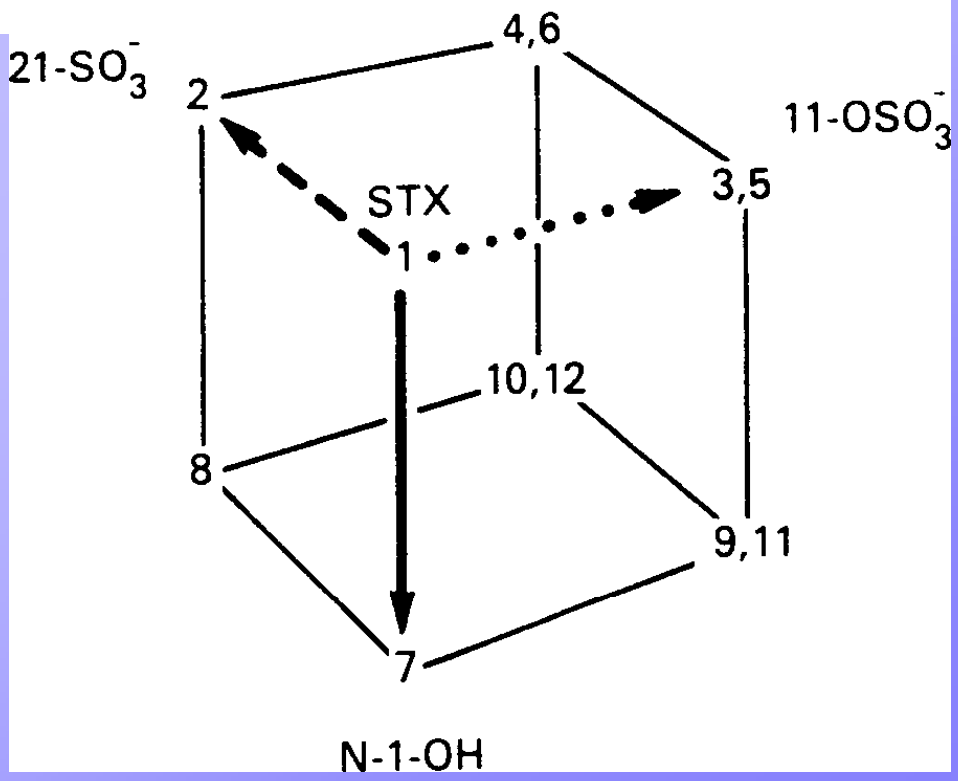
Alexandrium sp.

The ‘first 12’ of the saxitoxins



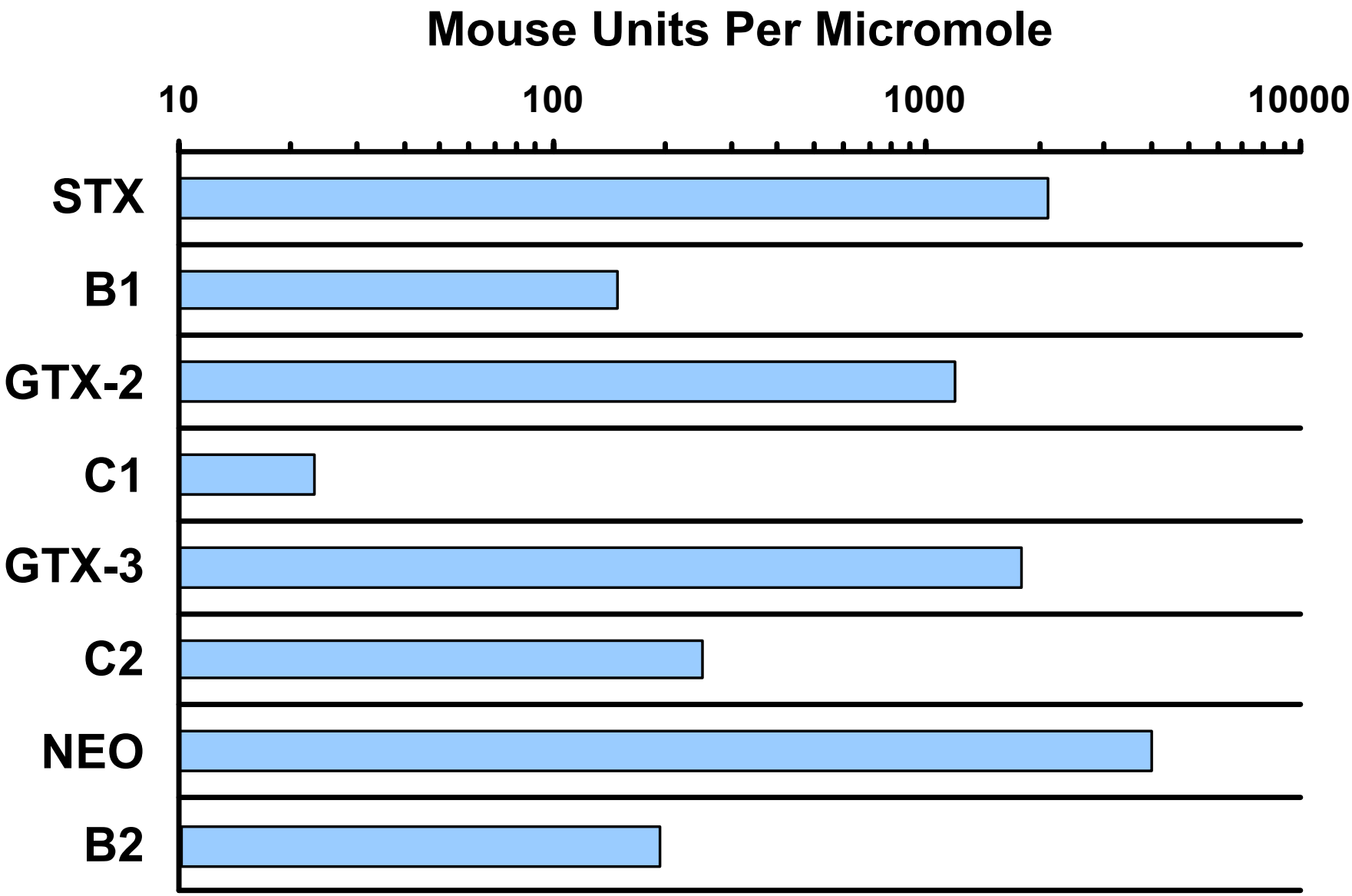
	R1	R2	R3	R4	
1	H	H	H	H	STX
2	H	H	H	SO_3^-	B1
3	H	OSO_3^-	H	H	GTX2
4	H	OSO_3^-	H	SO_3^-	C1
5	H	H	OSO_3^-	H	GTX3
6	H	H	OSO_3^-	SO_3^-	C2
7	OH	H	H	H	NEO
8	OH	H	H	SO_3^-	B2
9	OH	OSO_3^-	H	H	GTX1
10	OH	OSO_3^-	H	SO_3^-	C3
11	OH	H	OSO_3^-	H	GTX4
12	OH	H	OSO_3^-	SO_3^-	C4





Structural relationships among the ‘first 12’ Saxitoxins

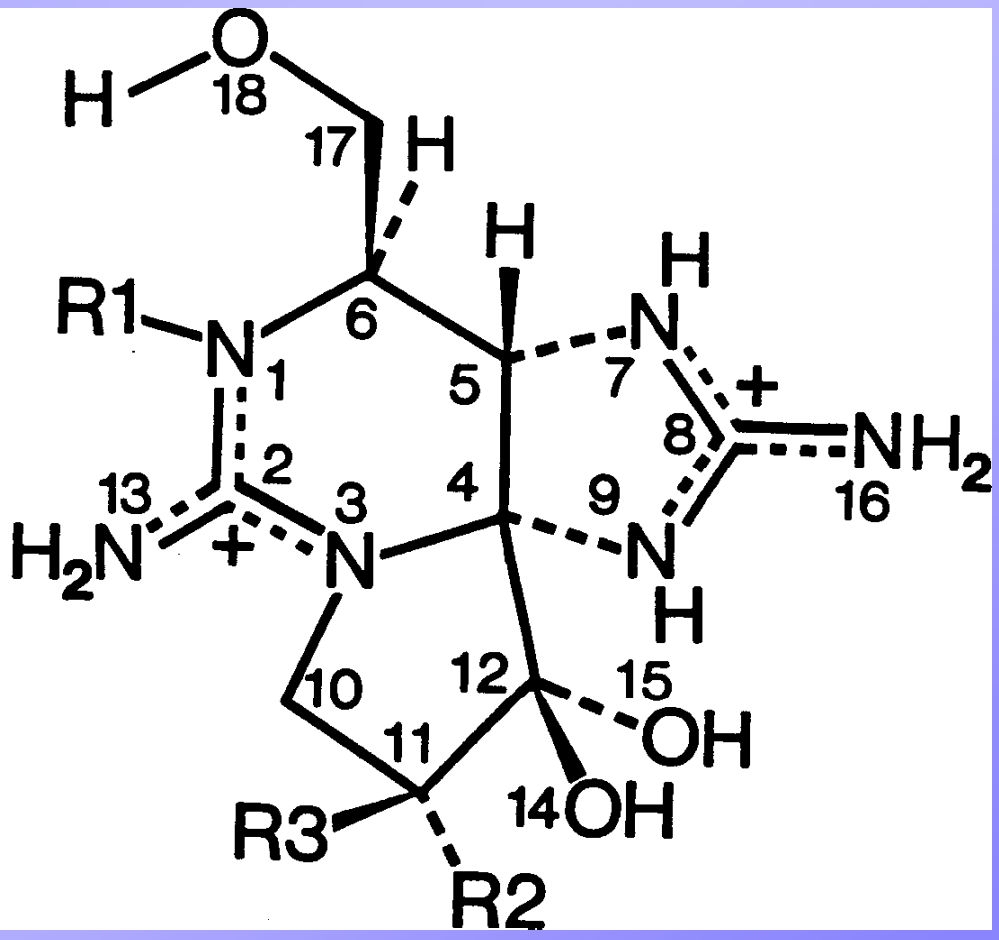
Specific Toxicities of the Saxitoxins



dcSTXs

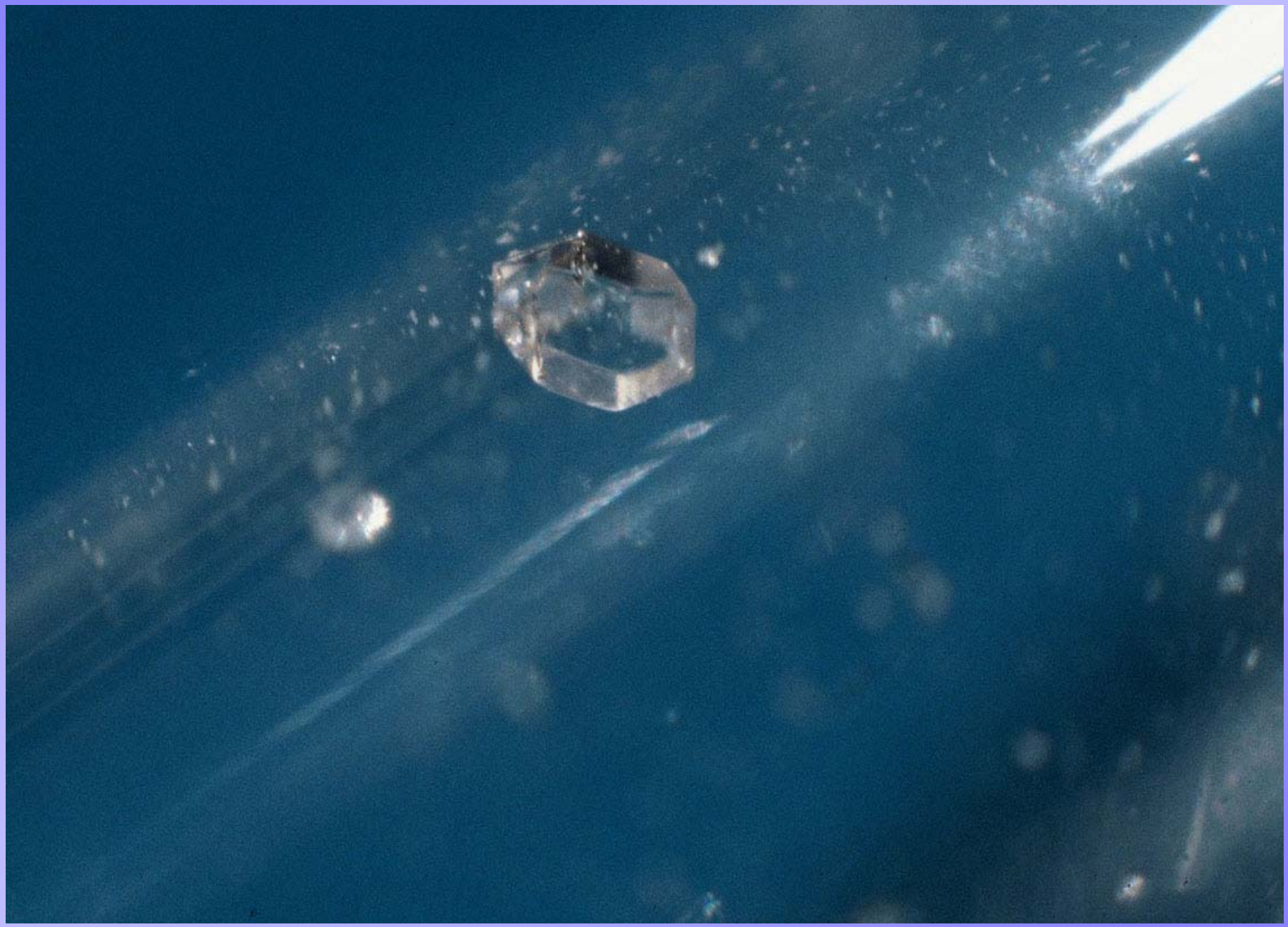
Decarbamoyl Saxitoxins

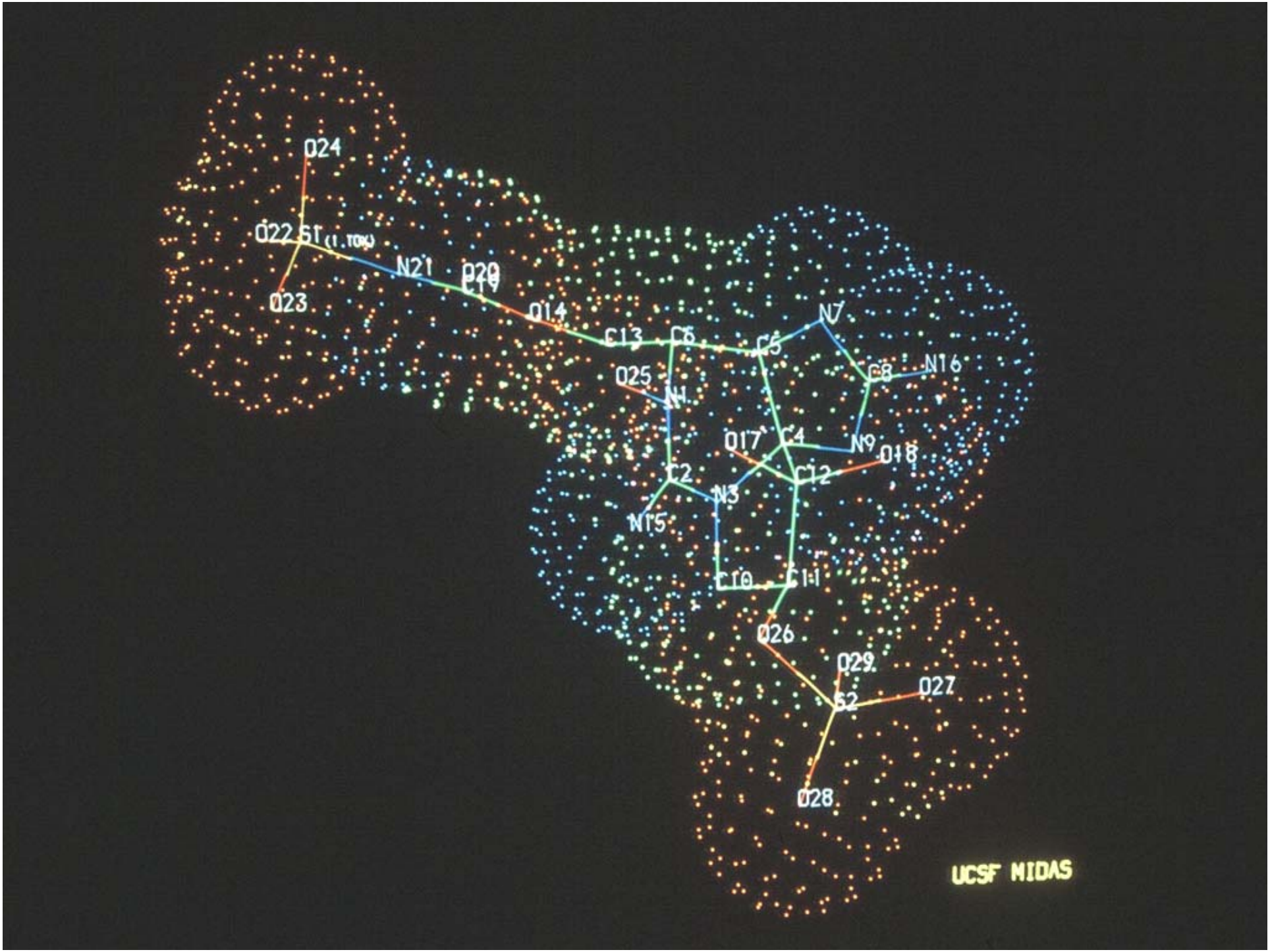
	R1	R2	R3
13	H	H	H
14	H	OSO_3^-	H
15	H	H	OSO_3^-
16	OH	H	H
17	OH	OSO_3^-	H
18	OH	H	OSO_3^-

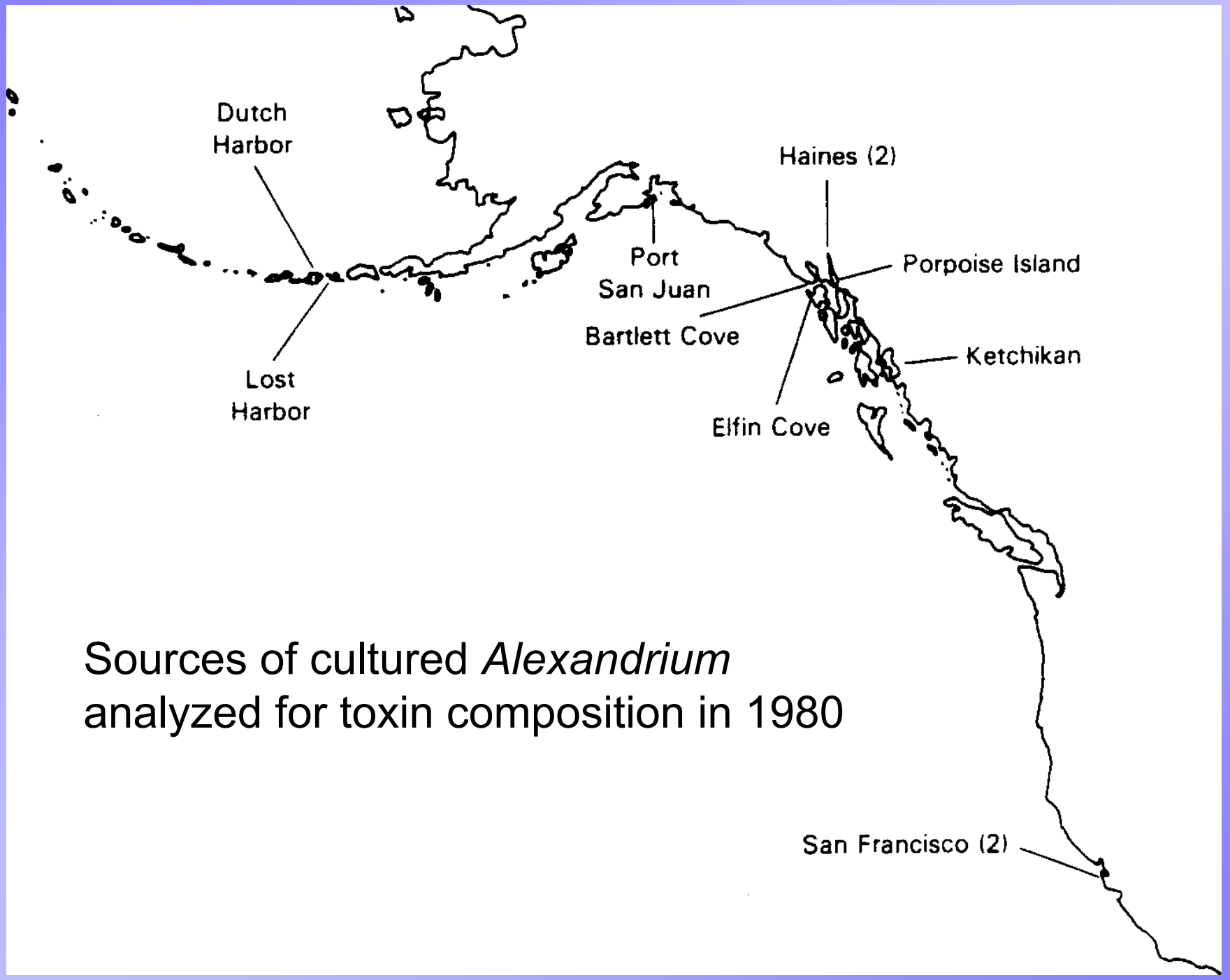


C1 crystals

C2 crystal




O24
O22
S1
N21
O20
O23
O14
C13
C6
C5
N7
O25
N1
C8
N16
O17
C4
N9
O18
C2
C12
N3
N15
C10
C11
O26
O29
S2
O27
O28
UCSF MIDAS



Sources of cultured *Alexandrium* analyzed for toxin composition in 1980

Diagenesis of toxin composition

Regional patterns of toxin composition

followed by accumulation and metabolism

such that toxin composition will vary with
time, location, and species.

Although there will be typical patterns and ranges, the
toxin composition of a sample cannot be safely assumed.

Detection Methods

ANALYSIS

ASSAY

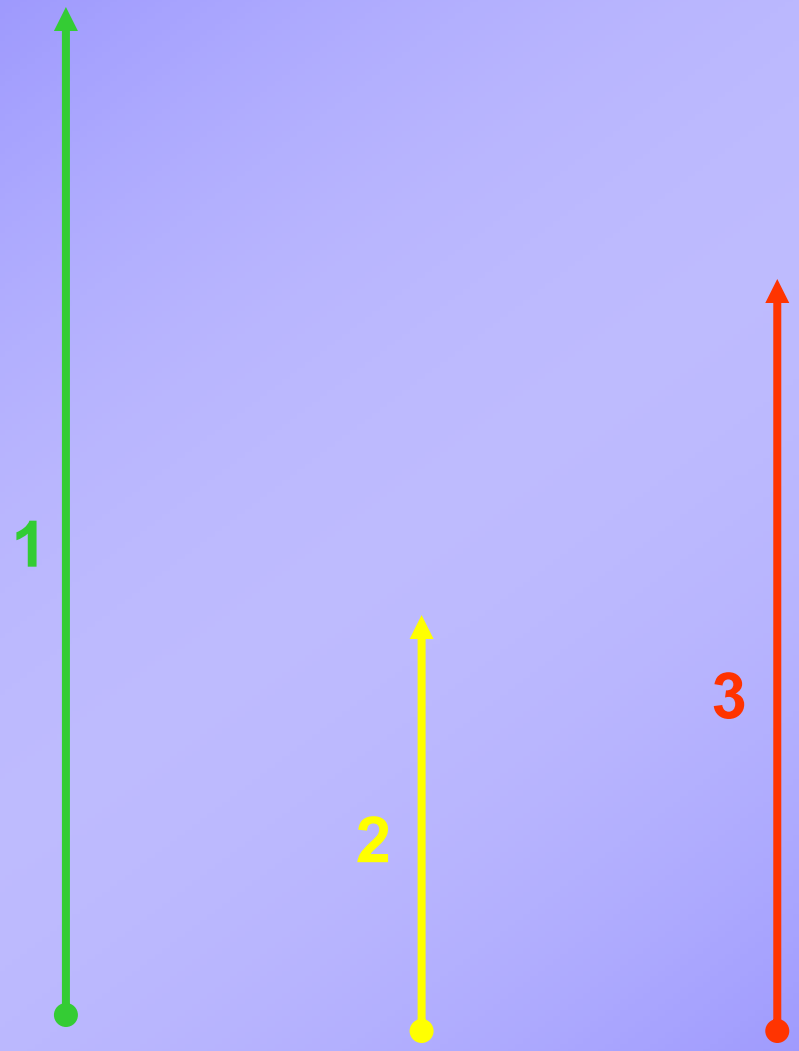
$$R = \frac{\text{Human Oral Potency}}{\text{Unit Response}}$$

ANALYSIS - SINGLE TOXIN

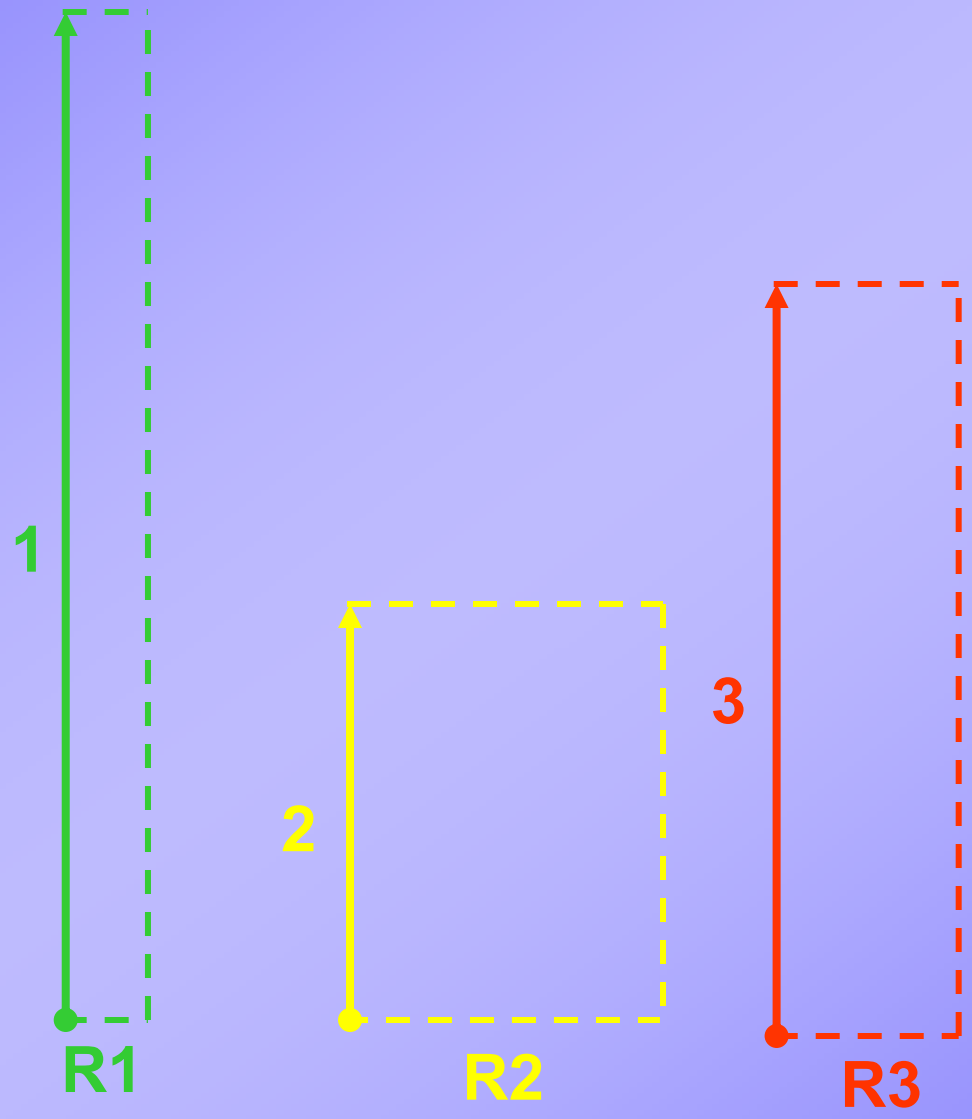
ANALYSIS
RESPONSE

ANALYSIS - SINGLE TOXIN

ANALYSIS - MULTIPLE TOXINS



ANALYSIS - MULTIPLE TOXINS



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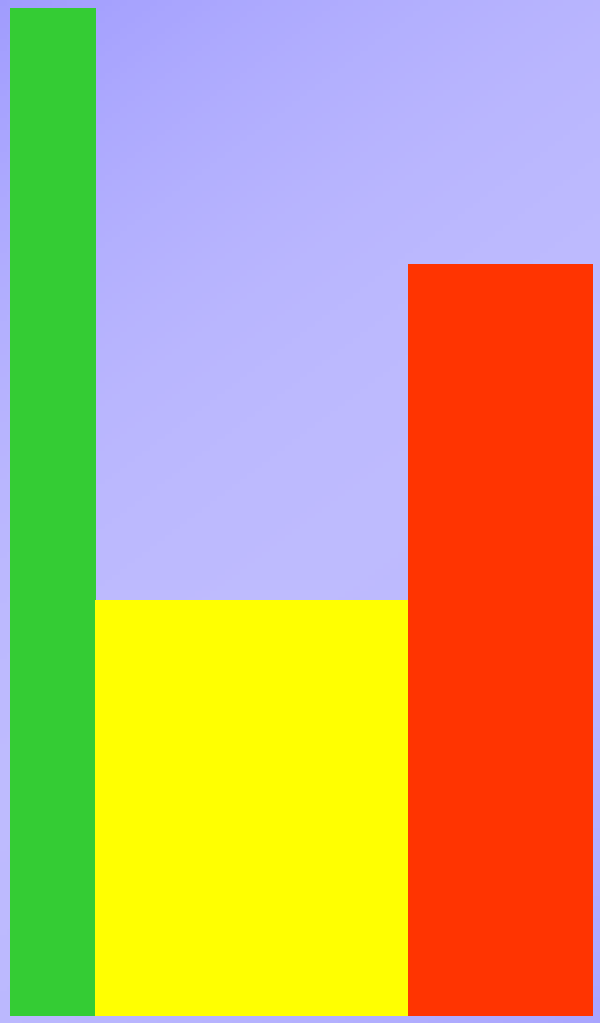
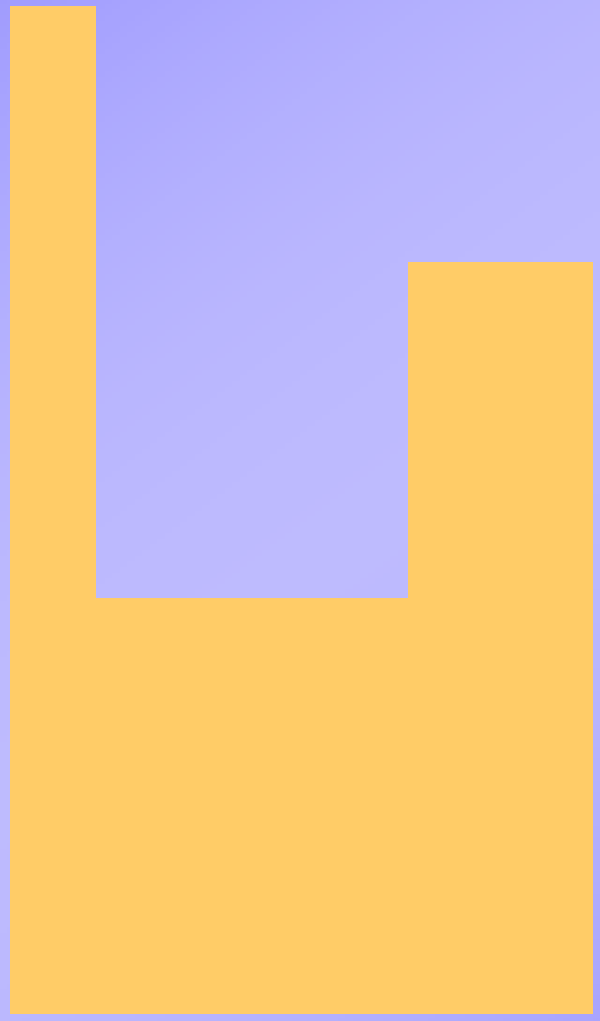
ANALYSIS - MULTIPLE TOXINS



TOTAL TOXICITY

ANALYSIS - MULTIPLE TOXINS

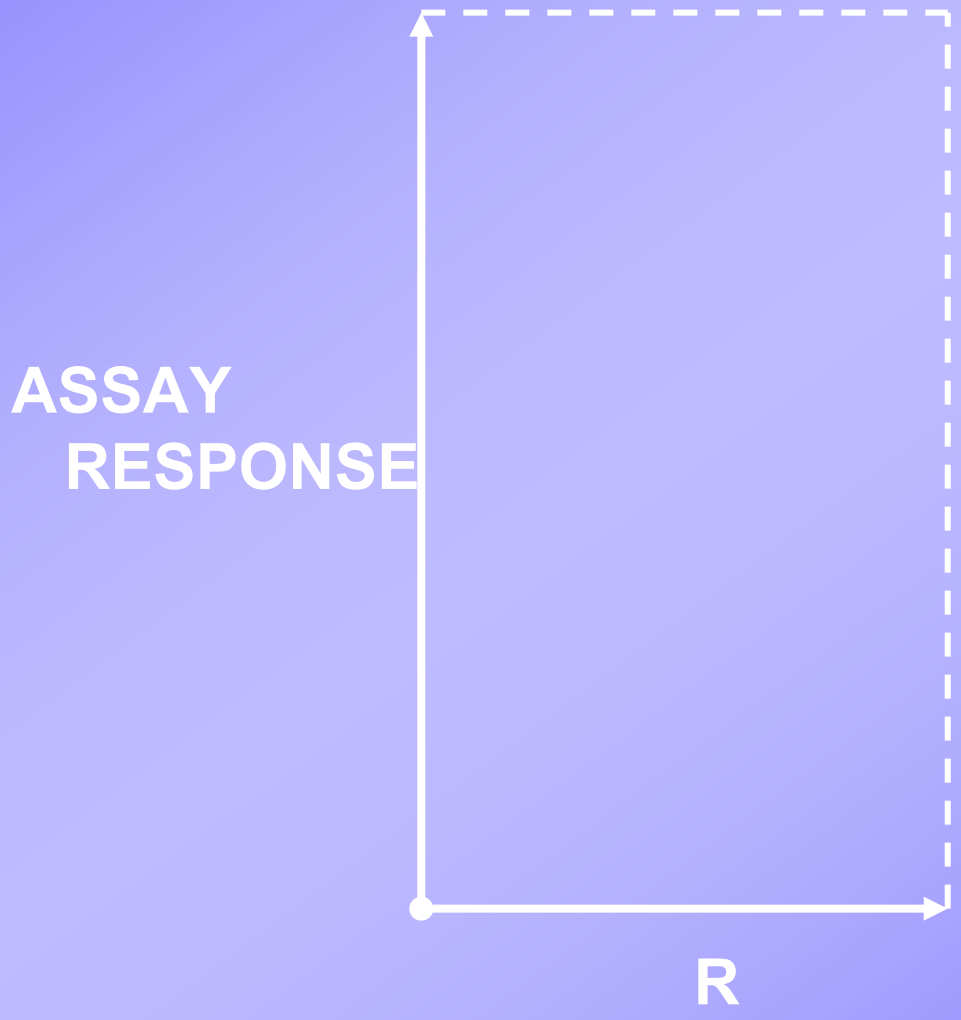


TOTAL TOXICITY

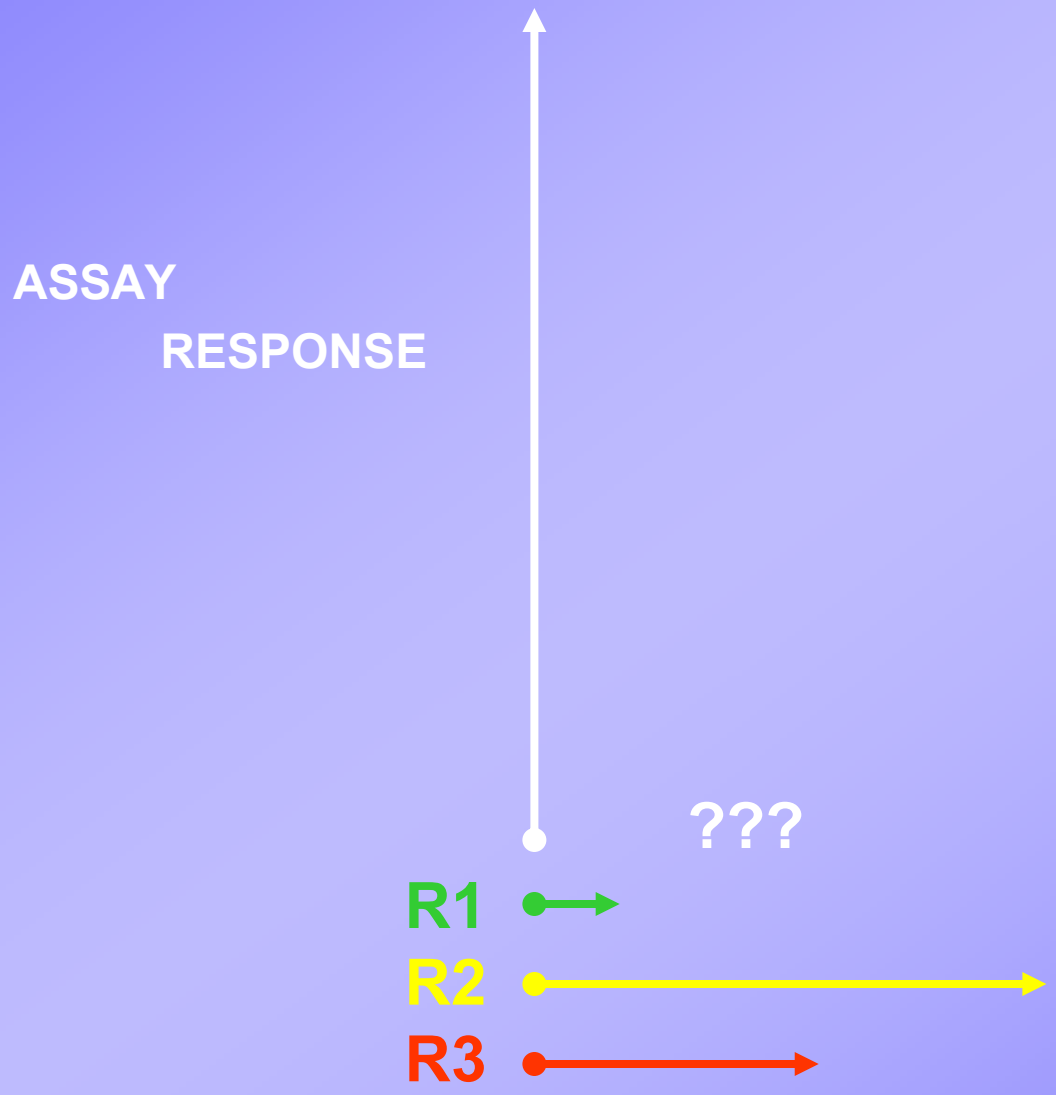
ASSAY - SINGLE TOXIN

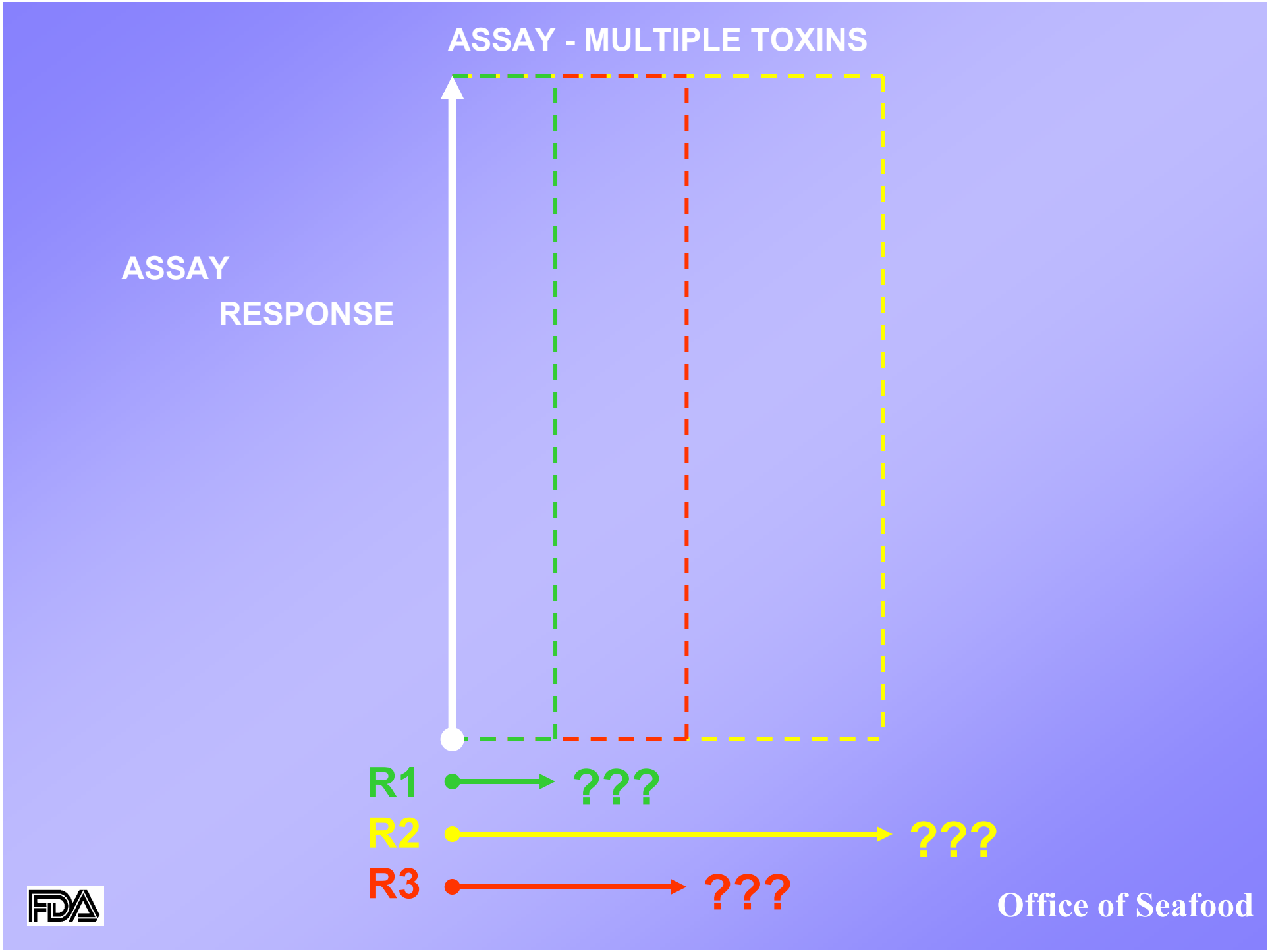
ASSAY
RESPONSE

ASSAY - SINGLE TOXIN

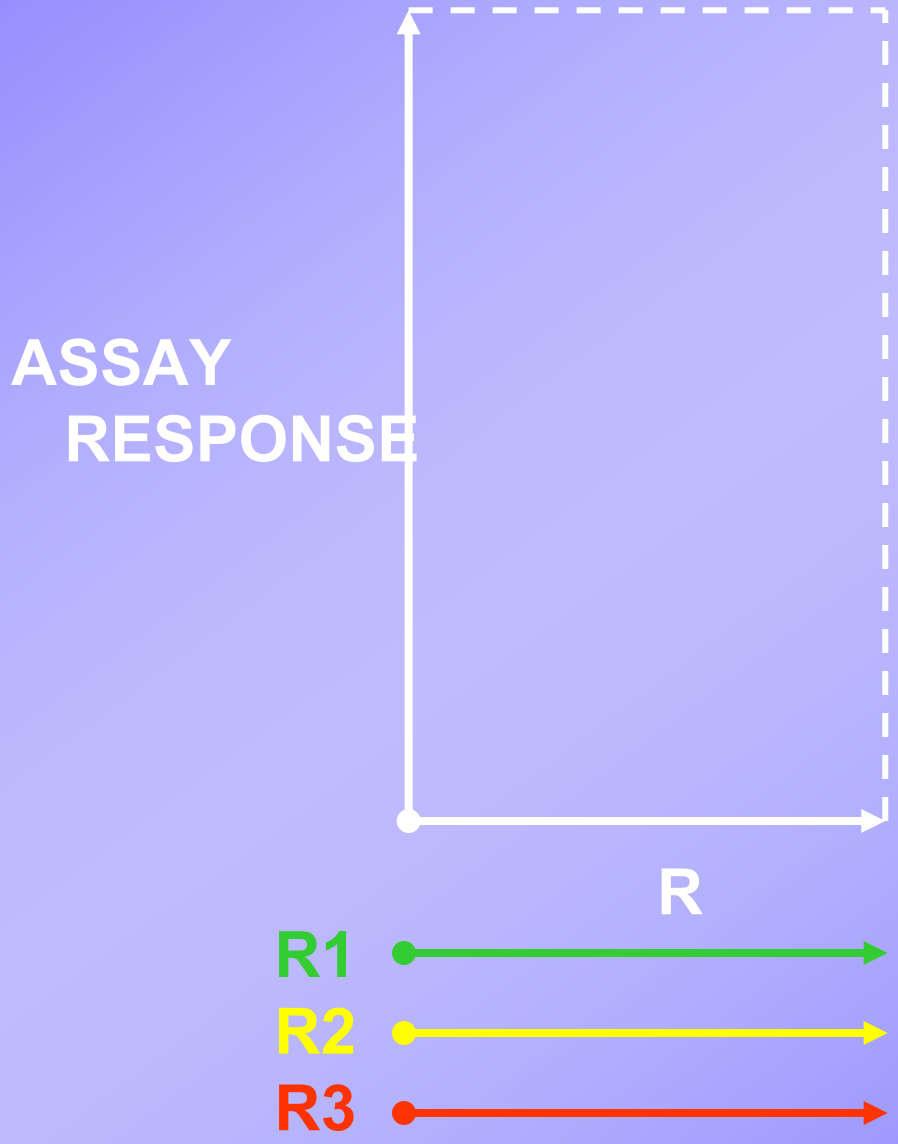


ASSAY - MULTIPLE TOXINS




ASSAY - MULTIPLE TOXINS
ASSAY
RESPONSE
R1
???
R2
???
R3
???
Office of Seafood

ASSAY - MULTIPLE TOXINS



Analyses

separate the sample so that the toxins present can be individually quantified

The appropriate response factor can then be applied to each toxin, and the sum of these calculated

Assays

provide a single result
that needs then to be correlated with
the net toxicity of the sample.

Since samples in general will contain several members of the toxin family that the assay is intended to detect, the response of the assay to EACH toxin must be such s to ensure that the assay provides an accurate measure of human oral potency.

Assay options

- Native and modified receptors
  - mouse bioassay
  - whole cell
    - electrophysiological
    - cytotoxicity
  - enzyme
  - receptor binding

- Immunoassays (‘artificial receptors’)

Immunoassays

have many advantages, but it is difficult to produce antibodies with the appropriate spectrum of responses to the various members of the family of toxins the assay is designed to detect.

Thus, while thy may be quite sensitive for some members of a toxin family, they may well be neither accurate nor even safe for toxicity monitoring.

Assays for the saxitoxins

which employ the selectivity of the native receptor of the voltage activated sodium channel appear to offer excellent sensitivity (more than 100x below that of the mouse assay and the regulatory limit) and the appropriate spectrum of responses.

However, it must be remembered that some subtypes of voltage activated sodium channels have different spectra of responses to the saxitoxins.

Events in the development of the rba:

Strichartz- Developed technique for tritium labelling of STX at high specific activity, making binding experiments possible. (The high affinity of STX for the receptor site implies that very low concentrations of STX, ca 1nM, must be used to observe the binding. Measuring these low concentrations requires a high activity label.)

Davio- Demonstrated that receptor binding could be used to measure STX concentration.

Hall and Strichartz- Demonstrated that the relative affinities of the saxitoxins in the receptor binding assay corresponded to their relative potencies in the mouse bioassay, implying that the receptor assay is a fundamentally reliable and valid alternative to the mouse bioassay for PSP.

Van Dolah- Developed a multi-well plate format for the receptor assay, vastly increasing the throughput of the assay with only a small reduction in sensitivity. This format makes the receptor binding assay practical for routine use in regulatory laboratories with a heavy workload.

RBA vs mouse bioassay:

The mouse bioassay gives a useful, approximate answer more quickly and will reliably detect a dangerously toxic sample.

The rba produces more results per day, can produce a large number of precise results much more quickly, and is much more sensitive.
(ca 0.5nM vs 0.5micromolar STX)

(We are currently waiting for for our rba to be set up to complete >1,300 research samples.)

RBA vs immunoassay

Response spectrum of an immunoassay:
Accuracy dependent on which STXs are present.

Immunoassays have the potential to be portable and to be performed by persons with little training, under field conditions.

RBA vs HPLC, LC/MS

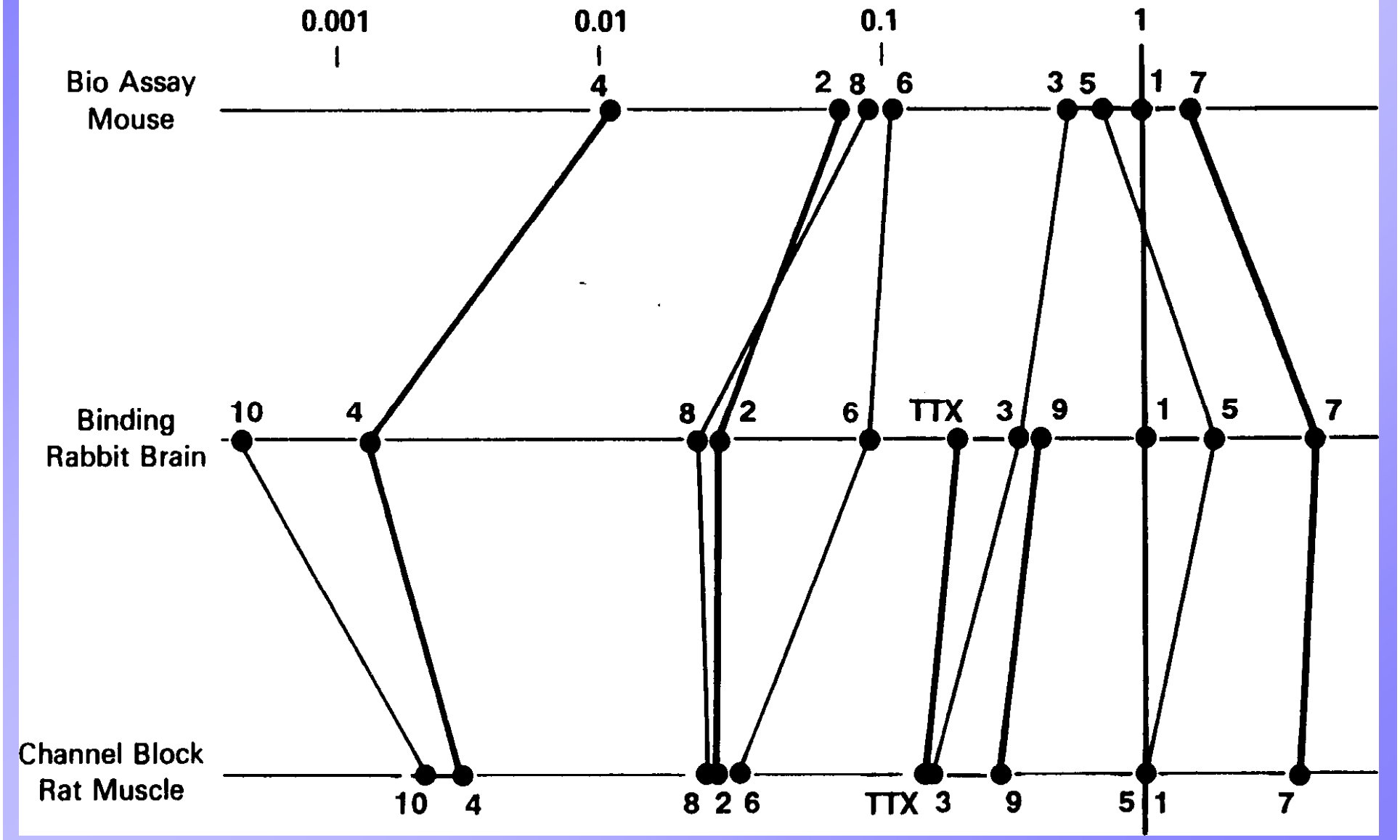
HPLC and LC/MS require careful filtration of the sample. This can be a significant cost.

Both methods provide a single channel, so throughput is dependent on run time.

Both methods are analyses and thus determine the concentrations of individual toxins. This information can be vital for research and useful in regulatory applications.

Equipment cost; operator skill.

Potency Relative to Saxitoxin



RBA work with Strichartz in 1984/4,
using tritiated STX:

The RBA is a useful and fundamentally valid assay for the saxitoxins in seafood. Its responses to the various toxins correspond satisfactorily to their potency in the mouse bioassay.

Receptor preparations are inexpensive and readily obtained. Bovine brain, available as a slaughterhouse waste, works well.

While exchange-tritiated STX works well as the labelled reagent toxin, improvements are possible:

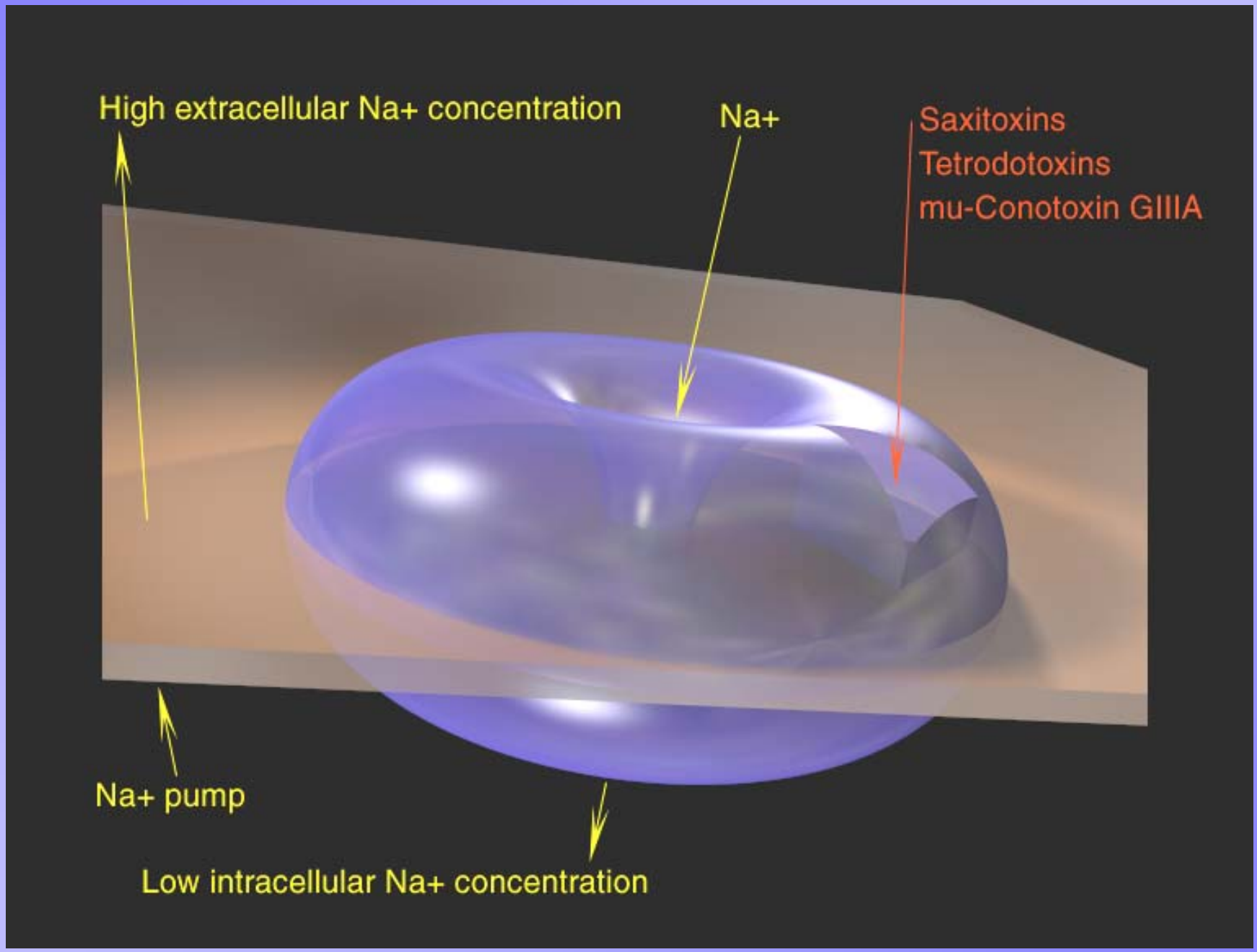
1. A label not capable of back exchange would be more durable and reliable in general use.

2. A non-radioactive label would make the assay more practical in some settings.

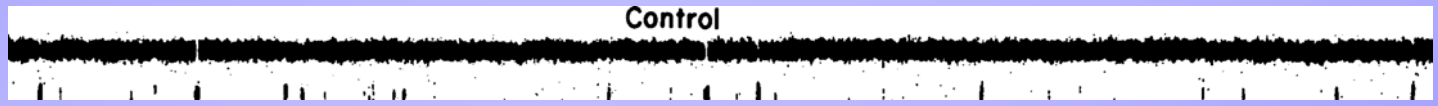
The reagent toxin must have suitable pharmacology, the dwell time being particularly important.

Thus, structure/activity studies.

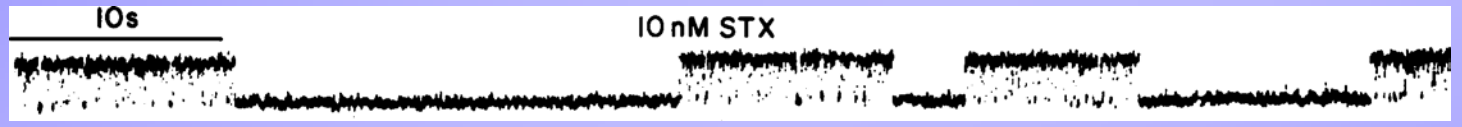
Na channel

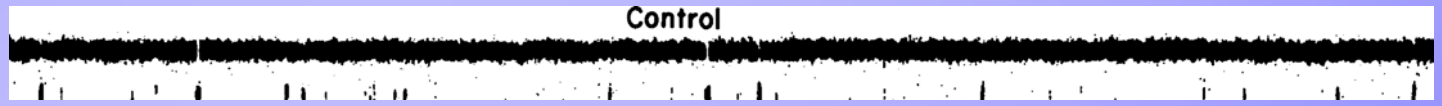

High extracellular Na+ concentration
Na+
Saxitoxins
Tetrodotoxins
mu-Conotoxin GIIIA
Na+ pump
Low intracellular Na+ concentration

Lipid bilayer + sodium channel + batrachotoxin

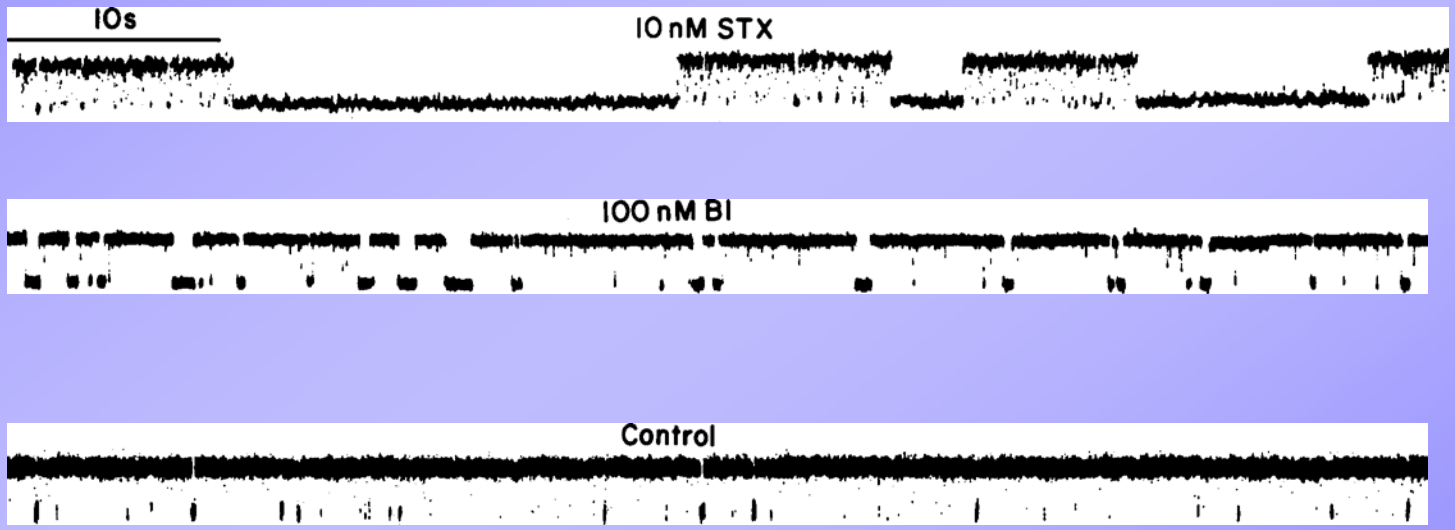


Lipid bilayer + sodium channel + batrachotoxin + saxitoxin

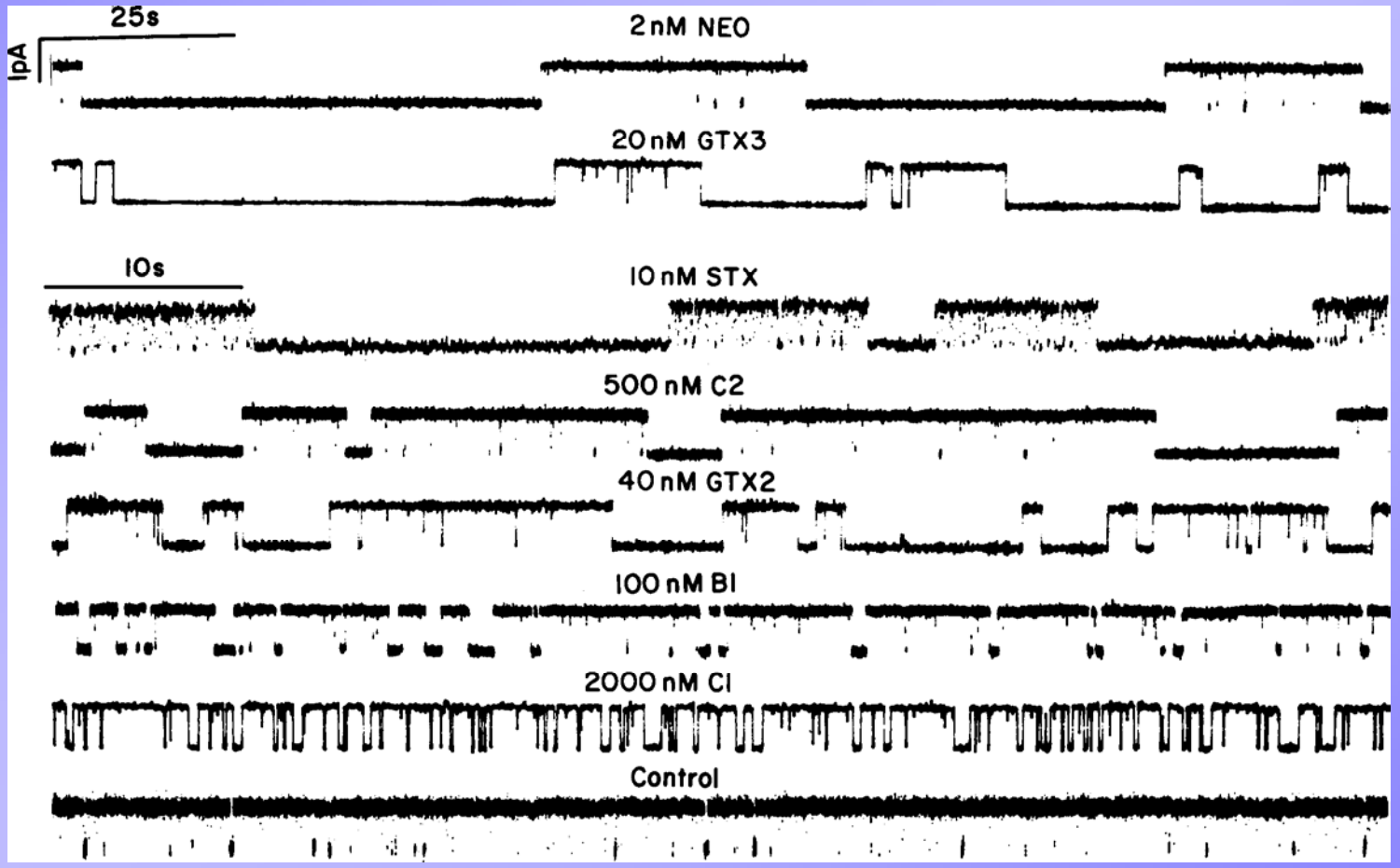




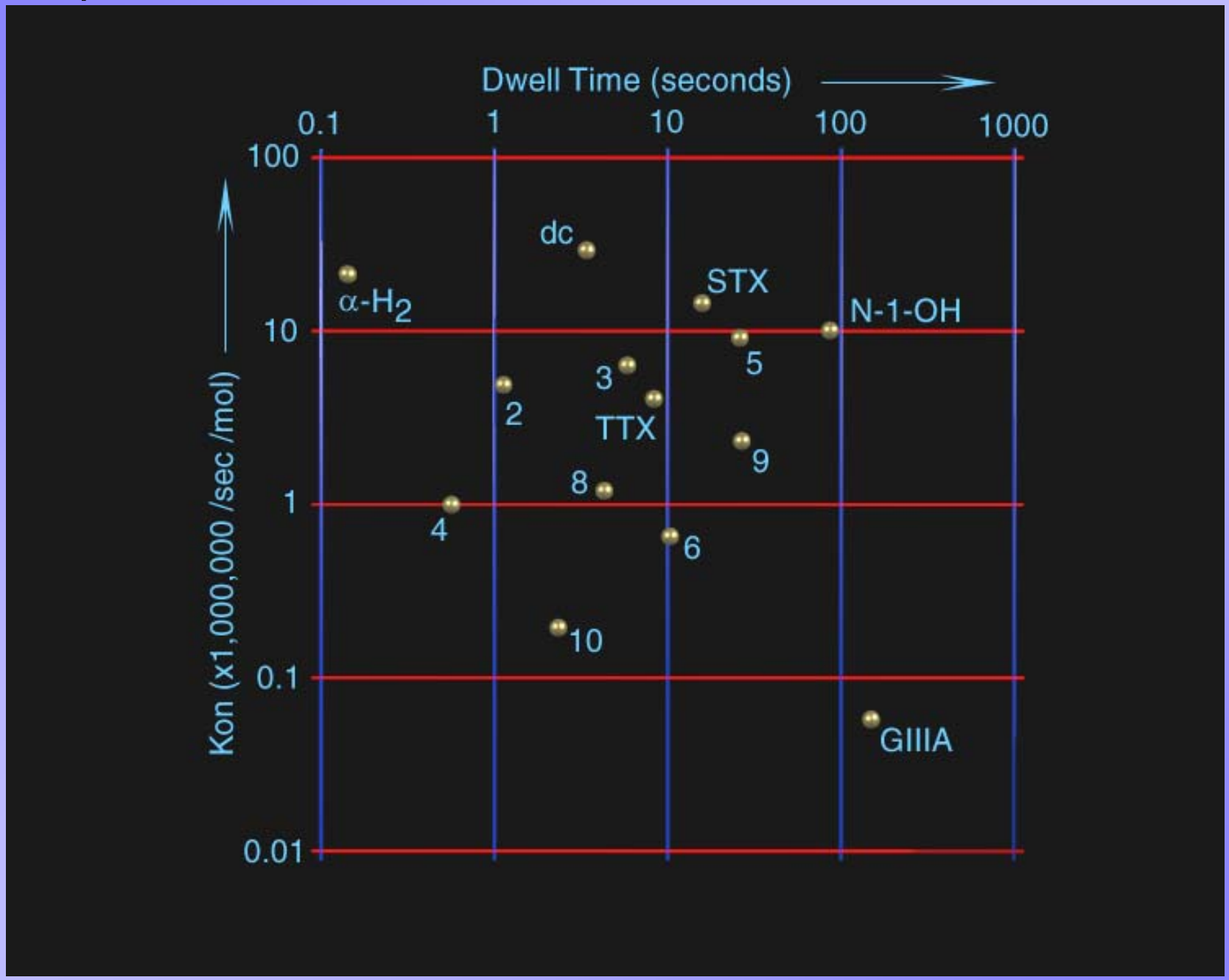
Lipid bilayer + sodium channel + batrachotoxin + saxitoxin and B1 (21-sulfosaxitoxin)



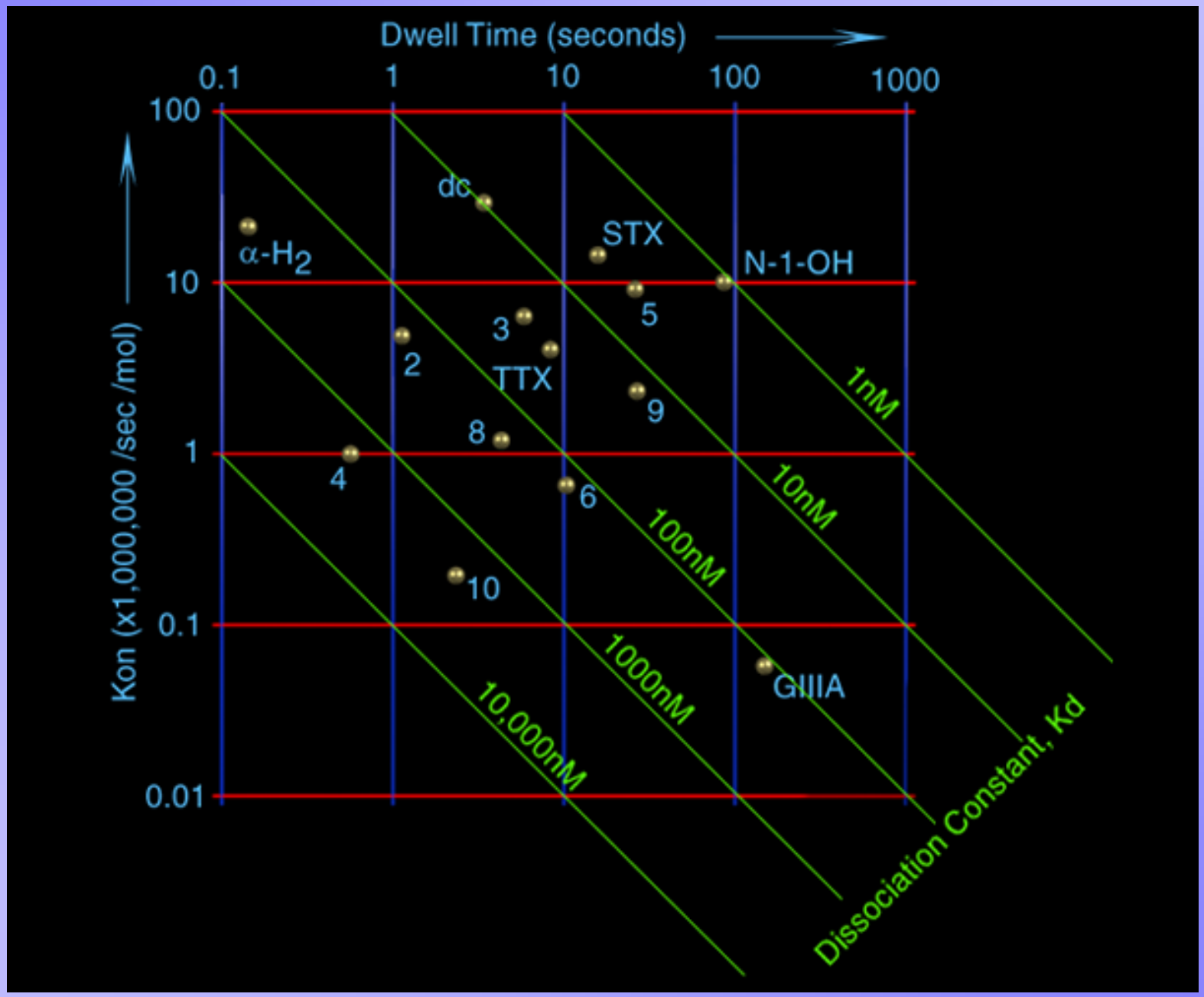
Bilayer experiments with several of the saxitoxins



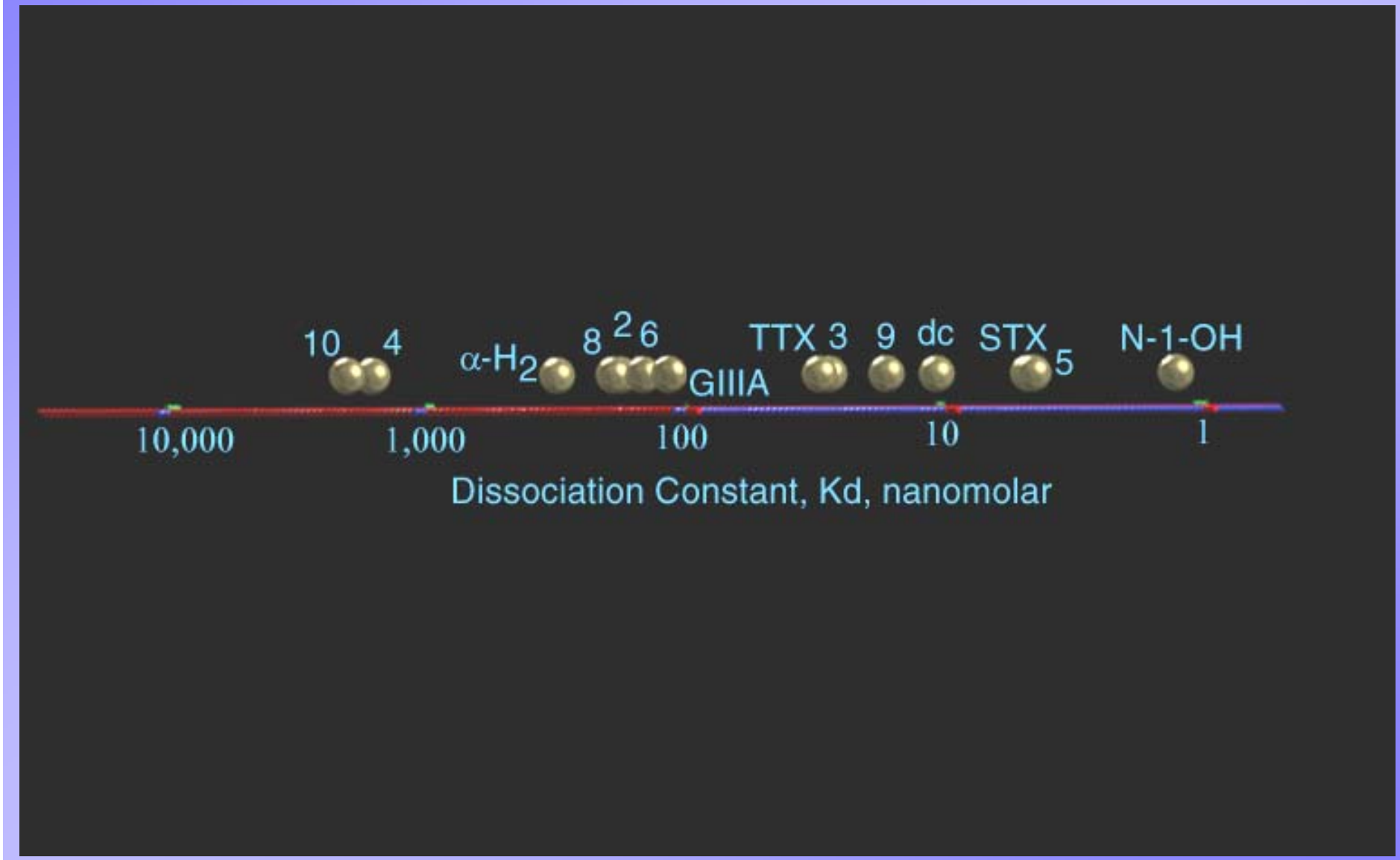
KE plot


Dwell Time (seconds)
0.1
1
10
100
1000
100
10
1
0.1
0.01
Kon (x1,000,000 /sec /mol)
dc
α-H2
STX
N-1-OH
5
3
2
TTX
9
8
4
6
10
GIIIA

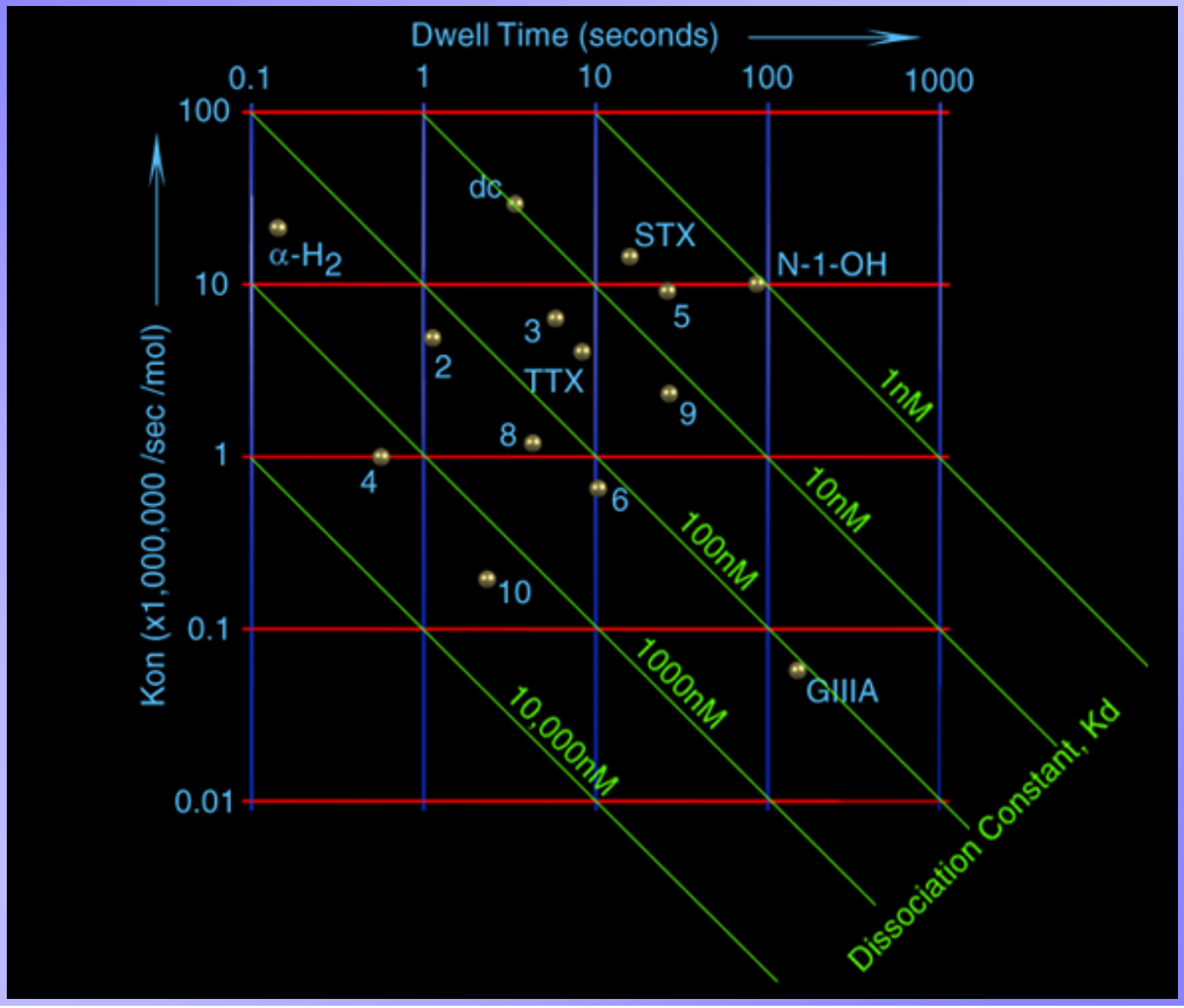
KE plot with Kd lines



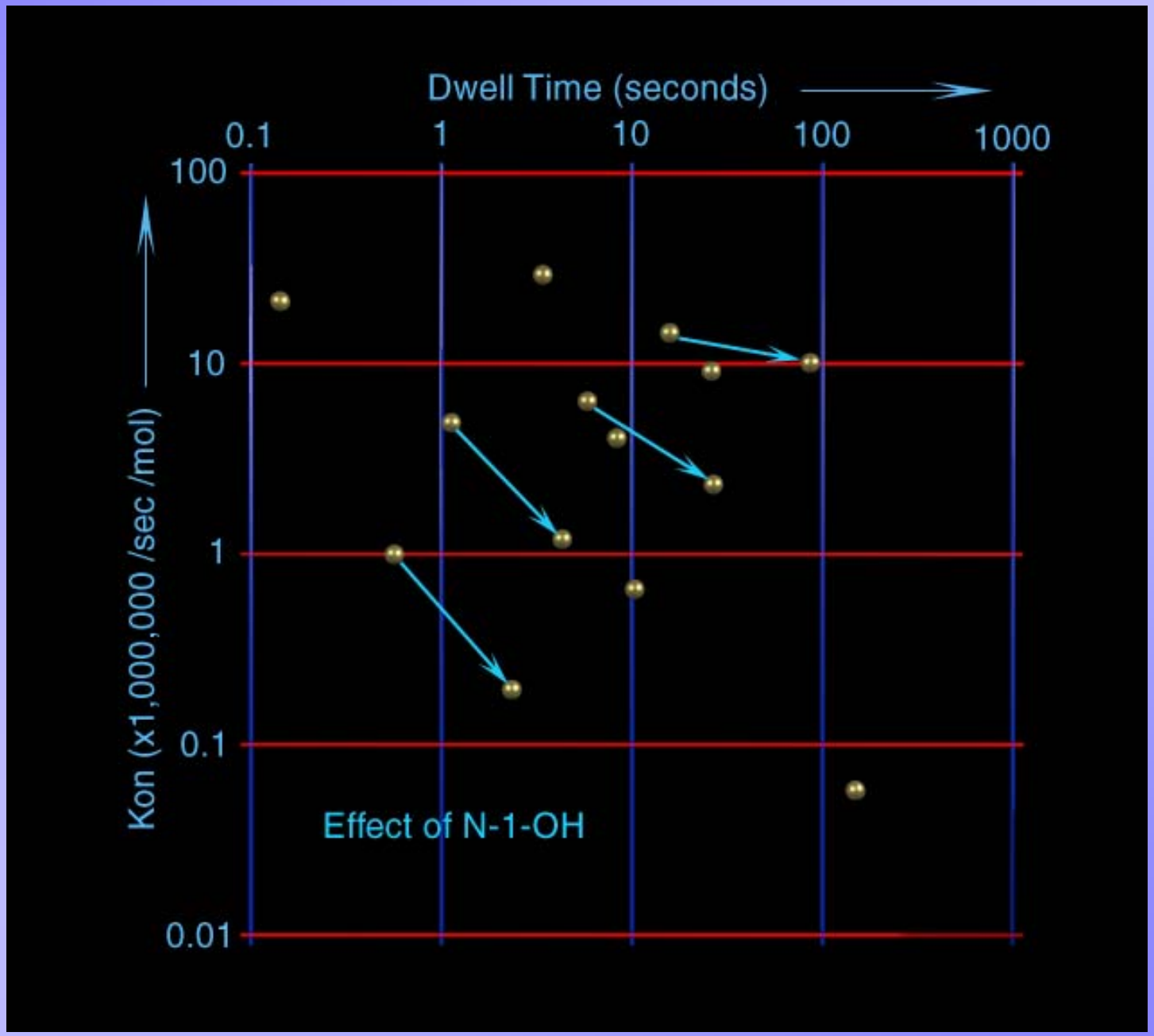
KE plot on edge



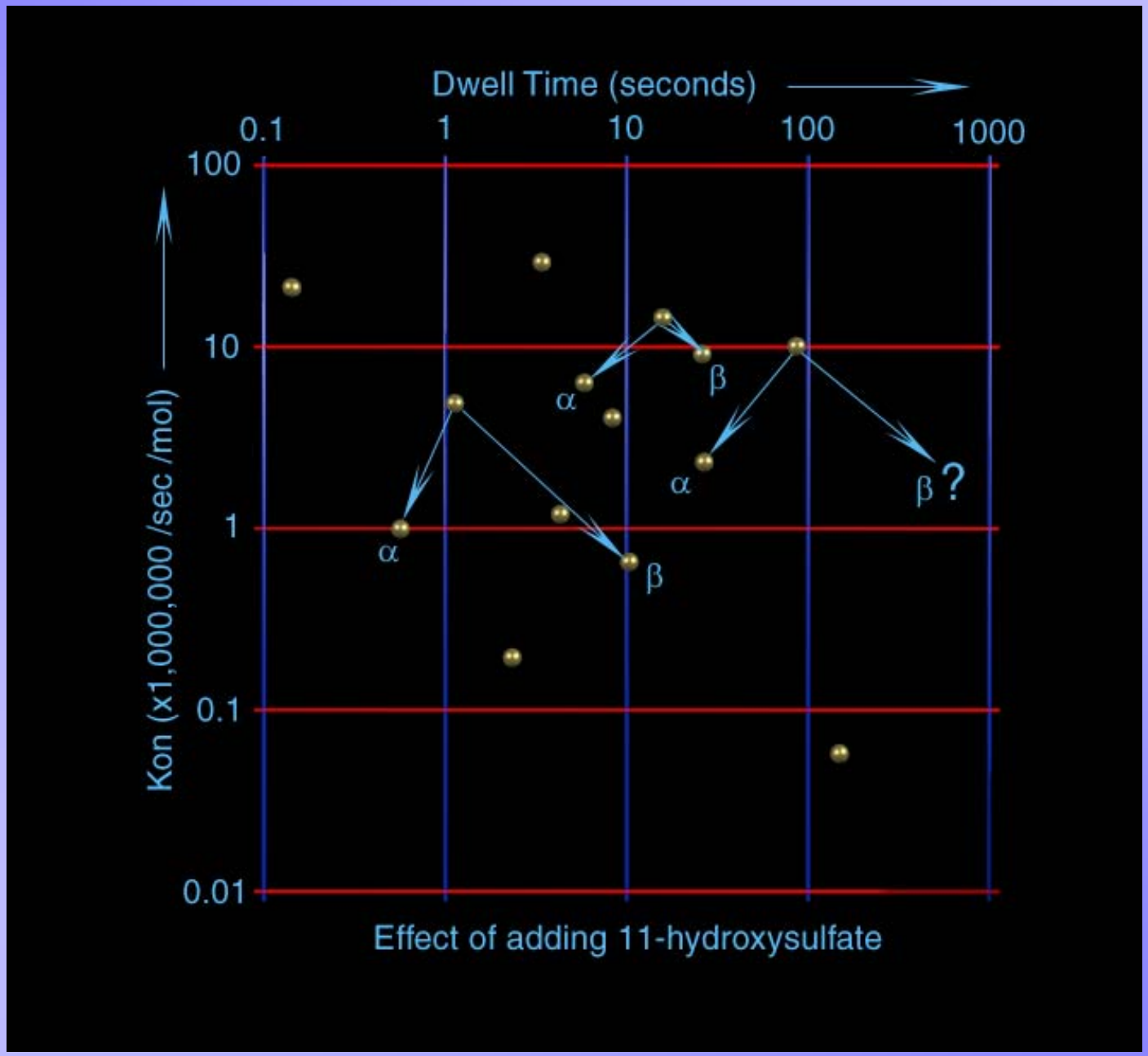
KE plot with Kd lines



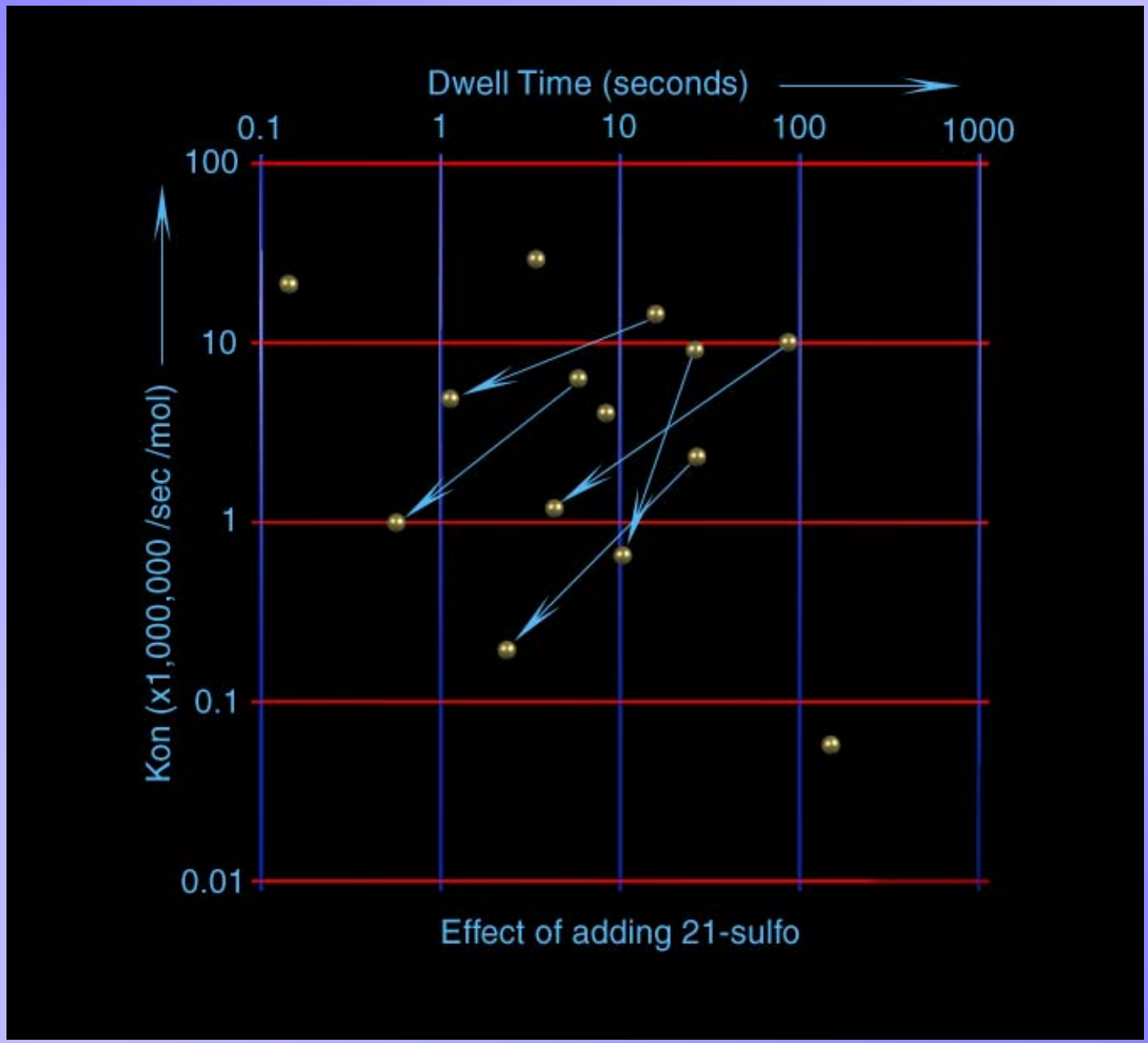
KE effect of N-1-OH



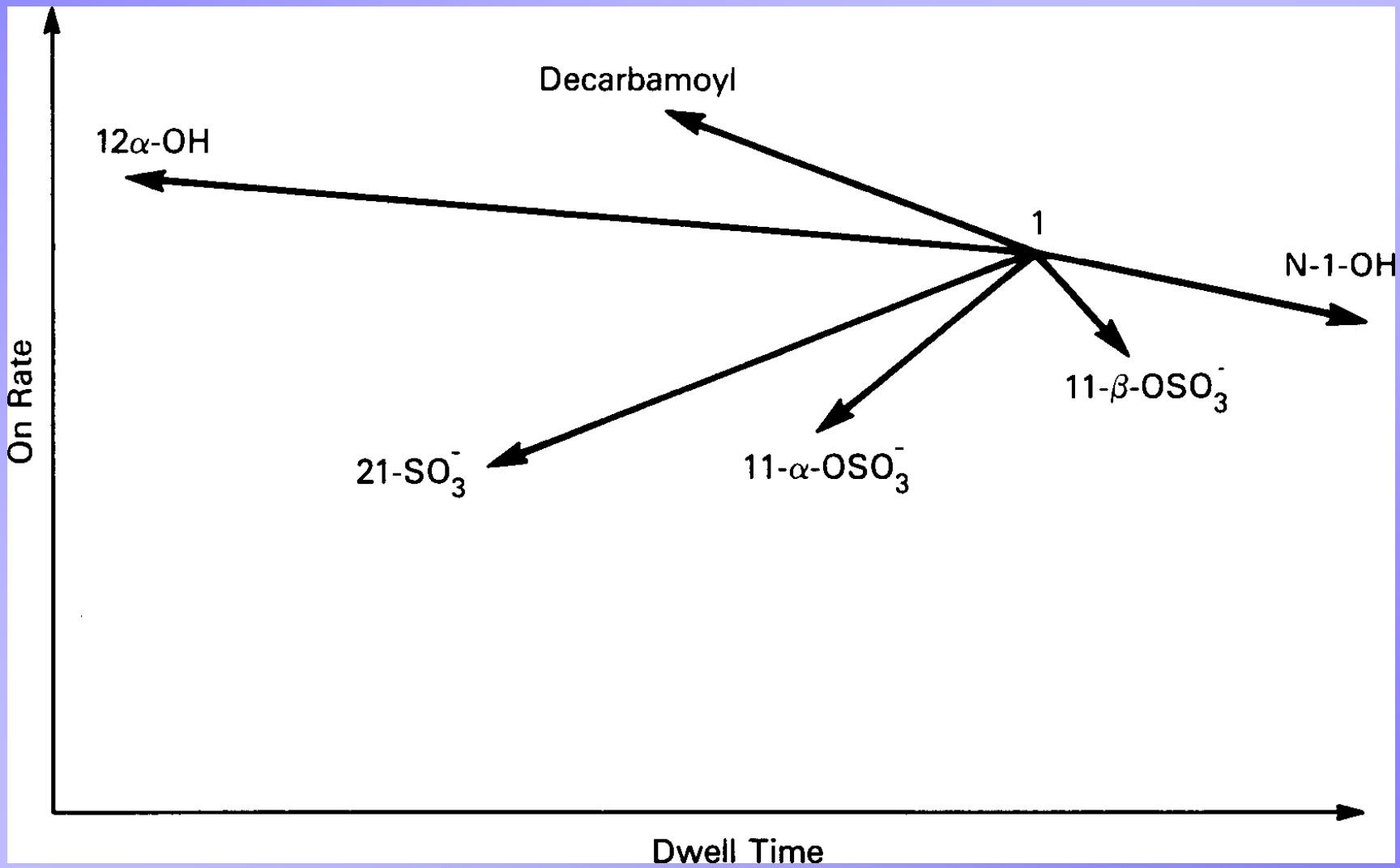
KE effect of 11-hydroxysulfate



KE effect of 21-sulfo



Summary of effects of structural modifications on the kinetics of saxitoxin binding



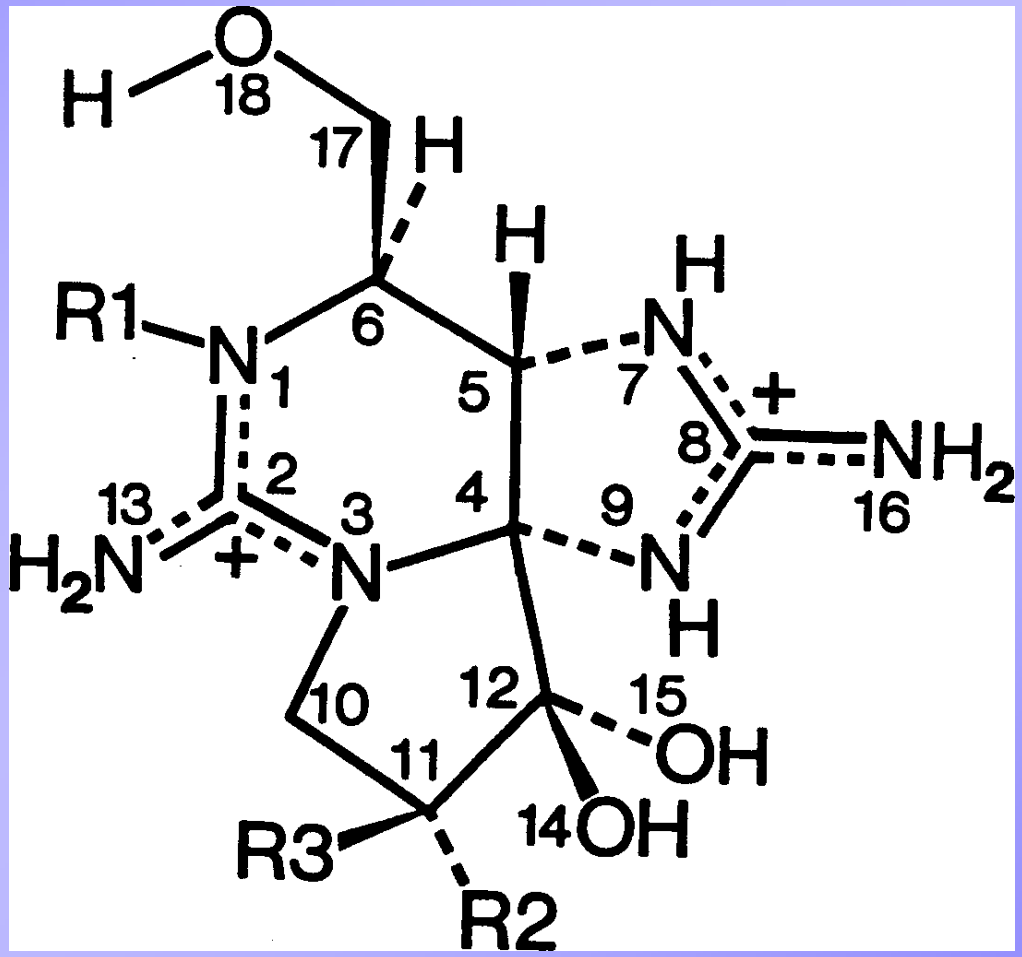
Derivative trials

- sulfation of dcSTX
- acdcSTX

Other options:

- 35-sulfation of 11-hydroxySTX
- H-13 oxidation/reduction cycle

Decarbamoylsaxitoxin (dcSTX)



5. The availability of labelled saxitoxin is assured in the long term. The current lack of labelled saxitoxin is a short-term problem, which is being solved.

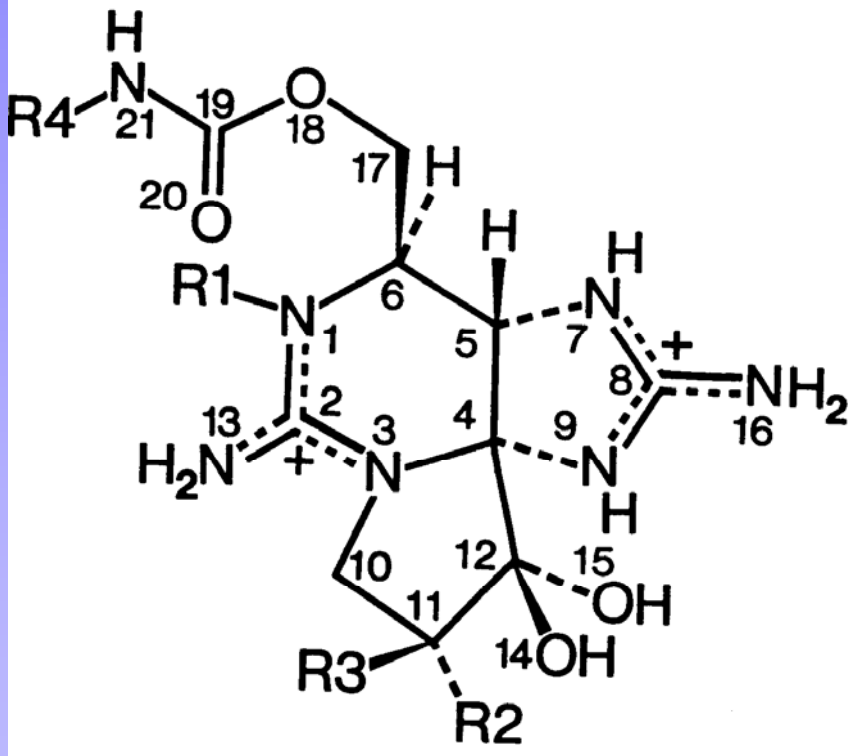
Current work on rabiolabeled reagent toxin for the receptor binding assay for the saxitoxins

assure the availability of exchange-labelled saxitoxin

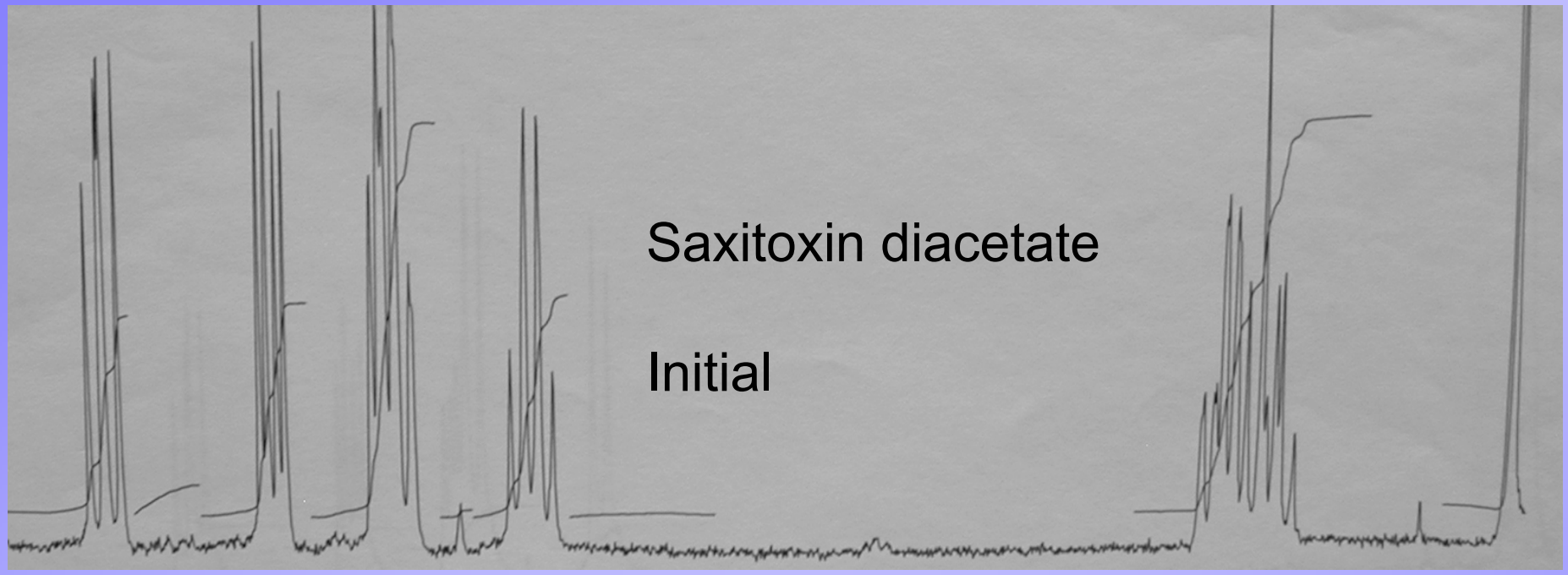
develop a non-exchangeable label

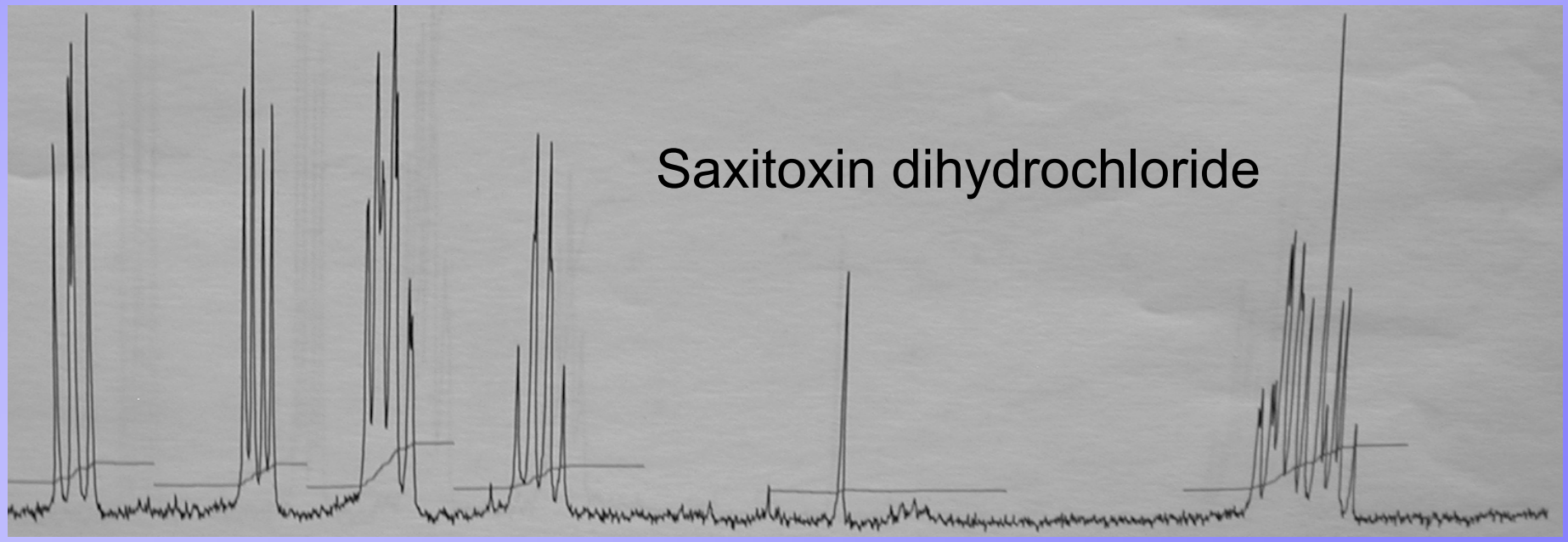
- STX derivatives
  - oxidation/reduction cycle at side chain
  - 11-hydroxysulfate
- TTX
- mu-conotoxin GIIIa

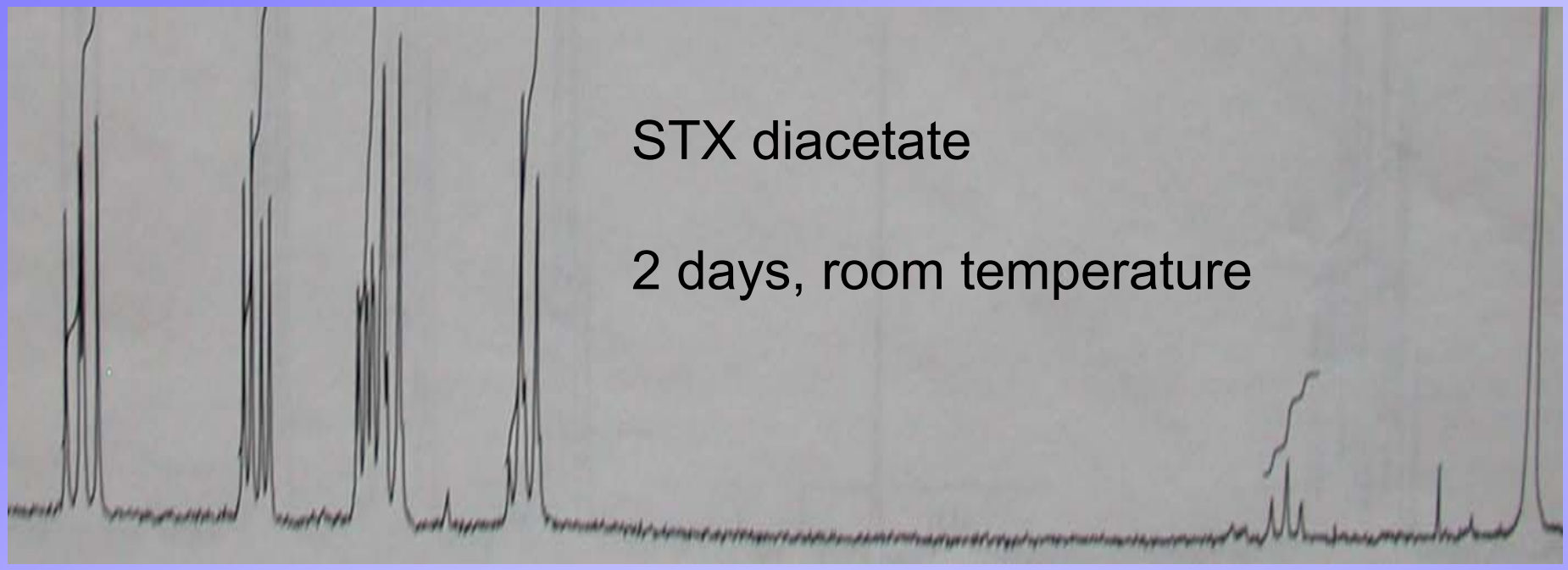
The ‘first 12’ of the saxitoxins

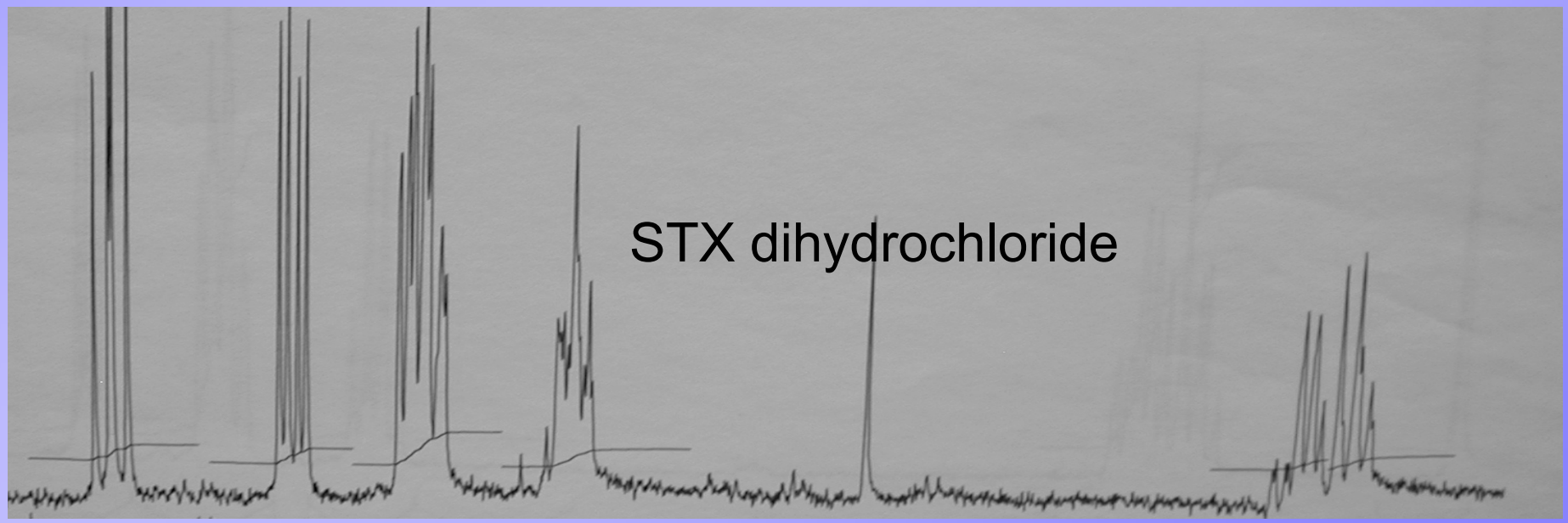


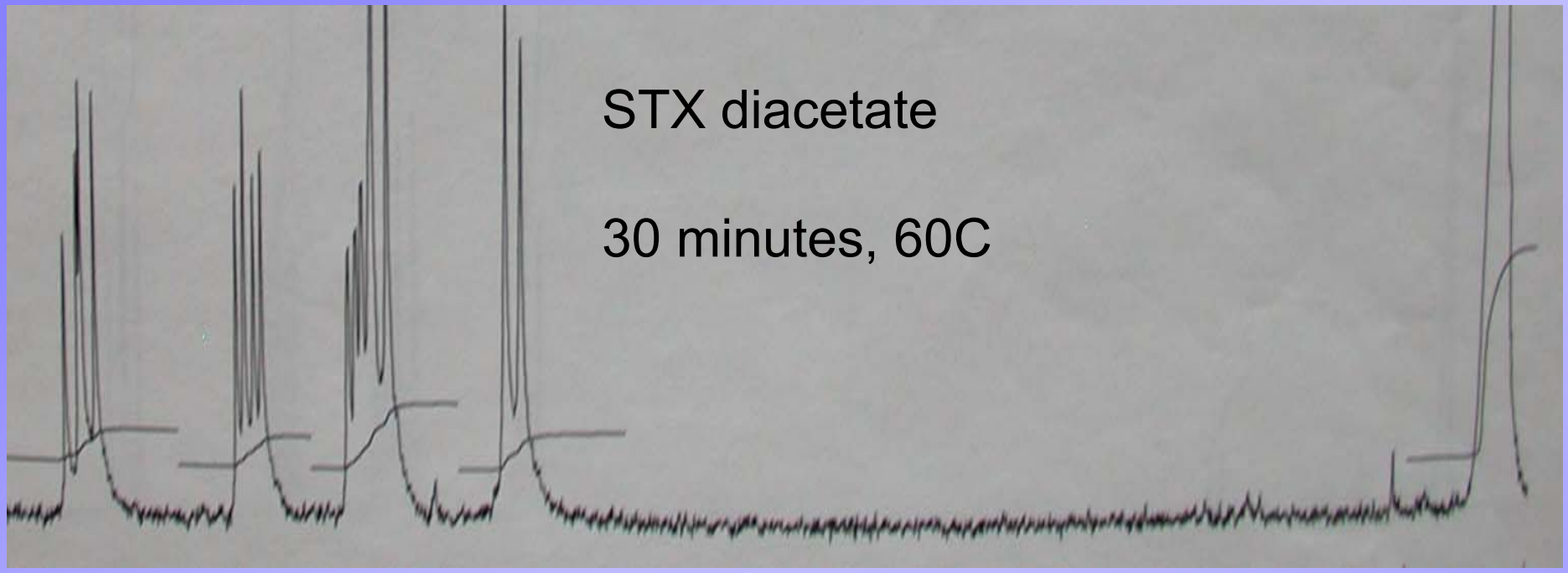
	R1	R2	R3	R4	
1	H	H	H	H	STX
2	H	H	H	SO_3^-	B1
3	H	OSO_3^-	H	H	GTX2
4	H	OSO_3^-	H	SO_3^-	C1
5	H	H	OSO_3^-	H	GTX3
6	H	H	OSO_3^-	SO_3^-	C2
7	OH	H	H	H	NEO
8	OH	H	H	SO_3^-	B2
9	OH	OSO_3^-	H	H	GTX1
10	OH	OSO_3^-	H	SO_3^-	C3
11	OH	H	OSO_3^-	H	GTX4
12	OH	H	OSO_3^-	SO_3^-	C4

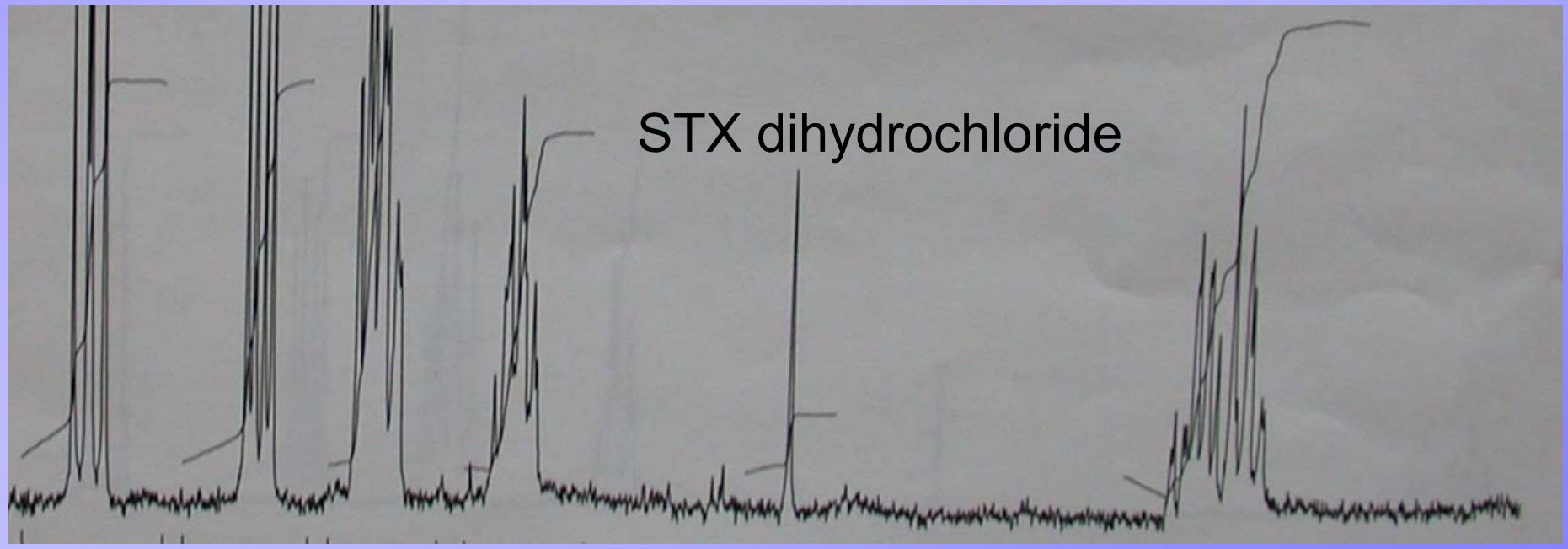

Saxitoxin diacetate
Initial


Saxitoxin dihydrochloride


STX diacetate
2 days, room temperature


STX dihydrochloride


STX diacetate
30 minutes, 60C


STX dihydrochloride

Exchange labelling stoichiometry

According to Dr. Van Dolah, labelled STX should have an activity of not less than about 0.01Ci/micromole to work well in the rba.

One micromole of STX.2HCl
weighs 0.372mg
would have an activity of 0.0584Ci if fully tritiated (2 atoms) at the 11 position.
This is about 6x the minimum required for the rba, so there is some latitude.

The 11-protons cannot be selectively exchanged.

All 13 exchangeable protons (two on the 11-carbon, 11 on the various heteroatoms) must be exchanged, then the 11 atoms of tritium on the heteroatoms exchanged off in subsequent washes.

Therefore, the one micromole of STX must be exchanged to 6.5 x 0.0584 = 0.380Ci to attain full labelling at the 11 position.

6.5 micromoles of water = 0.117 microliters.

If it were practical to manipulate as little as 10x this, 1.17 microliters,

1) The STX (which is extremely soluble in water) would dissolve in it, so the exchange would occur.

2) The resulting isotopic purity would be ca 0.9x that of the water used.

3) The total activity used would be 3.8Ci.

At a large facility (NEN or Amersham) quantities much larger than this may be practical, but:

1) The amount of activity is already large for most labs.

2) The practicality of manipulating this small a volume is doubtful.

3) The efficiency- quality of product per amount of label consumed- is poor. Better efficiency will be attained by using successive small portions of high activity tritiated water to ‘rinse away’ the protons.

One option is to use an aprotic carrier solvent to provide volume sufficient for manipulation without contributing protons that would dilute the activity of the tritiated water.

NMR experiments using 1% deuterium oxide in pyridine, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) show that all three solvents support efficient exchange of the two 11 protons. However, the solubility of STX in the aqueous pyridine is poor.

Once the details of the micromanipulation have been confirmed, this approach will be used at American Radiolabeled Chemicals (ARC; St. Louis) to label several small batches of STX using 10-50 microliter volumes of aprotic solvent with 1% carrier free tritiated water.

The resulting tritiated STX will be sent to Dr. Van Dolah for evaluation. If the activity is satisfactory, the material will be available for the IAEA project. An IAEA purchase order has been issued to ARC for this work. STX and expertise are being provided by the FDA/WSL.

Stocks of STX now on hand substantially exceed amounts required for this and similar applications.

Appropriate, non-commercial applications need not be limited by the availability of STX.

If this is so easy, why the delay?

Moving, after more than 15 years, from
- lab
- home

Regulatory impediments:
- The Faustian Contract
- CWC
- Australia Group
- US Patriot Act/CDC

6. Implementation of the receptor binding assay is practical, essential, and will have significant benefits to human well-being.

The rba in its current mode is best suited to use in a central lab to which shellfish samples are sent.

Since this is the way in which most toxin monitoring is now conducted, the rba can,with suitable equipment and training, be used as a direct replacement for the mouse bioassay in many existing biotoxin management programs.

AOAC Collaborative Study

AOAC collaborative study is a good thing, but is neither necessary nor sufficient for the use of a method in biotoxin monitoring.

AOAC collaborative study shows only that a detection method has the potential to work reliably.

A biotoxin monitoring program must continually confirm the performance of the toxin detection methods it uses.

Recommendations:

1. The FDA and the IAEA cooperate to ensure the availability of labeled reagent STX and other reagents for the rba to laboratories around the world, in the same way that the FDA now distributes reference standard toxins, free of charge, to all laboratories that need them.

2. The FDA, NOAA, IAEA, and other interested entities cooperate through training, technical guidance, and equipment grants, to ensure implementation of the receptor assay for PSP where it will benefit food safety and human well-being.

3. AOAC collaborative study should be considered a desirable goal and an asset, but not a requirement for implementation. On the other hand, a practical demonstration that the method works is essential for implementation, and must be followed by continual internal performance controls.