BCHEM 365
Lecture 10
November 26, 2018

Energy transfer in liquid scintillation counting

Role of the solvent and fluors

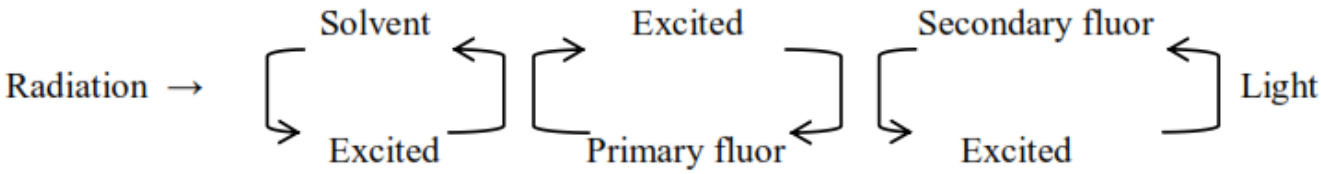
The solvent portion of a scintillation cocktail makes up about 60-99 % of the total solution. Properties required of a solvent are as follows:

1. The solvent must act as an efficient collector/absorber of energy
2. It must conduct that energy efficiently to the fluor molecules rather than dissipating it
3. The solvent must not quench the scintillation of the fluor
4. The solvent must dissolve the fluor to produce a stable and homogeneous counting solution

A small number of organic solvents fluoresce when bombarded with radioactivity, i.e., they also act as scintillators. However, the light emitted has a very short wavelength (Fig. 10.1) and cannot be efficiently detected by the photomultipliers, though they are gathered by the photocathode. If another compound is added to this cocktail that can accept the energy picked up by the solvent, and itself fluoresce at a longer wavelength, then the light emitted can be more efficiently detected. Such a compound is known as a primary fluor. The most frequently used primary fluor is 2,5-diphenyloxazole (PPO). It is included at a concentration of 0.3 % to 1 % of the total solution volume. Linked benzene rings rather than large aromatic systems make the best scintillators.

Unfortunately, the light emitted by PPO is not always detected with very high efficiency, so one needs to further aid the energy transfer by including a secondary fluor or wavelength shifter such as 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP). Combination of two fluors with the solvent makes the energy transfer process efficient. Because the solvent cannot transfer its energy directly to the secondary fluor, it becomes necessary to have both primary and secondary fluor in the scintillation system. Find below a path of energy transfer from

Sample ⟶ Solvent ⟶ Primary fluor ⟶ Secondary fluor ⟶ Photocathode



PPO and POPOP were among the first fluors used in liquid scintillation counting and are still commonly used. An alternative primary fluor 2-(4'-t-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole (Butyl-PBD) performs better as a primary fluor than PPO, but more expensive and is affected by extremes of pH. Bis-MSB is the most common secondary fluor. Napthalene can serve as both solvent and fluor. Fluors are organic and inflammable.

Most labs now purchase their scintillation cocktails already prepared and there are many different recipes on the market. Competition and increasing awareness of health and safety means that manufacturers are making less toxic scintillation cocktails with lower fire hazard.

Some cocktails are designed for organic samples and others for aqueous samples. For aqueous samples, emulsifiers such as Triton X-100 are added to the scintillation cocktail.

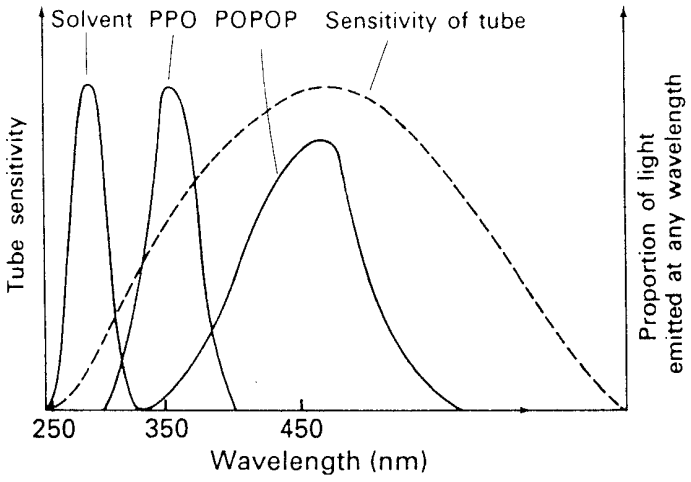
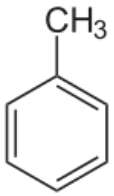


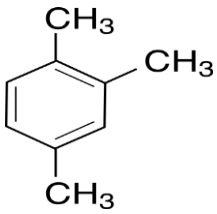
Fig. 10.1. Emission spectra of various fluors in relation to sensitivity of phototubes

The aromatic organics as solvents

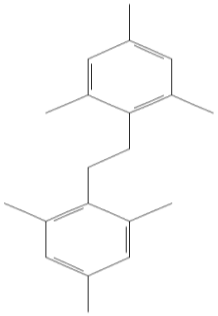
Aromatic organics are the best solvents for liquid scintillation counting. The π electron cloud of the toluene ring or any aromatic ring is a target for β-particle interaction, which captures the energy of the incident particle. The β-particle passing through toluene solution causes the production of energized toluene molecules. Energy from these molecules passes back and forth among the solvent ring system, allowing efficient energy capture by the dissolved fluors. The following solvents possess aromatic rings for efficient absorption of incident radiation.



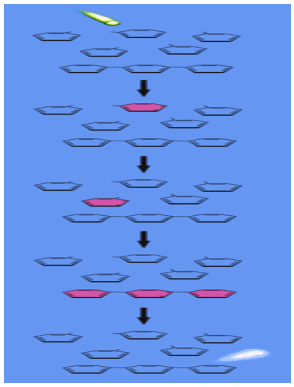
Toluene



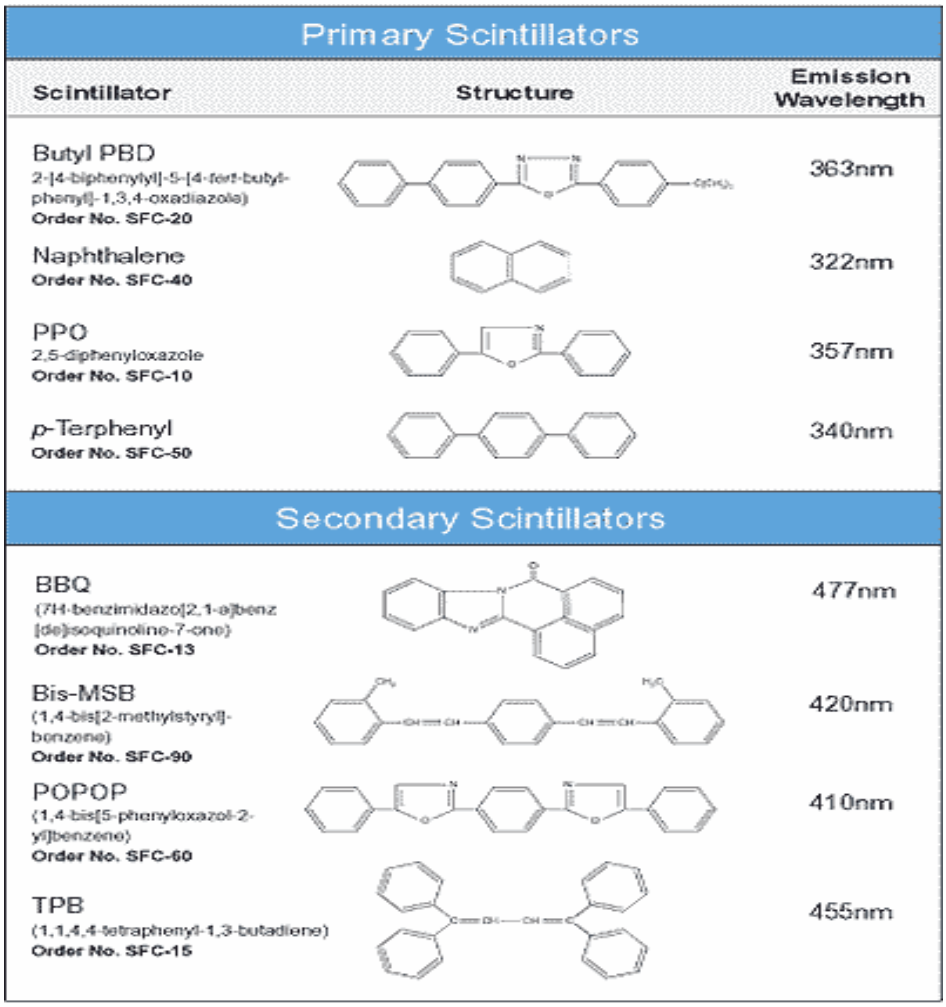
1,2,4-trimethylbenzene (pseudocumene)



Phenylxylyl ethane (PXE)



Solvent molecules in a scintillation cocktail absorb a portion of the alpha or beta particle's energy. The energy passes between solvent molecules until it reaches a fluor which absorbs the energy and re-emits it as light at a longer wavelength.



Advantages of scintillation counting

1. **No dead time** - Much higher count rates are possible with scintillation counting than gas ionization counting because of the speed of fluorescence decay (10^{-9} s). There is also no dead time as in a Geiger-Müller tube (10^{-4} s).
2. **No delay by end window** - Much higher counting efficiencies particularly for low energy β-emitters are obtained; over 50% counting efficiency for weak β-emitters, and over 90% counting efficiency for high energy emitters. This is because the negatrons do not have to travel through air or pass through an end-window of a Geiger-Müller tube dissipates much of the energy before causing ionization), but interact directly with the fluor so that energy loss before counting is minimal.
3. Ability to accommodate samples of any type, including polar and organic liquids, solids, suspensions and gels.

4. Ease of sample preparation.
5. Ability to count different isotopes separately in the same sample which means dual labeling experiments can be carried out.
6. Scintillation counters are highly automated and in-built computer facilities carry out many forms of data analysis, such as efficiency correction, graph plotting, radioimmunoassay calculations, etc.

Disadvantages of scintillation counting

Five disadvantages are associated with scintillation counting systems

1. **High cost**: High cost of counting per sample, however, sensitivity, versatility, ease and accuracy outweigh the cost for most applications
2. **Photomultiplier Noise**: At the high voltages applied to the photomultiplier, heat is generated and extraneous electronic events occur in the tube, which do not arise from the radioactivity, thus contributing to background count. This is called photomultiplier noise which must be reduced. There are four ways of reducing photomultiplier noise.

(a) **By Cooling** - Can be reduced by cooling the photomultiplier tube. Cooling results in improved counting efficiency.

(b) **Special design of the photomultiplier** - Use of low noise photomultipliers with greater temperature stability in ambient temperature systems.

(c) **Use of pulse height analyzer** – The data analysis tool can be regulated to electronically reject most of the photomultiplier noise which would usually have low energy. Regrettably, this approach also rejects the low energy pulses arising from low energy radioisotopes such as β-particles from ^{3}H (about 0.019MeV).

(d) **Use of coincidence counting systems** – this system employs two photomultiplier tubes (PMT) in one scintillation counter. When both PMTs produce pulse simultaneously, we describe it as coincidence pulse. An actual radioactive event, with its relatively higher energy than an electronic arising from heat would more often lead to simultaneously pulses (coincidence) in the PMT. The system is designed to allow only coincidence pulses to proceed to the scaler and reject single pulses. The photomultipliers are set in coincidence by mounting them opposite to each other to facilitate detection of only simultaneous pulses. In general, coincidence counting systems reduce photomultiplier noise to a very low level.

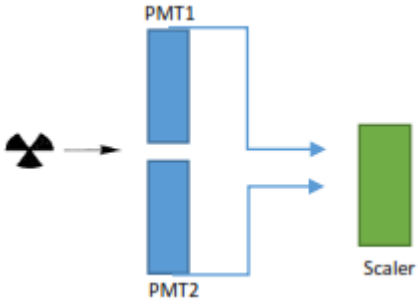


Fig. 10.2. Arrangement of photomultipliers for coincidence counting

3. **Quenching**: The greatest disadvantage of scintillation counting is quenching. Quenching occurs when the energy transfer process from solvent to fluor to photomultiplier tube is interfered with. Correcting quenching adds cost to scintillation counting.

Quenching

Quenching is the loss of counts due to some inherent property of sample, such as its components, and the properties of the cocktail reagents. There are three kinds of quenching: optical, chemical, and color quenching. Note that quenching is not a great problem in solid scintillation counting, but a major problem in liquid scintillation counting.

OPTICAL QUENCHING

This occurs if inappropriate or dirty scintillation vials are used. The dirt will absorb some of the light emitted by the fluors before it reaches the photomultiplier.

COLOR QUENCHING

This occurs if the sample is colored and absorbs light in the range of the wavelength emitted by the fluor. The light emitted is therefore absorbed within the scintillation cocktail before it leaves the sample vial. In this case the number of photons reaching the photomultiplier is reduced.

CHEMICAL QUENCHING

This form of quenching results when materials in the sample interfere with transfer of energy from solvent to primary fluor, or from primary fluor to secondary fluor. Quenchers absorb radioactive

energy before it is converted to light, hence reduction in the number of photons generated by each emission.

4. **Chemiluminescence and static electricity**

Some light emissions unrelated to excitation of the solvent and fluor system by radioactivity may arise from chemical reactions between sample components and reagents of the scintillation cocktail. These light emissions are generally low energy events and are rejected by the threshold setting of the photomultiplier in the same way as photomultiplier noise. Chemiluminescence can be overcome by storing samples for some time before counting, to permit the chemiluminescence to decay. In many modern instruments, chemiluminescence can be detected, subtracted or flagged on the print output.

Chemiluminescence can generate as high as 10^5-10^6 c.p.m., skewing both total c.p.m. data and counts ratio information. It is diagnosed by counting a sample twice within a period of about an hour between counts. A sharp decrease in counts over this period indicates presence of chemiluminescence. As the chemiluminescent reaction consumes its substrate, the rate of photon production decreases noticeably over an hour, and will usually decrease to zero over the course of 2-24 hours. By contrast, even a short-lived isotope like ^{32}P will decrease its emissions by only 5% over 24 hours (Fig. 10.3).

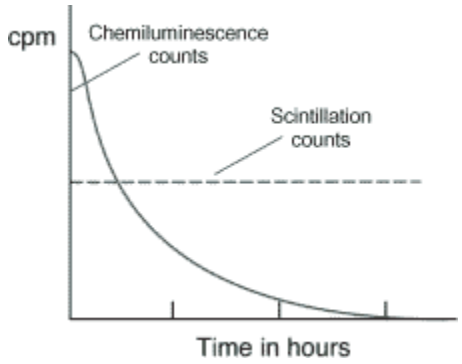


Fig. 10.3. Spurious counts due to chemiluminescence dissipates with time, while the true count stays nearly constant. With the common isotopes used in life science research, the rate of radioactive decay is much slower than the decay of chemiluminescent reactions.

Many scintillation counters use **coincidence counting** to eliminate counts due to chemiluminescence. Because chemiluminescence only generates one photon at a time, only one photomultiplier tube will be activated, hence, this count is rejected. In contrast, the burst of photons from a genuine decay event will activate both photomultiplier tubes simultaneously. Coincidence counting eliminates those emission events which do not appear at both photomultiplier tubes, thus

eliminating chemiluminescence counts. However, coincidence counting will also cause some low energy emission events to be missed.

A further source of false counts is static electricity. The energy from static electric buildup can be released as a burst of light from the cocktail. In dry environments, use of plastic vials and latex gloves cause build-up of high levels of static electricity, sufficient to give at least 10^4 c.p.m. Static is the likely cause if counts from an individual sample vary unpredictably from one measurement to the next. Static can be minimized by wiping the vials with wet paper towel (water dissipates static) or by wiping with an antistatic laundry dryer sheet or fabric softener.

5. **Phospholuminescence**: It results from components of the sample, including the vial itself, absorbing light and re-emitting it. Unlike chemiluminescence, which is a once-only effect, phospholuminescence will occur on each exposure of a sample to light. Samples that are pigmented are most likely to phosphoresce. To prevent this problem, samples should be adapted to darkness prior to counting and the sample holder should be kept closed throughout the counting process.

Despite the five disadvantages described above, scintillation counters are universal in Bioscience labs. This is because the instruments have automated systems for calculating counting efficiency.

Using scintillation counting for dual-labeled samples

A desirable feature of scintillation counting is that the size of electric pulse produced by the conversion of emissions to light energy and then to electrons in the photomultiplier is related directly to the energy of the original radioactive event. Because different β-emitters have different energy spectra, it is possible to quantify two isotopes separately in a single sample, provided their energy is sufficiently different. Examples of pairs of isotopes that have different energy spectra are ^{3}H and ^{14}C, ^{3}H and ^{35}S, ^{3}H and ^{32}P, ^{14}C and ^{32}P, ^{35}S and ^{32}P. The principle of counting dual-labeled samples is illustrated in Fig. 10.4.

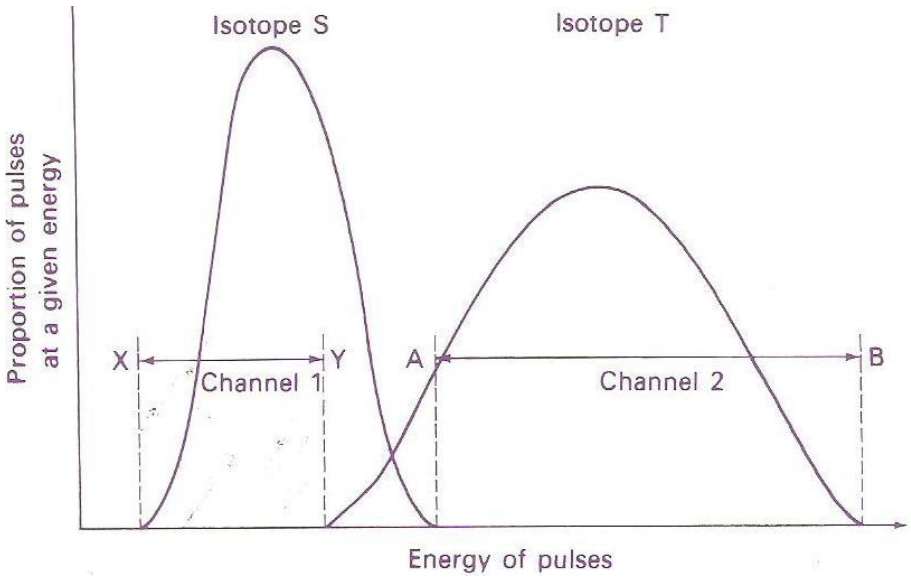


Fig. 10.4. Illustration of the principle of counting dual-labeled samples

In this diagram, the spectra of two isotopes, S and T overlap slightly. By setting up a pulse height analyzer to reject all pulses of energy below X (threshold X) and to reject all pulses of energy above Y (window Y) for isotope S,and also to reject below a threshold of A and accept pulses up to window B for isotope T, it is possible to separate counts from the two isotopes completely. A pulse height analyzer set with a threshold and window for a particular isotope is known as a channel.

Most modern counters operate with a so-called multichannel analyzer. They are based on an analog-to-digital converter; electronic signals from the photomultiplier are converted to digital signals and stored in a computer. Thus the entire energy spectrum is analyzed simultaneously. This greatly facilitates multi-isotope counting and in particular, allows the effect of quenching on dual-labeled counting to be assessed adequately.

Dual-label counting is useful in many aspect of molecular biology, such as nucleic acid hybridization and transcription; in elucidating metabolic pathways such as steroid synthesis, and in drug development.

Determination of counting efficiency

Quenching is a major problem encountered in scintillation counting and this problem makes it necessary to determine the counting efficiency of some samples in an experiment.

Counting efficiency can be determined by the following standardization methods:

1. Internal standardization
2. Channels ratio
3. External standardization

Internal standardization method

An internal standard is a known quantity of a substance that is added to every sample that is analyzed. The substance must behave in a similar way as the analyte and must provide a signal that can be distinguished from that of the analyte. Two approaches are employed in determination of quantitative measures by the internal standardization method: 1) A calibration curve, or 2) standard addition method.

The standard addition method

A sample is counted and gives a reading of, say, ***A*** in c.p.m. It is then removed from the counter, and a small amount of **standard** material of known d.p.m., say, ***B***, is added. The mixture is then recounted to get *C* c.p.m. and the counting efficiency of the sample is calculated.

$$\text{Counting efficiency} = \frac{C - A}{B} \times 100\% \quad \ldots. \qquad (1)$$

Worked example: The internal standard method for calculating counting efficiency

QUESTION

An experimental sample of ^{3}H on a filter paper in scintillation fluid gave a count rate of 1,450 c.p.m. in a liquid scintillation counter. The filter was removed and 5,064 d.p.m. added to it. On recounting, the filter gave a reading of 2,878 c.p.m. What is the d.p.m. of the experimental sample?

SOLUTION

A = 1,450 c.p.m.

B = 5,064 d.p.m

C = 2,878 c.p.m.

First use equation (1) to calculate counting efficiency.

$$\text{Counting efficiency} = \frac{C - A}{B} \times 100\%$$

$$\text{Counting efficiency} = \frac{2,878 - 1,450}{5,064} \times 100\% = 28.2\%$$

Now, use this value to calculate actual radioactivity present in disintegrations per minute.

If 28.2% efficiency gives 1,450 c.p.m.

therefore 100% counting efficiency will give what?

$$\frac{100\%}{28.8\%} \times 1,450 \text{ c.p.m} = 5,142 \text{ d.p.m.}$$

It is necessary to use an internal standard that contains the same isotope as the one being counted, and to ensure that the standard itself does not act as a quenching agent. Suitable ^{14}C-labeled internal standards include [^{14}C]toluene and [^{14}C]hexadecane. Suitable ^{3}H -labeled internal standards are [^{3}H] benzoic acid and $^{3}H_2O$. Note that, benzoic acid and water are themselves quenching agents and must be used in only very small amounts.

Perkin Elmer C-14 Internal Standard

Internal liquid scintillation counting (LSC) standard. ^{14}C Toluene, ~5 x 10^5 DPM/g, 10 mL, List price USD108.00.

Advantages of internal standardization

1. Simple and reliable and corrects adequately for all types of quenching.
2. When carefully carried out it is the most accurate way of correcting for quenching.

Disadvantages of internal standardization

1. It requires very accurate measurement of volumes of the internal standard
2. The method is time-consuming because each sample must be counted twice
3. Sample cannot be recounted in the event of error because it will be contaminated with the standard.
4. Changes in quenching characteristics may occur in sample during the interval between the first and second counts which can lead to considerable inaccuracies, nevertheless, internal standardization is the method by which a scintillation counter is calibrated.

Demerits of scintillation counting

1. No radiation detection system is perfect because of background count.
2. No instrument is capable of recording all of the atomic disintegrations within a scintillation vial because of the counting systems available are not 100 % efficient.

Spurious counts

Actual radioactive events are usually accompanied by spontaneous flashes of light that are also recorded as counts because they cannot be differentiated from genuine decay process. Such false counts in equipment arise from the following:

1. Cosmic rays
2. Beta particles arising from decaying potassium (Half life: 1.251×10^{9} years) in the glass vial
3. Spontaneous discharges from the sensitive photodetectors
4. Chemicals dissolved in the scintillation fluid: chemiluminescence and phospholuminescence

The c.p.m. attributable to such sources are called **background count**. Background counts are often so low relative to the actual decay events being measured and so they are ignored. However, if the number of "real" counts is low, background counts can contribute to experimental error. Therefore, a **control** vial must be prepared which contains everything except added radioactivity in order to determine the background level. Background c.p.m. is then subtracted directly from the c.p.m. from the experimental samples

Efficiency of counting: Owing to the geometry of the vial and photoelectric detectors some events go undetected. The maximum efficiency with which a low energy emitter such as ^{3}H can be detected is

about 70 %. Worse yet, the energy of some photons is absorbed by chemicals in the solvent before the photon can reach the detector. The latter phenomenon is an example of quenching. With the chemical quenching that is typical of most experiments, the usual counting efficiency for ^{3}H is 30 to 40%, and sometimes much less. The amount of quenching can vary from sample to sample therefore it is often necessary to estimate the efficiency of counting for each individual sample.

Remember that the amount of light detected is proportional to the energy of the beta particle that was released by the disintegrating nucleus. When you prepare to count samples, you select appropriate "**windows**", that is, ranges of light intensity that the instrument will record as counts, rejecting all others. The instrument records the amount of light detected following an event, and if that amount is within the energy range for a particular window, the event is recorded as one count. It is ignored if it falls outside the selected range. Each window is given a **channel number**, and the count for each window is given as c.p.m. for the corresponding channel.

Quenching causes scintillation process to be less efficient, that is, less light is produced for a given quantum energy of radiation. Thus, the energy spectrum for a sample appears to be lower than for an unquenched sample (Fig. 10.5)

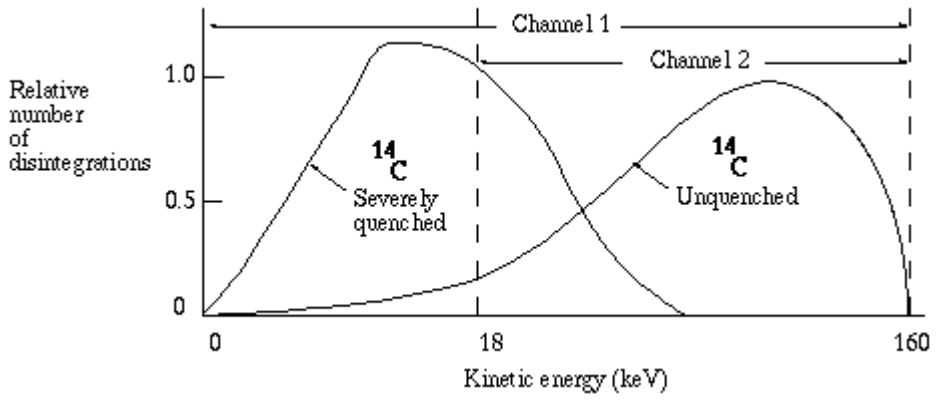


Fig. 10.5. Effect of quenching on a ^{14}C energy spectrum

The higher the degree of quenching, the more pronounced is the resulting decrease in the energy spectrum. As quenching takes place, the energy recorded for each event is less than it would be for an unquenched sample, since for each event the energy of some photons are absorbed before detection is possible. Counts in channel 2 therefore decreases with increasing concentration of

quenching material leading to the ratio of counts in channel 2 to counts in channel 1 becoming smaller after every addition. Similarly, counts in channel 1 decreases with increase in quenching material. The ratio of counts in the channels is the sample channels ratio, or SCR. This forms the basis of the **channels ratio** method for determining counting efficiency.

The channels ratio method

This involves preparation of a calibration curve based on counting in two channels that cover different but overlapping parts of the spectrum. As a sample is quenched, and the spectrum shifts gradually to lower apparent energies, the ratio of counts in each channel will vary.

To prepare the calibration curve

1. A standard (i.e., having known d.p.m.) is counted = unquenched standard. Because radioactivity in the standard is known, the efficiency of counting in each channel can be determined
2. Add **increasing concentrations** of a **quenching substance** to the standard and count after every addition. Calculate efficiency of counting.
3. As the energy spectrum of the quenched standard gradually moves to the left with increasing concentration of quenching substance, the appropriate windows are selected for channel 1 and channel 2 to pick energy ranges that correspond to the radiation being emitted.
4. Calculate standard sample channels ratio (SCR) for each concentration of quenched standard added and plot efficiency (*y*-axis) against SCR (*x*-axis) to produce the standard curve (Fig. 10.6).

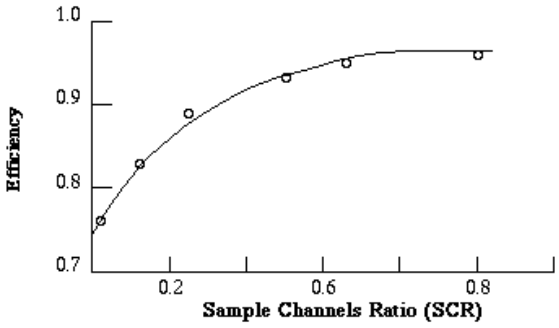


Fig. 10.6. Channels ratio quench correction curve for ^{14}C

Typical data for a set of ^{14}C quenched standards are shown in Table 10.1. It is important to note that one standard curve applies to only one circumstance, i.e., one radioisotope, counter and scintillation cocktail.

Table 10.1. Radioactivity recorded with gradually increasing concentration of quench substance (say, [^{3}H] benzoic acid) in two channels of a scintillation counter.

	c.p.m.		**Ratio**	**Counting efficiency**
Sample	Channel 1	Channel 2	Ch1 : Ch2	Counting efficiency in Channel 2 (%)
^{14}C standard (203,600 d.p.m.) unquenched	171,930	184,250	0.93	90.5= (184250/203600)
^{14}C standard (203,600 d.p.m.) With increasing quench	146,610	168,840	0.87	82.9 = (168,840/203,600)
	94,240	135,090	0.70	66.3 = (135,090/203,600)
	52,260	102,030	0.51	50.1 = (102,030/203,600)
	16,030	58,320	0.27	28.6 = (58,320/203,600)
	5,920	34,740	0.17	17.1 = (34,740/203,600)
	2,060	20,270	0.10	9.9 = (20,270/203,600)
	1,130	13,260	0.08	6.5 = (13,260/203,600)

Once the standard curve is prepared, the efficiency of counting experimental samples can be determined. Samples are counted in the same two channels, the ratio is calculated and efficiency is read from the graph. In practice, all the data can be stored in the counter's computer and corrected values printed automatically.