Chapter 22

Liquid Scintillation Counting of Biological Samples using External Standardization and Automatic Data Processing

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INTRODUCTION

The purpose of this communication is to describe the method by which we routinely process biological samples by liquid scintillation counting. We have used three different counters to compare standardization methods for a variety of samples with wide degrees of quenching. The samples are processed through one of the following counters: the Packard Tri-Carb model 3324, the Beckman model LS-200B or the latest Intertechnique model ABAC SL40-4K, all of which are fitted with a teletype for computer processing. We found that the automatic external standard (AES) channels ratio method, which is a built-in feature of the Beckman, was unsatisfactory for carbon-14 samples with counting efficiencies lower than 70%. We therefore modified the counter using the auxiliary power supply and fitting an external switch to drive the caesium-137 γ-source into position to obtain gross counts as required. Both the Tri-Carb and Intertechnique counters are fitted with an AES, but in addition the Intertechnique has its own built-in 4K computer. This gives the option with this counter of using either its own or our ICL 1903A computer which is used routinely for off-line applications.

The AES process in the modified Beckman is different from that in the Tri-Carb and Intertechnique counters. In the modified Beckman, the samples are all counted and then the AES source is placed in position and each sample is counted again. In the Tri-Carb and Intertechnique, after the β-emission of each individual sample has been recorded, the γ-source is automatically brought out of its lead shielding and the total external standard counts are measured for that particular sample in a separate channel before counting the next sample. To overcome earlier troubles caused by variable positioning of the AES source, a magnetic latch system is now available for the Tri-Carb and has been fitted to our machine. Thus the geometry of counting varies in the three systems.

In our laboratory the punched tape output from the counters is relayed to the ICL 1903A computer by an off-line system, the program language is Fortran IV and the average turn round time is 1 h.

METHOD

The basic method depends on the production of a quench curve (Fig. 1). Eight

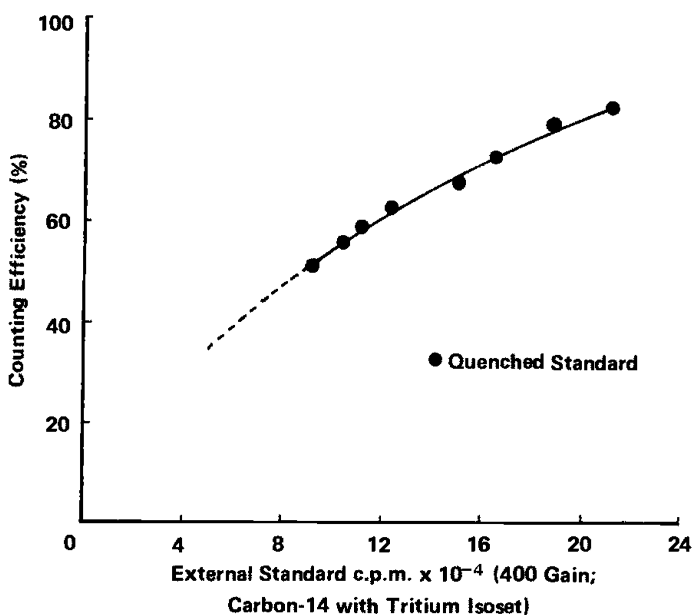

Fig. 1. A typical Beckman external standard quench curve – Hyamine scintillator (10 ml) plus bile/water mixtures (1 ml) plus ^{14}C-hexadecane.

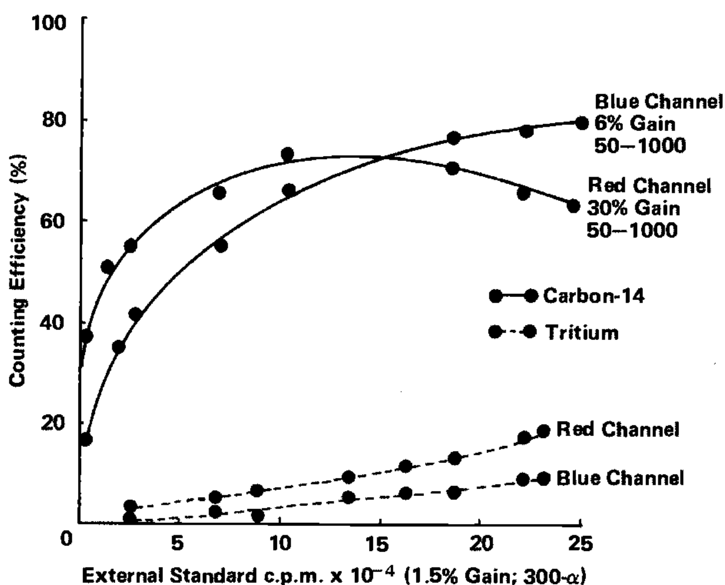

Fig. 2. A typical Tri-Carb dual-labelled quench curve.

standards (or sixteen for dual-labelled determinations) are prepared containing the same volume of scintillator and 10 μl of ^{14}C- or ^{3}H-labelled hexadecane. These are counted to ensure that the accuracy of pipetting of the hexadecane is within ±1%. These standards are quenched to varying degrees either by using non-radioactive aliquots of the biological

sample to be counted or with carbon tetrachloride to produce a curve in which the counting efficiency is plotted against the external standard counts observed.

The counting efficiencies of the eight standards are calculated and equated with their corresponding external standard counts to give a best fit curve by the method of least squares. For single-labelled samples this is achieved by using the exponential of a third order of the logarithms. The curves obtained from the standards for a typical dual-labelled isotope experiment are shown in Fig. 2. The polynomial function used is the square of a fourth order of the square roots and the d.p.m. values are determined by solving two simultaneous equations. The values of the coefficients obtained from these expressions are entered into the data store for the subsequent calculations.

Table 1. Routine biological samples, scintillators and quenching agents.

Sample	Scintillator	Quenching Agent
Urine Plasma Bile	Triton X-100/xylene phosphor[a] (1 : 2) or 14.5% (w/v) hyamine chloride in butyl-PBD–toluene[b]	Dilutions of the biological sample
$^{14}CO_2$	Ethanolamine/methyl Cellosolve (3 : 7) in butyl-PBD–toluene[c]	CO_2 and CCl_4
$^{3}H_2O$	Koch-Light scintillator 354[d]	CCl_4

[a]DPO (0.6%) + POPOP (0.12%) in AR xylene.
[b]Butyl-PBD (0.75%) + Oxitol (7.5%) in AR toluene.
[c]Butyl-PBD (1.0%) in toluene/methyl alcohol (2 : 1).
[d]1,4-dioxan based.

Choice of quencher

Quench curves of the type described above are generated for various biological samples and scintillator systems as illustrated in Table 1. The correct choice of quenching agent is of prime importance. We have compared colour and chemical quenching and have shown a distinct difference between the two at low counting efficiencies (Fig. 3). Walter and Purcell[1] came to a similar conclusion using carbon tetrachloride and lycopene, whereas Higashimura *et al.*[2] found no difference using acetone and Sudan III. This is probably due to the choice of quencher. However, there can also be a difference for the counting of biological samples (e.g. urine and plasma) with or without a chemical quencher (Fig. 4). It can be seen that there is a variation between the curves especially at low counting efficiencies. If, however, the activities of urine samples are calculated from a standard curve prepared from the urine of different species, the results are acceptable (Table 2). We obtain high counting efficiencies with human urine which is probably due solely to the cleaner conditions of collection of such samples, with one or two exceptions! A particularly important example of the effect of the biological sample itself has been experienced in the counting of expired $^{14}CO_2$. Using ethanolamine as trapping agent from small animal metabolic studies it has been found that the ethanolamine quenches differently from the carbamate formed during the carbon dioxide absorption. To overcome this, the

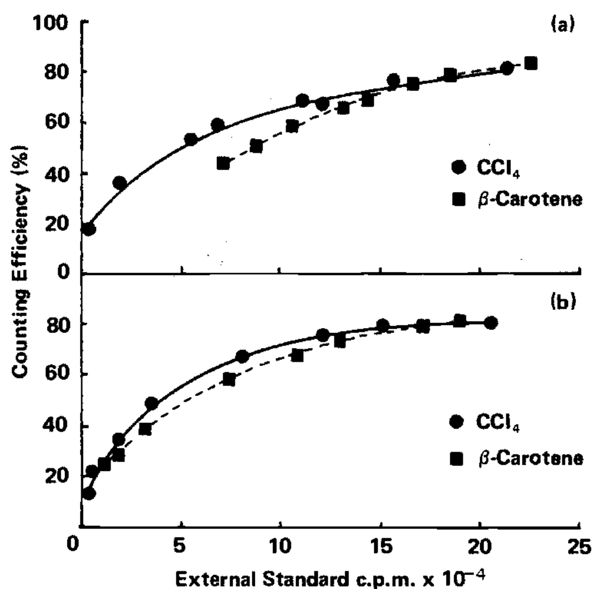

Fig. 3. Colour versus chemical quenching. (a) Beckman – Hyamine scintillator (10 ml) plus 0.5 ml urine plus ^{14}C-hexadecane (10 μl); (b) Tri-Carb – Koch-Light scintillator 354 (10 ml) plus 0.5 ml water plus ^{14}C-hexadecane (10 μl).

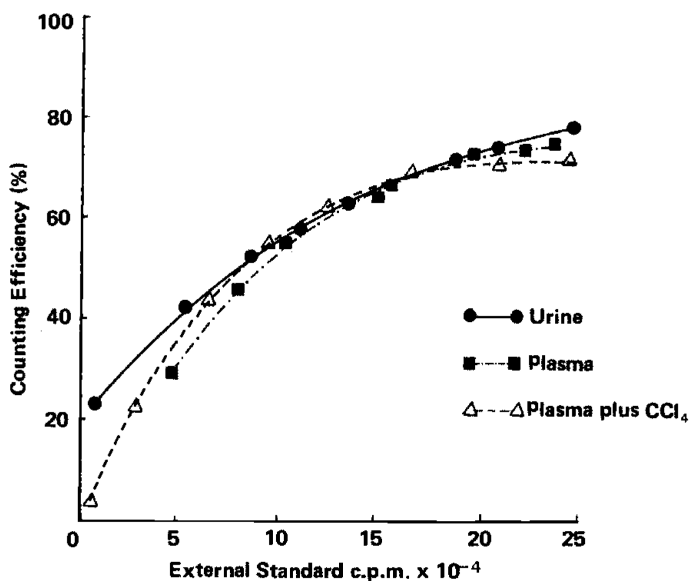

Fig. 4. A comparison of urine, plasma and carbon tetrachloride quench curves. Tri-Carb – Triton X-100 scintillator (10 ml) plus urine or plasma (1 ml) plus ^{14}C-hexadecane (10 μl).

Table 2. Calculation of d.p.m. values from a combined urine quench curve.

Species	Counting efficiency	d.p.m.	% Error
Sheep	76.8	18061	−0.1
	67.0	17769	−1.7
	60.4	18054	−0.2
	52.8	18168	+0.5
	42.0	18059	−0.1
Rat	74.3	18176	+0.5
	67.0	18140	+0.3
	60.8	17515	−3.1
	54.3	18107	+0.1
	46.3	18198	+0.6
	35.1	17644	−2.4
Human	81.3	17906	−0.4
	78.3	18177	+0.5
	75.1	18574	+2.7
	74.9	18499	+1.7
	76.7	17727	−1.9
	75.7	17792	−1.9

The standard curve was prepared from hyamine scintillator (10 ml) plus dilutions (1 ml) of rat, sheep and human urine. Each sample contained urine (1 ml) plus ^{14}C-hexadecane (18,083 d.p.m.) and was counted in the Beckman to an accuracy of 1%.

Table 3. The effect of expired carbon dioxide on the ethanolamine/EGME quench curve.

Sample	CCl_4 standards			(CCl_4 + CO_2) standards		
number	Efficiency	d.p.m.	% Error	Efficiency	d.p.m.	% Error
1	57.6	14256	−21.8	45.4	18110	−0.7
2	26.6	19375	+ 6.2	28.4	18122	−0.6
3	25.9	19578	+ 7.3	27.9	18168	−0.4
4	17.9	17692	− 3.0	17.8	17698	−3.0
5	10.5	17990	− 1.4	10.1	18330	+0.5
6	8.7	18429	+ 1.0	8.9	18016	−1.2

The standard curves were prepared from ethanolamine/EGME absorber plus butyl-PBD scintillator and contained either CCl_4 or CCl_4 + CO_2 as quenching agent. All samples were authentic pre-dose absorber/scintillator mixtures containing ^{14}C-hexadecane (18,240 d.p.m.) and were counted in the Tri-Carb.

carbon-14 standards must be saturated with carbon dioxide as well as containing carbon tetrachloride as quencher (Table 3).

The shelf-lives of various sets of quenched scintillator standards have also been investigated (Table 4). It appears that the stability of these standards is dependent on the scintillator, the biological sample and the quenching agent and in general is independent of storage temperature. Unstable samples such as bile are normally used only on the day

Table 4. The shelf-lives of scintillator standards.

Sample	Scintillator	Isotope	Shelf-life 0°	R.T.
Plasma	Triton X-100	Carbon-14	8 weeks	8 weeks
		Tritium	2 weeks	1 week
Urine	Hyamine	Carbon-14	> 12 weeks	> 12 weeks
	Triton X-100	Tritium	–	12 weeks
Bile	Hyamine	Carbon-14	–	1 day
Carbon dioxide	Ethanolamine/butyl-PBD	Carbon-14	4 days	4 days
Water	Koch-Light No. 354	Tritium	–	> 8 weeks

All samples were stored in the dark, either at room temperature (R.T.) or at 0 to 4°C (0°). The shelf-lives were determined by counting freshly labelled hexadecane and using the original standard scintillator sets to calculate the d.p.m. values. The shelf-life normally refers to the maximum period for which the standards gave a counting accuracy of ±2.5%.

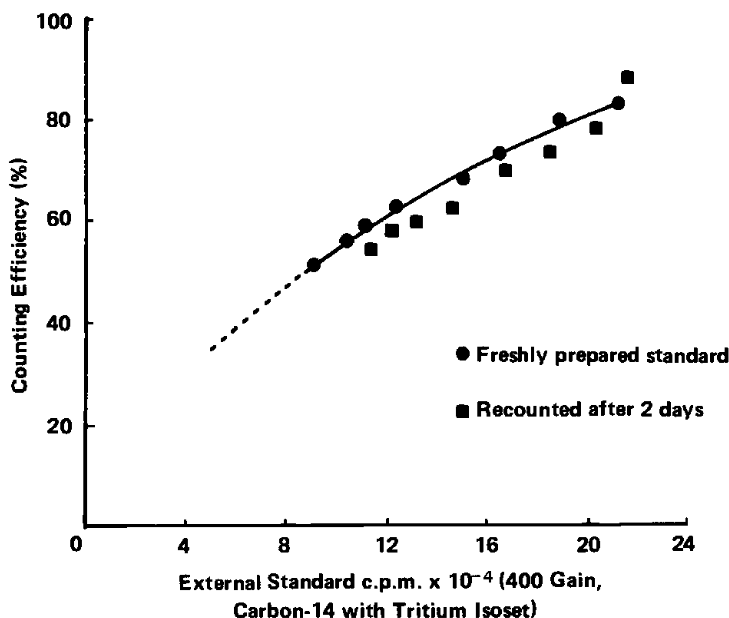

Fig. 5. The shelf-life of bile quenched standards.

of preparation (Fig. 5). For the stable scintillator sets both the counting efficiency and external standard values decrease with increasing storage time. An example of this is shown in Fig. 6. It might be argued that the coefficients derived from these stable sets could be stored in the program and this would certainly be possible given stable counting conditions. However, this would involve the inclusion of various sub-routines in the program and time and expense to remove and replace programs in the computer library file when the standards needed to be changed. One method reported to have overcome these difficulties associated with a stored standard curve is to make slight adjustments of the attenuation

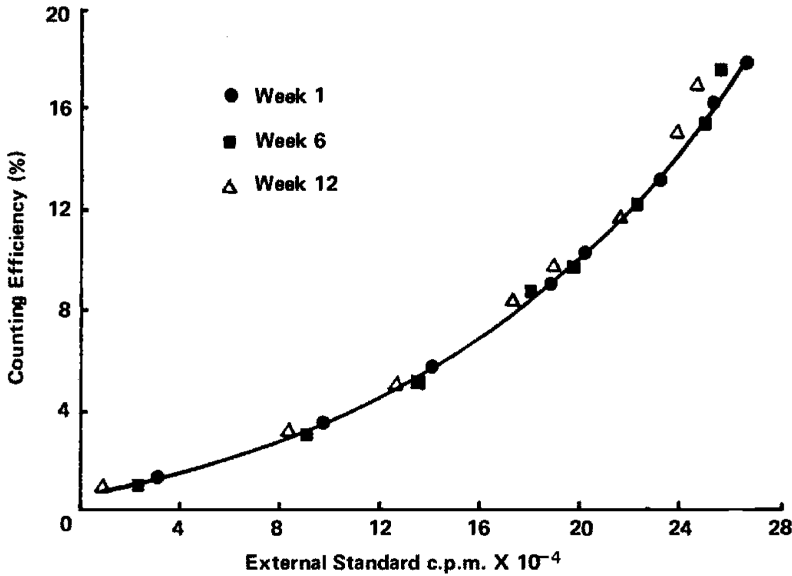

Fig. 6. Shelf-life of ^{3}H-hexadecane/urine standards. Beckman – Triton X-100 scintillator (10 ml) plus urine dilutions (1 ml) plus ^{14}C-hexadecane (10 μl).

settings (Figdor,[3] Jordan, Kaczmar and Köberle[4]). However, in our opinion the best way to overcome any day-to-day variations and drift of the liquid scintillation counter is to count the relevant standards on each occasion.

Print outs

A typical print out for a single isotope experiment is shown in Table 5. It can be seen that there is a minimal deviation between measured and curve efficiency which shows a correct choice of polynomial function – the same is true for dual-labelled determinations. The d.p.m. values of the hexadecane standards are written into the program with a half-life correction for tritium. All our samples are counted in duplicate and the average d.p.m. for each sample is calculated. For the print out of d.p.m. from dual-labelled experiments (Table 6) a cautionary message was incorporated into the program. This was required very occasionally when the tritium counting efficiency was below 1% and the corresponding curve was extrapolated by the computer to infinity rather than to zero.

Comparison of methods

As yet there is no all-purpose quench correction method and like other methods the use of the external standard gross count method has been reported to have certain disadvantages. These have been adequately resolved for our purposes:

1. The problem of variable external standard geometry does not really arise in the Beckman. The standard deviation of the external standard count rate under several quenched conditions in the Tri-Carb has been observed. When calculated in terms of efficiency this deviation is minimal at low quenching and at high quenching (20% efficiency for carbon-14 and 2% efficiency for tritium) is approximately ±1% for carbon-14 and ±0.1% for tritium.

Table 5. A typical printout for a single isotope experiment.

PROGRAM RRC6 LINK WRL8 PAR
00 0 DATE 23/06/71 TIME 12/32/44

14C URINE TEST SAMPLES IN TRITON SCINTILLATOR
C 18083.0

CALIBRATION CURVE

COUNTS STD. IN	COUNTS STD. OUT	MEASURED EFFICIENCY	CURVE EFFICIENCY
190838.	14024.	77.55	77.59
176439.	13663.	75.56	75.80
107952.	11510.	63.65	63.73
171393.	13705.	75.79	75.12
128805.	12376.	68.44	68.05
70366.	9897.	54.73	54.86
143657.	12655.	69.98	70.79
86015.	10650.	58.90	58.65

BACKGROUND = 50.5

SAMPLE NO.	COUNTS	EFFICIENCY	D.P.M.
11	1425.1	76.55	1795.7
12	1482.7	78.97	1813.7
MEAN			1804.7
13	1478.8	78.61	1817.0
14	1388.5	73.68	1815.9
MEAN			1816.4

Table 6. The d.p.m. of the ^{14}C- and ^{3}H-labelled samples.

BACKGROUND, RED CH. = 48.0 , BLUE CH. = 41.0

SAMPLE NO.	RED CH. C.P.M.	BLUE CH. C.P.M.	RED C EFF	BLUE C EFF	RED T EFF	BLUE T EFF	C D.P.M.	T D.P.M.
1	17293.0	15770.0	64.53	75.55	17.45	7.73	18270.3	33227.9
2	17257.0	15707.0	64.86	75.31	17.19	7.56	18347.4	34943.1
STD. FOR NEXT LINE IS OUTSIDE RANGE OF ONE OF CALIBRATION CURVES								
3	17756.0	17025.0	62.04	76.94	18.87	8.60	18356.9	33743.4
4	14708.0	11745.0	67.67	60.74	7.29	2.24	18092.8	33794.0
5	13680.0	10871.0	64.79	54.93	6.16	1.93	18017.9	32056.1
6	12359.0	9234.0	60.75	49.09	4.56	1.37	18003.5	31205.8
STD. FOR NEXT LINE IS OUTSIDE RANGE OF ONE OF CALIBRATION CURVES								
7	12810.0	10046.0	61.51	50.09	4.92	1.14	19624.3	15031.2

2. Since the AES count rate depends also on the sample vial material and wall thickness being the same, we always use the same type of counting vial and it has been shown that a 10% variation in wall thickness causes a count rate error of less than 0.2% (Higashimura *et al.*[2]).
3. Variation in the scintillator volume causes large errors. In our system the scintillator volume is therefore kept constant.
4. New calibration standards are prepared for each new scintillator batch, thus overcoming small batch variation.
5. Since the external standard method is not normally suitable for emulsions or suspension counting it is not used under these conditions.

Table 7(a). A comparison of standardization methods – 20 urine samples in Triton X-100 scintillator, each containing 1858 d.p.m., were standardized by five different methods.

Method	External standard gross count Tri-Carb	Beckmann	Intertechnique	External standard channels ratio	Internal
Average	1877	1845	1873	1863	1847
Range	1811-1989	1788-1928	1798-1943	1761-1996	1755-1919
SEM	9.2	8.7	9.6	12.0	10.5
Average % deviation from theoretical value	1.90	1.93	1.95	2.00	2.12

Table 7(b). A comparison of standardization methods – the d.p.m. values reported represent a range of 65 ^{14}C-labelled rat urine samples in Hyamine scintillator from a typical metabolism experiment.

External standard gross count Tri-Carb	Beckman	Intertechnique	External standard channels ratio	Internal
57198	57336	56637	57183	55952
37233	36825	36195	36770	35951
20855	20531	20069	20315	20860
6269	6178	6305	6211	6391
399	451	424	435	468

We have compared our method of external standardization with internal standardization for most of the biological systems used and have generally found agreement to within 3% or better. Examples of these comparisons are shown in Tables 7(a) and 7(b). In Table 7(a) the average percentage deviation from the theoretical value, which might appear relatively high, was calculated on the basis that the activity of all samples was identical. The calculated

d.p.m. values of a number of samples were for all methods consistently either above or below the theoretical activity. Also all samples were only counted to an accuracy of ±1%. Therefore this percentage error would probably be lower under more suitable conditions. The values in Table 7(b) represent five typical samples from a metabolic experiment covering the whole range of activities. Again there were only small variations in the d.p.m. values between the different methods. Some counters (e.g. the Intertechnique used in the present study) use a combination of the channels ratio and external standard methods. This removes the disadvantages of the channels ratio method and also reduces some of the shortcomings of the external standard method since the fluctuations in wall thickness, the distance between the specimen and the γ-source and the scintillator volume change the total count rate more than the channels ratio (Frampton[5]). Figdor[3] using this method for counting various carbon-14 and sulphur-35 biological samples on a Nuclear Chicago model 6860 reported a percentage difference from internal standardization of −6.3% to +4.0%. From our own studies (unreported) on the external standard channels ratio method using the Tri-Carb, this error seems large, and we are more in agreement with Parmentier and ten Haaf[6] who in a recent review concluded that for modern counters this combination method was the best for standardization. However, one of the disadvantages of using this method with the older models of counter is the alteration of window and gain settings which would be involved when both carbon-14 and tritium samples are continuously being counted.

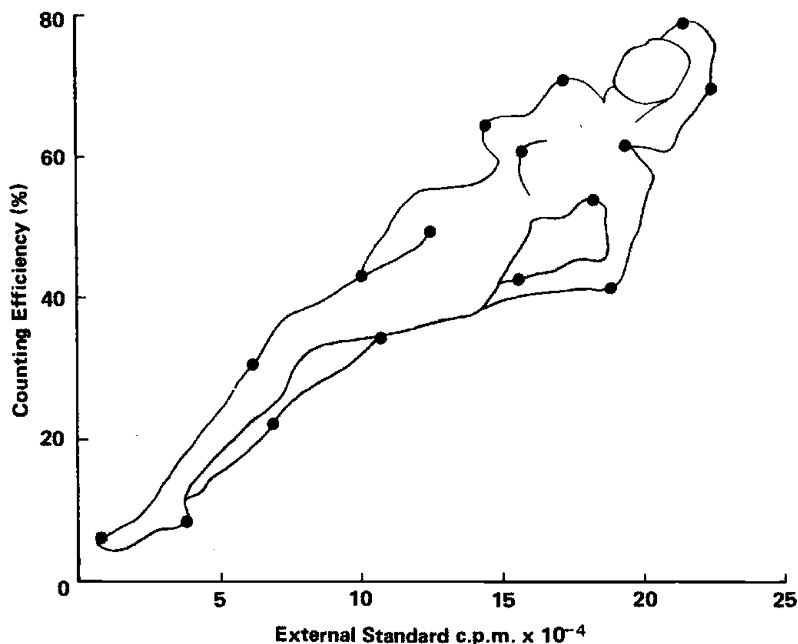

Fig. 7. Miss Plot 1971.

CONCLUSIONS

There is a wide range of quench correction methods available. For routine counting of biological samples in older models of counter we recommend the use of the method described; that is, the external standard gross count method. Other than the obvious need for computer facilities, the success of the method depends on the careful preparation of the quenched standards. If, however, care is not taken in the preparation of the quenched standards, our computer puts its own interpretation on the data input and the quench curves shown in Fig. 7 are obtained.

ACKNOWLEDGEMENT

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REFERENCES

1 W. H. Walter and A. E. Purcell, *Anal. Biochem.* **16**, 466 (1966).
2 T. Higashimura, O. Yamada, N. Nohara and T. Shidei, *Intern. J. Appl. Radiation Isotopes* **13**, 308 (1962).
3 S. K. Figdor, *Comput. Biomed. Res.* **3**, 201 (1970).
4 P. Jordan, U. Kaczmar and P. Köberle, *Nucl. Instr. Methods* **60**, 77 (1968).
5 J. A. Frampton, Fachtagung Nuclex, Basel, 8-14.9, Vortrag 7/25 (1966).
6 J. H. Parmentier and F. E. L. ten Haaf, *Intern. J. Appl. Radiation Isotopes* **20**, 305 (1969).

DISCUSSION

F. E. L. ten Haaf: Comment: This paper has shown that external standardization can be an excellent method provided the necessary precautions are observed.