

SOME RECENT DEVELOPMENTS IN LIQUID  
SCINTILLATION COUNTING OF BIOCHEMICAL SAMPLES

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The purpose of this presentation is to review and comment upon a few of the more recently developed techniques that have been found useful in applying liquid scintillation counting to the assay of beta emitter activity in samples typical of biochemical research. This does not attempt to be comprehensive and represents this author's experience.

A year and a half ago we reported (1) on our use of suspensions of barium carbonate in toluene, gelled with Thixcin, for counting  $C^{14}$  derived from hemoglobin. Use of this technique has continued and well over 2000 such samples have been prepared. Our technique evolved from replacing the aluminum stearate, used by Funt and Hetherington (2), with the Thixcin as employed by White and Helf with gels for substances other than barium carbonate (3). The resultant counting samples look opaque and bizarre with all sizes of clumps of precipitate but they count reproducibly. We attribute this to the probability that the barium carbonate is really in microcrystalline form of rather uniform size, that the large aggregates of such crystals are permeated by scintillator solvent, and that the solid is an efficient reflector for the light of the scintillations. The absolute efficiency of the system depends upon the amount of barium carbonate in the bottle and runs linearly from 60 per cent with no suspension to 41 per cent with one gram of barium carbonate in 20 ml. of gel. The method is especially convenient for samples that inherently arise as  $CO_2$  gas. The barium carbonate is weighed into the bottles and this weight provides the appropriate efficiency factor from previously established data.

We have tried the Cab-O-Sil gels described by Ott et al. (4). The efficiency did prove to be somewhat superior to that of Thixcin. For our particular production line this seemed less important than the convenience of the single-step addition of fluid Thixcin-toluene

to the barium carbonate, as compared with separate additions of Cab-O-Sil and toluene. We have also been concerned over the possible health hazard with Cab-O-Sil because it is silica, it tends to dust into the air, and it could readily be inhaled. Cab-O-Sil, however, would seem to be a real convenience for those needing only an occasional suspended sample--a sort of "instant" scintillator gel. The choice between barium carbonate gel counting of CO<sub>2</sub> and counting the CO<sub>2</sub> as Hyamine-carbonate in toluene solution (5, 6) seems to be very much one of personal preference and economics. The counting efficiencies, the quantities of carbonate that can be incorporated per bottle and the labor of sample preparation seem very similar. The Hyamine technique is significantly more expensive. It costs about nine cents per millimole for the commercially prepared solution of the Hyamine base in contrast with a half a cent for the Thixcin necessary to gel 20 ml. of toluene and support 5 millimoles of barium carbonate.

Both gel counting and Hyamine counting have numerous applications beyond carbonate assay. Helf, White and Shelley (7) have reported the use of gels for counting various solid compounds involving a number of isotopes. Shapira and Perkins have recently used Thixcin gels for counting aqueous materials in emulsion form (8). Hyamine is equally versatile since it can solublize in toluene almost any acidic sample material as well as compounds such as proteins and polysaccharides that are susceptible to alkali digestion (9, 10, 11, 12).

In instances where specific activities are sufficiently high for small samples to be adequate, specially tailored scintillation solvent mixtures may be ideal. Along these lines the author reported the use of a dioxane-anisole-dimethoxyethane mixture (DAM) for counting purines dissolved in water or ethylene glycol (13). This same mixture has been extensively employed since for counting tritium water and a variety of water-soluble compounds. Like other mixtures of organic solvents with water, the water is prone to separate out if there are appreciable quantities of salts in the sample. This, for example, caused us difficulties in testing for radioactivity in fractions from a Dowex-1-formate ion exchange column being eluted with formic acid-ammonium formate. Formic acid up to 4 normal made fine counting samples with 4 parts of DAM but when ammonium formate-formic acid buffer was used to elute columns, similar DAM counting samples all became diphasic. Treatment of the eluates with sufficient Dowex-50 hydrogen resin to remove the ammonium ion resulted in solutions that were again miscible with DAM.

In another instance a 30 per cent methanol in toluene scintillator solution was employed to prepare samples containing up to 0.1 mg. of C<sup>14</sup>-dextran and C<sup>14</sup>-carboxylinulin. These difficultly soluble substances were in 0.1 ml. of aqueous solution that was counted with 10 ml. of the organic

scintillator with 58 per cent absolute efficiency. The same type of alcohol-toluene scintillator has been employed at the level of 18 ml. to dissolve 0.2 ml. of aqueous alkali containing  $\text{CO}_2$  or digests of protein (14). Herberg has used a blend of toluene, dioxane, methanol and naphthalene to dissolve alkali digests of tissue samples (10).

Another point to keep in mind is the fact that some non-scintillation solvents, which may be useful as extractants to isolate material for counting, can themselves be mixed with a scintillation solvent without much loss of counting efficiency. Dr. Thomas Butler and I employed this device for counting dimethylloxazolidinedione- $\text{C}^{14}$ , DMO. This compound was readily extractable into ethyl acetate from aqueous homogenates of tissues. One part of such ethyl acetate extract was counted in 5 parts of toluene scintillator solution with nearly the same efficiency as a pure toluene solution. It did require two taps higher voltage on the Tri-Carb photomultipliers to show the peak differential counting rate.

One of the more agreeable developments of the past few years, so far as our counting in DAM solvent goes, is the availability of adequately pure solvents from commercial sources. The former need for elaborate purification of the ethers discouraged many workers from using this system. Now we find that the spectroscopic grade of dioxane manufactured by the Matheson Co. yields very satisfactory results when used with 1, 2-dimethoxyethane from the same manufacturer and anisole from Distillation Products Industries.

Dr. Daniel Steinberg is responsible for a novel and most useful approach to the problem of counting aqueous samples (15). He explored the possibility of reversing the phases of the suspended sample technique and put his aqueous samples in bottles containing solid scintillator particles. The object is to provide a large surface area of scintillator to come in contact with the sample solution. The best form of scintillator to date is blue-white fluorescence grade anthracene. For  $\text{C}^{14}$  solutions this can provide 54 per cent absolute efficiency when the anthracene is less than saturated with sample. This efficiency is difficult to control so Dr. Steinberg recommends using a fixed excess of sample solution with a specific weight of anthracene, a procedure analogous to infinitely thick barium carbonate Geiger-Mueller counting. For 3.0 ml. of aqueous sample and 1.00 gm. anthracene the absolute efficiency is about 22 per cent. A nonionic wetting agent is added in preparing the samples. This technique seems applicable to any water solution of adequate optical clarity and its rather moderate efficiency is compensated for by its relatively large capacity for aqueous sample material. Full details have recently been published (16) and I shall merely vouch for the fact that we have confirmed the reported efficiency and reproducibility.

Last fall a brief note by Wang and Jones (17) reported that radioactivity deposited on squares of filter paper could be counted by immersing them in especially constructed bottles containing conventional toluene scintillator mixture. This came to our attention six months ago when we were separating C<sup>14</sup>-labeled purines and nucleotides on Whatman 3 MM paper by high voltage electrophoresis. We tested a 1" x 1 1/4" piece of paper wedged vertically in a standard 5 dram vial and were amazed at the 50 per cent counting efficiency. This technique quickly became our standard one and the 2-11 per cent variation in counting rate for repeated automatic counts on the same samples was not excessive for much of our work. In April, Geiger and Wright (18) published a study of this counting technique and reported their experience with a manual Tri-Carb counter, where the orientation of the paper in the bottle with respect to the photomultiplier tubes could be controlled. Their data showed 10-17 per cent of the mean as the range of observed counts at different angles of rotation of the sample. The correlation of low values and high values with certain orientations supported our belief that much of our variation was due to the random positioning inherent in automatic counting. Last month we received the June issue of "Atomlight", issued by New England Nuclear Corporation. This carried an article by Dr. Robert Loftfield in which he reported attempts to obviate the sample paper orientation problem by forming the paper into a vertical cylinder within a 10 mm. diameter test tube filled with solvent. This very substantially reduced the variability of the counting rates with a concomitant 20 per cent reduction in efficiency. At just this time we had also given a little thought to the asymmetry problem characteristic of the vertical square type of paper sample and decided that a recumbent circle or disc of paper on the floor of the bottle might look the same to the phototubes from any rotational angle. The fact that we, like Loftfield, lost only 20 per cent of our counts in going to a disc sample was encouraging. A set of ten replicate paper disc samples was prepared using a few micrograms of hypoxanthine-C<sup>14</sup> in 10 lambda of water as the load. Papers were dried, put in bottles of toluene scintillator solution and counted for ten cycles of the automatic Tri-Carb counter with the accumulation of sufficient pulses to provide for a 95/100ths error of less than one per cent on each count. The data in terms of the variation between the highest and lowest of the ten counts on each bottle, as a percentage of the average of the ten counts, are given in Table 1 in the "circles" column. The maximum variation for any ten counts on random repositioning of a bottle was 2 per cent. The column headed "squares" shows analogous data for 10 of our wedged-in, 1" x 1 1/4", vertical pieces of paper. The average variation here is over three times as great as with the circles. Truly homogeneous, dissolved C<sup>14</sup> samples under these conditions show variation that amounts to the statistical error of the counting or about 1 per cent in this case. The absolute efficiencies are 70 per cent for dissolved samples, 50 per cent for

squares and 40 per cent for circles. To obtain this last efficiency for circles it is necessary to count them at one or two high voltage taps (85-170 volts) greater photomultiplier voltage. The disparity in efficiencies for dissolved as compared with "on paper" samples makes it essential to know whether samples do dissolve. If they dissolve readily off paper, samples will count as homogeneous samples and the phototubes seem to be unaware of the presence of either a square or circle of paper. A simple test for what has happened is to count, remove the paper and count again. With truly insoluble compounds the "used" sample bottle and solvent give a blank counting rate and may be used for the next piece of paper.

A number of the factors influencing this counting technique have been examined. The volume of toluene overlying a paper disc is inconsequential between 5 and 15 ml. except as larger volumes increase the background. At 18 ml. of toluene about 4 per cent of counts is lost. If solvent and bottle are reused, the background of the solvent eventually builds up due to small bits of fiber accumulating. Simple filtration removes these and restores the original background. Reuse of solvent also accumulates color. Our sample bottles for electrophoresis papers, which are dried from Varsol, showed this and any color extractable from sample papers might be expected to accumulate similarly. Such color betrays itself by a shift of the pulse spectrum. Small pencil notes on sample papers, on the other hand, do not influence the counting rates. The translucency of filter paper to scintillation photons is so good that a blank disc of Whatman 3 MM on top of a disc containing a  $C^{14}$  sample causes only a 3 per cent reduction in count rate.

Since this paper counting appears to be an infinitely thin sample technique we sought to determine the maximum mass of a safe load. With glucose solutions and  $C^{14}$ , 10 lambda spots applied to blotted water-wet papers showed no self-absorption up to 5 per cent glucose in the loading solution; this was the highest concentration tested. In one calibration of our disc counting efficiency, 0.1 ml. of sodium hydroxide-carbonate containing 5.1 mg. of salts was dried on a 24 mm. disc and gave the same 40 per cent efficiency calibration as did a 3  $\mu$ g. sample of hypoxanthine- $C^{14}$ . The 24 mm. disc may be conveniently cut from papers using a silver quarter dollar as a template. When spots on papers from electrophoresis or chromatography exceed this size, overlapping circles can be employed to count the large spots as several samples. This is not as convenient as dicing papers into squares, which should suffice for manual counter operators, who can provide uniform orientation of their samples. As might be expected, color in a paper sample does quench its counting and this cannot be measured with an internal standard in solution.

Table 1

COMPARISON OF VARIABILITY OF AUTOMATIC  
COUNTS ON PAPER SAMPLES IN BOTTLES

Sample No.	<u>Squares</u>	<u>Circles</u>
	(% variation)	
1	11	1.4
2	8	1.2
3	4	1.6
4	1	2.0
5	11	1.0
6	4	1.6
7	11	1.0
8	7	1.4
9	10	2.0
10	4	1.6
Average	7.1%	1.5%

Ten samples each of C<sup>14</sup>-hypoxanthine on 1" x 1 1/4" squares mounted vertically and 24 mm. diameter discs lying horizontally in 20 ml. bottles were counted for 10 automatic cycles in a Tri-Carb counter. All bottles contained toluene with 0.3% PPO and 0.05% POPOP, 18 ml. for the squares, 15 ml. for the circles. 40,000 or more counts were recorded at "peak" high voltage thru a 10-100 volt window for each counting. The % variation is the % ratio of the difference between the highest and lowest values of ten counts on the sample to the mean of the ten counts.

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