

ROUND-TABLE ON
'METHODS OF COUNTING ACIDS AND OTHER
SUBSTANCES BY LIQUID SCINTILLATION'

NORMAN S. RADIN

*Radioisotope Service, Veterans Administration Research Hospital and Biochemistry
Department, Northwestern University Medical School, Chicago, Illinois*

THE Radioisotope Service in the Veterans Administration Research Hospital Chicago, had the privilege of receiving the first or nearly first commercial model of the Packard Tri-Carb counter 3 years ago. At that time, toluene seemed to be the solvent of choice and we knew that some water could be dissolved if ethanol were added. The technique of liquid counting was therefore limited to T_2O , many lipids, and trace amounts of water-soluble substances. From the standpoint of the biochemist, it seemed that the liquid counter would be much more useful if fairly large amounts of carbon dioxide (whether respiratory or the product of a combustion), as well as amino acids, carbohydrates, etc. could be dissolved in a non-quenching form.

The problem of solubilization has been of great commercial importance for many years, and some of the principles involved are now known, if only empirically. In the case of salts, it is generally known that changing one of the ions can produce enormous changes in solubility characteristics. Thus, zinc sulfate is insoluble in toluene but is soluble in water, while zinc oleate shows the reverse properties. As a matter of fact, I think one could count radioactive zinc and other metals by liquid scintillation by making the oleate or similar salts. It seemed likely, on the basis of these observations, that a toluene-soluble salt of carbonic acid could be prepared with an organic base of high molecular weight. A strong base, such as a guanidine or quaternary amine, would be necessary to form a stable salt. Drs. John Passmann and John A. D. Cooper and myself chose for the first trials a typical quaternary ammonium compound, cetyldimethylethylammonium bromide. The free base was generated by ion exchange, the bicarbonate salt was formed, and the first test showed that the compound was indeed soluble in toluene and did not quench excessively in the scintillation system.

The highly promising first result kept us chasing after an elusory finished method for 14 months. We discarded the use of ion exchange as a means of preparing the free base because of the large volumes of solutions that would have to be handled and eventually evaporated down. The use of KOH was tried and dropped. Finally, we turned to silver oxide, the more traditional

reagent for forming such free bases, but found that some of the silver dissolved in the basic solution, forming a light-sensitive mixture. The initially colorless solution of the free base slowly turned brown or yellow. The darkening was much faster if the solution was concentrated, even by vacuum evaporation. I suppose the quaternary amine contains some tertiary and secondary amines which form complex ions with the silver, just as ammonia does. The use of alcohols other than methanol as the solvent for the base produced somewhat worse darkening, and here I think the problem is aggravated by the traces of aldehydes present in the higher alcohols. Methanol may form formaldehyde on standing, but it may be that the formaldehyde condenses readily to form formal (dimethoxymethane), which should be stable in alkali. Attempts at removing aldehydes and lower order amines were of no avail. Finally we decided that, if we could not beat the light effect, we could join it. Exposure of the base solution to a bright light for a few days precipitated the complexed silver and gave us a stable, colorless (or nearly colorless) solution.

In the course of these experiments, we learned from Bill Holland, of Helene Curtis Industries, that Rohm and Haas sells high quality quaternary amines called Hyamine. We chose Hyamine 10-X, which is *p*-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium chloride monohydrate. It appears to be the cheapest and purest available amine in this class, and possesses branched methyl groups, which should contribute to the toluene-solubilizing activity. This material showed the same drawbacks as did the other amines, but to a somewhat lesser extent.

The methanol used in preparing the hydroxide reduces the counting efficiency, but we found that evaporating off the MeOH often produced dark material. Moreover, a solution stronger than 0.5 M is rather viscous for pipetting (due partly to the high molecular weight). Consequently, we evaporate down to a solution that is 1 M and then dilute with an equal volume of toluene. I have the impression that the methanol in the solution tends to stabilize the amine. There is nevertheless a slow degradation of base strength. I have a Hyamine solution that has lost half its titer in almost 2 years, but is otherwise satisfactory.

Radioactive carbon dioxide can be absorbed by the Hyamine by aeration techniques, but it seems likely that a variable and differential amount of solvents would be lost, so we considered only a diffusion technique. We use a flask made from two 50 ml Erlenmeyers, which are joined by a short, V-shaped tube of 16 mm o.d. The V is inverted, but it might be more effective in trapping spray if it were not. The Erlenmeyer, which contains sodium carbonate, has a side arm for holding sulfuric acid. The other Erlenmeyer contains the Hyamine base, in at least 10% mole excess. Rubber stoppers are used, as toluene absorption during the 90 min diffusion period is negligible. We agitate the flasks on a rotary shaker to speed the diffusion. Excessive

diffusion time, whether shaking is or is not used, results apparently in diffusion of methanol into the aqueous solution while water diffuses into the Hyamine side. Water, as you all know, is an undesirable component of liquid scintillation systems.

We have tried to simplify the diffusion technique, particularly to avoid the transfer of the Hyamine carbonate into the counting vial. Tests were made with large jars in which the carbonate solution was on the bottom and the counting vial, containing Hyamine, was suspended from the jar's rubber stopper. Acid was added by a stopcock, and the jar was shaken by a rotator. Unfortunately, variable results were obtained, probably due to excessive absorption of toluene by the large rubber stopper. The use of an all-glass device might solve this problem.

Dr. John P. Miller, working in Dr. Cooper's laboratory, has run experiments with tissue homogenates and C^{14} acetate in Warburg flasks, using Hyamine in the center well, and apparently there was no harmful effect on the enzymes. Conway diffusion vessels could probably be used with Hyamine also.

The use of Hyamine for counting $C^{14}O_2$ has the convenient characteristic of giving counting efficiencies which are independent of the amount of carbonate in the Hyamine. In other words, the Hyamine base and Hyamine carbonate (and bicarbonate) have the same quenching activity. This obviates the need to work with systems free of unlabeled CO_2 and to know the amount of CO_2 present, provided sufficient Hyamine is available. If it is of interest to know the exact amount of CO_2 being counted, one could titrate the excess Hyamine hydroxide. It is unfortunate that increasing the amount of Hyamine in a counting vial decreases the efficiency; how much is due to the methanol and how much is due to the amine, I do not know. It might be wise, if large amounts of CO_2 are to be counted, to try evaporating off more of the MeOH before diluting with toluene.

Another convenient characteristic is the ease of getting complete transfer of CO_2 samples into the counting vials, whether from combustion, sodium carbonate, or enzyme systems. The more recent $BaCO_3$ suspension systems¹⁻⁴ pose a bit of a problem in this respect.

We have worked with PPO as our scintillator, but it is possible that terphenyl with POPOP would be superior. One old batch of PPO produced a dark solution when added to the Hyamine. The impurity was removed by recrystallization.⁵

In measuring background values with Hyamine and varying amounts of unlabeled CO_2 , we observed that CO_2 -free solutions gave erratic background values. It may be that the latter solutions, which are very basic compared to the CO_2 -buffered solutions, are sensitive to extraneous variables. In our paper describing the Hyamine method,⁶ we specified the use of a blank that contains some unlabeled carbonate. It seems advisable in other applications

of Hyamine to avoid having too great an excess of free base. If it is necessary to use a large excess of free base to dissolve something, some acid such as acetic should be added subsequently.

We have tried to adapt the silver persulfate wet combustion technique^{7,8} to the double flask Hyamine diffusion method, but found that heating the system caused excessive diffusion of water into the Hyamine. Certain substances, however, are oxidized even at room temperature, particularly when a fresh bottle of potassium persulfate is used. Dr. Cooper and I modified that technique for use with C¹⁴-carboxyininulin,⁹ a compound that has been proposed for inulin kidney clearance studies. It was found that this material was rapidly decarboxylated at room temperature and our ordinary double Erlenmeyer diffusion flask could be used. In the case of urine and plasma, the chloride present made necessary the use of extra silver nitrate. The method finally adopted was as follows:

One to 3 ml of plasma, 1 ml of 1 N sulfuric acid, and 12 ml of water containing 500 mg of potassium persulfate are added to one flask. The other flask receives 1 ml of Hyamine base and 4 ml toluene and is stoppered with an ordinary rubber stopper. Next, 1 ml of 20% silver nitrate is quickly added to the sample side and this is quickly stoppered to prevent loss of CO₂. The flask is then swirled 2 hr and the Hyamine carbonate transferred and counted at 11° in the usual way, which, in our case, includes the pulses ranging from 10 to ∞. High sensitivity and precision are obtained. Actually, a great deal more than the carboxyininulin is oxidized. In the case of urine, we add a little glucose to furnish carrier CO₂.

For those substances which require heating for combustion, the CO₂ can be caught in NaOH and then transferred by a second diffusion step to the Hyamine.

The ability of Hyamine base to solubilize acids is not limited to carbonic acid. Amino acids can be dissolved, as well as polyhydroxy acids like mucic acid. Proteins are generally insoluble in cold Hyamine, although it is likely that low molecular weight protein will dissolve. However, partial hydrolysis of proteins can be accomplished by heating with Hyamine base an hour or two at about 60°. The crude proteins I have used in this way give a yellow solution, but quenching measurements with an internal standard indicate the loss of efficiency is not too bad. If higher efficiency is desired, acidic hydrolysis can be carried out, the excess HCl can be removed by vacuum evaporation (toluene addition seems to aid the removal of the acid), the mucin (if any) can be removed, and the hydrochlorides of the amino acids and peptides can be dissolved readily in Hyamine base. We used this method in a study of the distribution of C¹⁴-D-alanine in rat tissues.¹⁰ While Hyamine chloride is not too soluble in cold toluene, the small amounts involved here seem to give no trouble. Perhaps the other compounds present increase its solubility. Acidic polysaccharides, such as chondroitin sulfate, may be expected to dissolve in Hyamine.

The Hyamine base is a very strong base, particularly in non-aqueous systems, and one would expect a wide variety of very weak acids to dissolve in it, even enolizable substances. Also, volatile compounds like H_2S and SO_2 dissolve very nicely. However, in the few trials we made with these compounds, there was appreciable darkening and the counting efficiency was impaired. The darkening may be due to traces of silver still left in the Hyamine, and possibly they could be removed by preliminary treatment with a bit of H_2S and centrifugation. However, it might be easier to convert radioactive sulfur compounds to sulfate and count this.

We have not studied the problem of recovering radioactive samples from Hyamine solutions, but it should be possible to use a cation exchange resin to take up the Hyamine. It probably would be better to add alcohol and water and pass the resultant solution through a column rather than just add water, as the Hyamine makes a fine emulsion with toluene and water. Removal of the DPO should also be simple as the DPO is probably unable to stick to ion exchange resins. In some cases, we have removed the DPO by sublimation at about 85° in oil pump vacuum.¹¹

We have been interested also in counting labeled sulfate, as part of a study of brain lipid sulfate esters. This problem is still in an uncompleted state, but in an encouraging one. It was reported some years ago¹² that HCl and other acids could be extracted from water by a solution of trioctylamine in chloroform. Later, the use of di(2-ethylhexyl)amine, a much cheaper amine, was reported for removing HCl from protein hydrolysates.¹³ We have found that the same amine, in toluene containing terphenyl and POPOP, will extract sulfate from sodium sulfate which has been acidified with tartaric acid. It is necessary to use an acid solution, but one should use an acid, I think, which will not extract too readily into the toluene. The efficiency of such a system is excellent, even when 10 mg of sodium sulfate are used. We add 0.1 ml of water to the vial to speed the equilibration; if more water is used, the efficiency of counting is somewhat reduced, probably due to an unfavorable partition coefficient. The technique requires further study, as the activity decreases several per cent each day.

We have also had occasion to count C^{14} -hydroxy acids, particularly the α -hydroxy acid, cerebronic acid. Although this acid possesses 24 carbon atoms, its hydroxy group greatly lowers the solubility in toluene. In this case, we have found that the acid can be made soluble by heating 30 min with a little acetic anhydride at 100° in a closed screw-cap counting vial (with a cap fitted with a Teflon liner). The excess anhydride is readily removed by vacuum evaporation. I suppose the compound is acetylated, but it might also be converted to an anhydride or even dehydrated.

Incidentally, I should like to recommend the use of our 'swirler' vacuum evaporator¹⁴ for evaporating samples or reaction mixtures to dryness in counting vials. We can handle up to ten vials at once. The rotary types of

vacuum evaporators, in our experience, tend to give splashing with the vials, and are more suited to flasks.

We have also been counting tritium water for some while, in order to determine body water content. In these experiments, the patients also receive radioactive sodium, and, we hope eventually, other radioisotopes. Rather than resort to differential counting of deproteinized plasma, using different photomultiplier voltages, we decided to separate the isotopes physically. This is done by lyophilizing the water from the whole plasma in a special device which permits recovery of the water.¹⁵ A few ml of plasma are shell-frozen in a small flask, the flask is attached to a V-shaped tube which dips into dry ice-isopropanol, and the other end of the V-tube is attached to a manifold connected to an oil pump. The lyophilization is complete in about 3 hr, but no isotopic enrichment occurs in this process, so this time is not adhered to rigidly. Two 1 ml aliquots of sublimate are dissolved in 49 ml of toluene-ethanol-PPO, and the samples are counted in 2½ oz screw-cap jars, preferably after waiting a day. We found that there is a slight drop in activity during the first day, but that the count rates were quite steady afterward.

Actually, there is fluctuation in counting efficiency due to machine variation, and it is necessary to count each sample alternating with a similar standard. Using the standard permits one to correct away the fluctuations. We found also that the 5 dram counting vials which are so useful for C¹⁴ work give somewhat greater variability in counting efficiency. This was true with the Wheaton snap cap vials and the Kimble screw cap vials. The snap cap vials, incidentally, gave us occasional leaks and many of them showed appreciable evaporation during storage.

The lyophilization method of counting body water is useful compared to the use of TCA filtrates because there is no need to make corrections with an internal standard and there is also no dilution with the TCA solution itself. We find, with this method, that the TCA-dioxane-naphthalene method¹⁶ of counting gives lower count rates per ml of patient's blood.

Another technique we have been using is an adaptation of the idea of suspension counting. Frequently it is desirable to count an aqueous solution with a minimum of manipulation. This can be done by emulsifying the solution in toluene and a scintillator. Tween 80 and Span 80, in the ratio of 1 : 9, produce an excellent water-in-oil emulsion with almost no shaking, but the counting rate of such a system deteriorates rapidly, apparently because there is a certain fraction of the droplets which coalesce. We next tried the addition of a thickening agent to prevent the coalescence. Aluminum stearate, which forms an excellent gel in toluene,² forms a poor one when water and the emulsifiers are present. Thixcin³ is somewhat more resistant to the action of these substances, and moderately good counting can be done, but there is still an objectionable amount of drop-off in count rate. Glycerol was found

to slow down this drop-off and also improve the counting efficiency. At present,¹¹ we use a mixture of 250 ml toluene, 2 ml emulsifier, 2.5 ml glycerol, 6.25 g Thixcin, and 590 mg PPO, homogenized in a high speed blender. The thick mixture is poured into 5 dram vials containing 0.2 ml aqueous radioactive solution and is then shaken to emulsify the water. Improved stability seems to be obtained by shaking on a machine in the cold room for 90 min before placing in the counter. The count rate still drops with time, about 5% per day. The efficiency of this method is about 45%. Perhaps it would be easier to combust such samples, and count the CO₂. Tritium cannot be counted in this emulsion system.

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