Pitfalls in Measurement of $^{14}CO_2$ Activity from Glucose-6-^{14}C and Two Corrective Procedures[1] (37013)

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Radioactively labeled glucose is routinely used to study glycolysis in a tissue. Many investigators have made use of Warburg flasks to trap the evolved radioactive carbon dioxide in an alkali and measure its activity by liquid scintillation counting (1–3). Due to the widespread acceptance of this method, most researchers assume this procedure to be valid. So far as Hyamine is concerned as a trapping agent, the validity of this method is undeniable. But the use of KOH or NaOH does lead one to doubt the authenticity of this procedure.

The present report is of a serendipity during our investigation on halothane inhibition of glucose metabolism in rat cerebral cortex slices. The $^{14}CO_2$ trapped in 3.5 *N* KOH was counted on the day of experiment in Bray's scintillation cocktail (4) and for 6 consecutive days. The counts obtained were low, unstable and statistically poor. On the basis of these findings, alternate counting techniques were explored to correct this problem. Although Hyamine gave high reproducible counts, the use of Cab-O-Sil (a thixotropic gelling agent) in combination with KOH proved to be a desirable procedure.

Methods. Counting of $^{14}CO_2$ activity trapped in KOH. Male Sprague–Dawley rats weighing 180–200 g were decapitated and the cerebral hemispheres were quickly removed. The hemispheres were placed in a bicarbonate-free Tris–glucose medium of the following composition (m*M*): Tris, 25.0; glucose, 1.0; NaCl, 145.3; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.33, and $CaCl_2 \cdot H_2O$, 1.22. The pH of the medium was 7.4. It was chilled and aerated for 0.5 hr with 100% oxygen before receiving the cerebral hemispheres. The two lobes of each hemisphere were separated and, using a Stadie-Riggs slicer, a 0.5 mm thick slice was obtained from each lobe. These slices were aerated until transferred to the Warburg flask. In a separate volumetric flask enough glucose 6-^{14}C (Amersham/Searle, sp act 2.9 mCi/mmole) was added to the bicarbonate-free Tris–glucose medium to obtain an average of 27,000 dpm/0.1 ml of the medium. Two milliliters of this radioactive medium were placed in each Warburg flask. The center well and the side arm of the flask received 0.2 ml of 3.5 *N* KOH and 0.2 ml of 70% (w/v) $HClO_4$, respectively. A slice of the cerebral cortex was placed in each flask and it was stoppered immediately. The flasks were placed in a Dubnoff metabolic shaker and were incubated at 37° for a period of 100 min at which time the reaction was stopped by acidifying the medium with $HClO_4$ from the side arm of the flask. The flask was agitated for another hour for complete trapping of the $^{14}CO_2$ into the KOH. An aliquot (0.1 ml) of the KOH was then removed and radioactivity was counted in 10 ml of Bray's scintillation cocktail using standard counting procedures. The counting of these samples was repeated for 6 consecutive days. The samples were kept at room temperature (25°) between each counting. After each experiment, the cortical slices were fished out of the Warburg flask, placed in an aluminum foil and dried at 110° for 24 hr. These were weighed to determine the dry weight of the tissue. The activity of $^{14}CO_2$ from each

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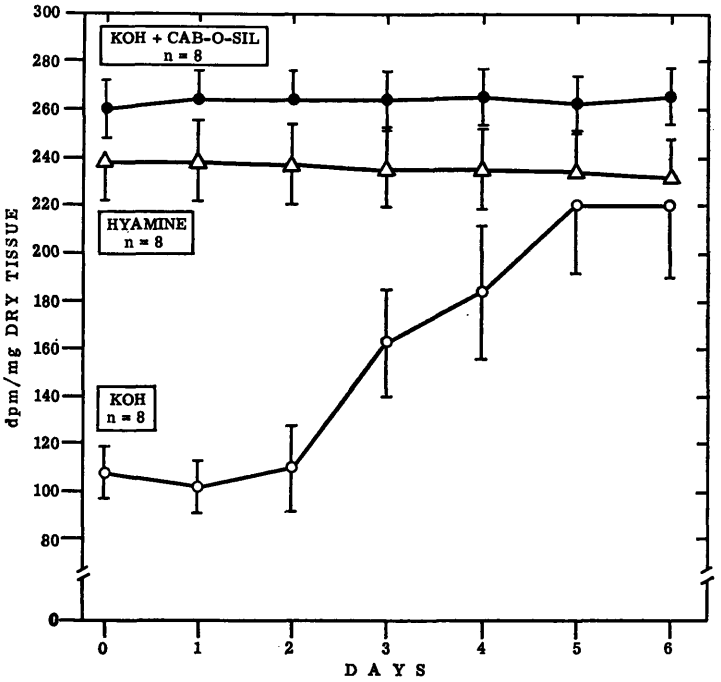

FIG. 1. Changes in measured activity of $^{14}CO_2$ when rat cerebral cortex slices were incubated in glucose-6-^{14}C, activity trapped in KOH, Hyamine or KOH in Cab-O-Sil and samples counted in Bray's solution for 6 consecutive days.

slice was then calculated on a milligram dry weight basis.

Corrective procedures. In order to improve the low, unstable and statistically poor counts, two corrective procedures were attempted. In one case, instead of KOH, 0.2 ml of Hyamine was used to trap the evolved $^{14}CO_2$. The rest of the procedure stayed the same.

In another procedure, the scintillation vials were loosely filled with 0.4 g of Cab-O-Sil (Research Products International Corp., Elk Grove Village, IL). A 4% gel was then prepared by adding 10 ml of Bray's scintillation cocktail to these vials. The 0.1 ml of KOH with the trapped $^{14}CO_2$ from the center well was then added to this thixotropic gel and the samples were counted. For comparison, controls utilizing KOH as the trapping agent, were run along with Hyamine and Cab-O-Sil experiments.

Effect of temperature on counting. To determine the effects of temperature on the counts from control and Cab-O-Sil experiments, samples of each were counted every day for a period of 6 days. Between countings, these samples were kept refrigerated at 5°. At the end of this period, the samples were kept at room temperature (25°) for 2 days and recounted. The recounting was continued each day, keeping the samples at room temperature between countings. This procedure lasted for an additional 6 days.

Determination of f value. According to the method of Hays, Rogers and Langham (5), a ratio of Cab-O-Sil suspension counting efficiency to the homogeneous internal standard counting efficiency (called the *f* value) was determined using ^{14}C-toluene (Packard Instrument Co., radioactivity $5.72 \times 10^5 \pm 3\%$ dpm/g) as the internal standard. Four such *f* values were determined and an average of these was calculated.

Results. The line with open dots in Fig. 1 was generated by the counts from controls obtained each day for 6 consecutive days when the samples were kept at room temperature between counts. The graph indicates that the counts were low, unstable and statistically poor yielding large standard error of the means with the lapse of time. They showed

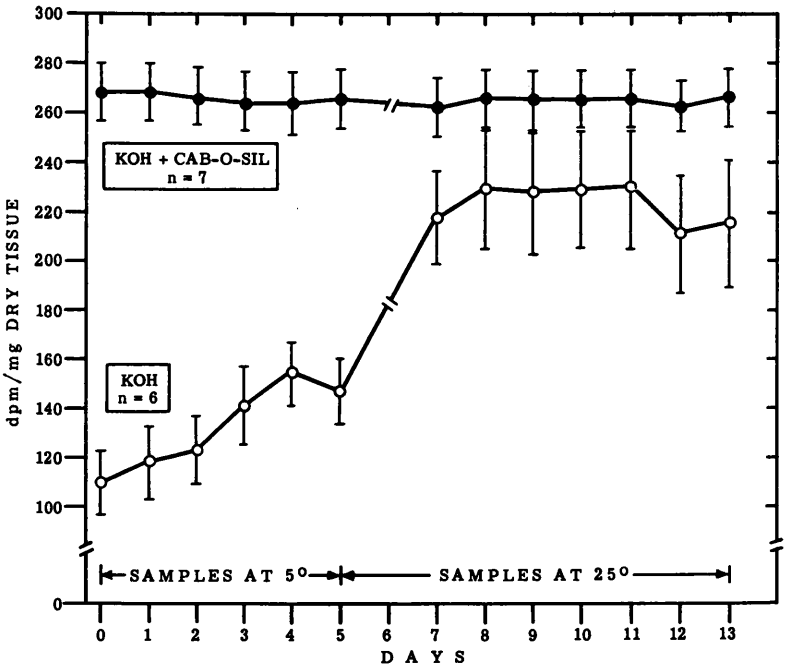

FIG. 2. Effect of temperature on $^{14}CO_2$ measured activity when rat cerebral cortex slices were incubated in glucose-6-^{14}C, activity trapped in KOH or KOH in Cab-O-Sil and counted daily in Bray's solution at 5 or 25°.

a progressive increase until Day 5 from which onward they were statistically comparable with Hyamine and Cab-O-Sil suspension counts.

The triangles in the graph indicate the activity of $^{14}CO_2$ when Hyamine was used as a trapping agent. It counted 238 dpm/mg dry tissue on the very first day of counting which was significantly higher from the control counts of 108 dpm/mg dry tissue. The activity remained high and essentially unchanged for the remainder of the experiment. The reproducibility of the counts in Hyamine and the high initial counts compared to controls is obvious in this graph.

Finally, the closed dots of the Fig. 1 represent the counts obtained when $^{14}CO_2$ trapped in KOH was suspended in 4% Cab-O-Sil suspension and counted. The daily counts of these samples gave high, reproducible counts (264 dpm/mg dry tissue) for 6 days which were statistically comparable with the counts obtained with Hyamine.

Figure 2 depicts the counts of control and Cab-O-Sil suspensions when they were kept at 5 and 25°. The controls (open dots) showed no progressive rise in counts with the lapse of time at 5° and were significantly different from Cab-O-Sil suspension counts. When kept at 37° for 2 days, these counts went up and became statistically comparable yet unstable with the counts from Cab-O-Sil suspensions. In contrast to the controls, the Cab-O-Sil suspension counts (closed dots) were unaffected by the change in temperature and remained high and stable throughout the experimental period.

Table I lists the *f* values of the Cab-O-Sil suspensions. An average value of 0.92 ± 0.02 indicates a loss of 8% in counting efficiency due to self-absorption.

Discussion. The results of this investigation demonstrates that when KOH is used to trap $^{14}CO_2$ from glucose-6-^{14}C, the initial counts do not represent the true activity of $^{14}CO_2$. Even though the counts rise with the lapse of time and become rather stable after 5 days, they are not reliable and show large standard error of means. The implication of these findings in the study of glycolysis are

TABLE I. Cab-O-Sil Suspension *f* Values.

Sample	Percentage of homogenous internal standard[a] counting efficiency	Percentage of Cab-O-Sil suspension counting efficiency	*f* values[b]
1	94	88	0.94
2	92	83	0.90
3	87	83	0.95
4	92	80	0.87
Av			0.92
SEM			±0.02

[a] ^{14}C-Toluene (Packard Instrument Co., radioactivity 5.73×10^5 dpm ± 3%/g).

[b] Ratio of suspension counting efficiency to homogenous internal standard counting efficiency.

quite apparent and reporting the initial values of $^{14}CO_2$ activity can lead to erroneous results.

Although the explanation for the observed changes in $^{14}CO_2$ counts is not completely clear, it seems to be a solubility problem. According to Woeller (6) a compound to be a suitable trapping agent should be soluble in both reacted and unreacted form in the scintillation cocktail. It seems likely that initially the resultant $K_2{}^{14}CO_3$ after the trapping of $^{14}CO_2$ in KOH remains insoluble in Bray's scintillation cocktail. It gradually becomes solubilized if kept at room temperature for a period of at least 5 days. The results in Fig. 2 also substantiate this viewpoint. Here again the $K_2{}^{14}CO_3$ did not go into solution when kept at 5° and hence the counts did not become equal or comparable to Hyamine or Cab-O-Sil suspension counts. On the other hand, Hyamine upon reaction with $^{14}CO_2$ seems to form a dioxane-soluble bicarbonate and therefore produces high, reproducible counts, but there are some shortcomings in the use of Hyamine as a trapping agent. Due to the fairly viscous nature of the solution, problems arise in removing an aliquot of Hyamine carbonate for counting. Rapkin (7) has also noted a similar objection. Apart from this, the high cost of Hyamine and its appreciable quenching properties do not favor its use as a trapping agent.

On the assumption that variability in the measurement of $^{14}CO_2$ activity was due to the poor solubility of $K_2{}^{14}CO_3$ in Bray's scintillation cocktail, suspension counting was considered a possible solution. This approach was also described by Greene (8) in his review of heterogeneous system and suspension counting. Ott and co-workers (9) had reported the use of Cab-O-Sil as a thixotropic gelling agent which requires neither heating nor blending and is useful with both toluene and acqueous dioxane solutions. Gordon and Wolfe (10) had also reported excellent results with Cab-O-Sil. The results of this study documents the claims of these researchers. The $^{14}CO_2$ counts in this suspension were high, stable and reproducible for at least 2 wk. A similar stability of Cab-O-Sil counting system has been reported by Gordon and Wolfe (10).

Three important factors which tend to reduce the efficiency of suspension counting have been described by Helf (11). These are the settling of suspension, opacity, and self-absorption of the low energy *betas* within the individual solid particles of the suspension. In our experiments, no settling effects were observed as the counting rate of the samples remained stable for at least 2 wk without additional shaking. So far as opacity is concerned, Gordon and Wolfe (10) noted that Cab-O-Sil produces an optically clear thixotropic system. The phenomenon of self-absorption is perhaps the most interesting factor in suspension scintillation counting. The mean *f* value for Cab-O-Sil suspension obtained by the method of Hays, Rogers and Langham (5) was so close to unity that it indicated almost no self-absorption by this system. It would thus appear that the most practical and appropriate method for counting $^{14}CO_2$ activity would be the use of Cab-O-Sil as a suspending agent. Subsequent use of this procedure during halothane inhibition

of glucose metabolism in rat cerebral cortex slices has confirmed this view.

Summary. Studies on the validity of measured activity of $^{14}CO_2$ from glucose-6-^{14}C trapped in KOH, Hyamine or KOH suspended in Cab-O-Sil were carried out in dioxane-based scintillation solutions. The counts obtained with KOH were low, unstable and showed a progressive increase becoming rather stable after 5 days. Contrary to this, Hyamine, or KOH in Cab-O-Sil produced high counts which were reproducible for at least 2 wk. Poor solubility of $K_2{}^{14}CO_3$ in Bray's solution is a likely reason for the initial low counts of $^{14}CO_2$ trapped in KOH. The use of Hyamine or KOH in Cab-O-Sil is therefore a better method for the measurement of $^{14}CO_2$ activity. In addition to counting stability, Cab-O-Sil suspensions have low settling rates, no opacity and almost negligible self-absorption and hence, would be considered as a method of choice.

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