# Determination of the Pattern of Labelling of Carbon Atoms 1, 2, 3 and 6 of D-[U-$^{14}$C] Glucose by Chemical Methods *

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## SUMMARY

*Uniformly labelled D-[$^{14}$C] glucose obtained from* Canna Indica *and from* Chlorella *was converted into several derivatives and degraded by chemical methods to yield products that separately contained carbon atoms 1, 2, 3 and 6. The specific activities of the various compounds were determined by liquid scintillation counting and from the results the pattern of labelling in carbon atoms 1, 2, 3 and 6 of the two glucose samples was obtained. The labelling was found to be uniform within the limits of experimental error.*

## EXPERIMENTAL

Uniformly labelled D-[$^{14}$C] glucose prepared from leaves of *Canna Indica* (Product Code Number CFB.2), uniformly labelled D-[$^{14}$C] glucose prepared from cell walls of *Chlorella* (Product Code Number CFB.35) and D-[1-$^{14}$C] glucose (Product Code Number CFA.204), were all from the Radiochemical Centre, Amersham, England.

The radiochemical purities of these compounds were as follows : CFB.2, by dilution analysis, 99%; by paper chromatography in two solvent systems 99 and 99%. CFB.35, by dilution analysis, 98%; by paper chromatography in two solvent systems 98 and 98%, CFA.204, by dilution analysis, 97%; by paper chromatography in two solvent systems 99 and 99%. All three products had been crystallized to ensure good chemical purity.

The products were separately diluted with inactive anhydrous D-glucose (Fluka Chemical Company) to give resulting specific activities of approximately 1.8 μc/mM.

The diluted D-[U-$^{14}$C]glucose samples were separately recrystallized four times from water/methanol/isopropanol and the purified products were

desiccated *in vacuo* over phosphorus pentoxide. Yields of purified diluted D-[U-$^{14}$C] glucose varied between 29% and 45%. This D-[U-$^{14}$C] glucose was used for the determination of the percentage activity in carbon atoms 1 and 6. Separate dilutions and preparations of pure diluted D-[U-$^{14}$C] glucose were made for the determination of the percentage activity in carbon atoms 2 and 3.

PREPARATION OF DERIVATIVES.

*Potassium-D-[U-$^{14}$C] arabonate.*

D-[U-$^{14}$C] glucose (0.5 g) in water (5 ml) was frozen onto one wall of a 50 ml conical flask and 2N potassium hydroxide (5 ml) was frozen onto another portion. Air was displaced from the flask with oxygen and the flask was attached to a gas burette containing oxygen. The solutions were allowed to melt and mix and the flask was shaken vigorously (mechanical shaker) and warmed with a hair-drier from time to time. Oxygen uptake was usually complete after ~5 hr and the solution was then shaken overnight at room temperature. The solution was then concentrated to a thin syrup, using a rotary-film evaporator. The syrup was diluted with methanol (75 ml), stirred well and allowed to stand for several hours at room temperature. The resulting crystalline precipitate was filtered, washed well with methanol, dried and recrystallized three times from aqueous methanol. The pure product was desiccated *in vacuo* over phosphorus pentoxide. Yields of pure product varied between 40% and 50%. This method was adapted from that of Frush and Isbell [1].

*Potassium-D-[U-$^{14}$C] gluconate.*

D-[U-$^{14}$C] glucose (1 g) and resublimed iodine (2.83 g) were dissolved in hot methanol (53 ml). A solution of potassium hydroxide in methanol (4% w/v, 58 ml) was added dropwise, over a period of 20 minutes, to the stirred solution which was maintained at 40° C. The mixture was cooled to room temperature and the product was filtered, washed with methanol and ether and dried. The crude product was recrystallized four times from aqueous methanol to give the final pure product which was desiccated *in vacuo* over phosphorus pentoxide. Yields of pure product varied between 42% and 54%. This method is essentially that of Moore and Link [2].

*Methyl α-D-[U-$^{14}$C] glucopyranoside.*

D-[U-$^{14}$C] glucose (8 g) was boiled under reflux for 3 hours with methanolic hydrogen chloride (3% w/v, 200 ml). The solution was cooled, diluted with three volumes of water and neutralized with stirring, by the addition of portions of Amberlite XE 78 ($OH^-$) anion-exchange resin. The resin was filtered, washed with methanol and the combined filtrate and washings concentrated to a syrup, using a rotary-film evaporator. The syrup was crystallized by the

addition of ethanol and the resulting crude product was recrystallized three times from methanol/ether. The pure product was desiccated *in vacuo* over phosphorus pentoxide. Yields of pure product were about 33%. This method is essentially that of Neish *et al.* [3].

## DEGRADATION OF DERIVATIVES TO YIELD CARBON ATOMS 1, 2, 3 AND 6 OF THE ORIGINAL D-[U$^{14}$C] GLUCOSE.

### *Carbon atom 1.*

D-[U-$^{14}$C] glucose from *Canna Indica* and from *Chlorella* was converted into potassium-D-[U-$^{14}$C] gluconate as described above. The potassium-D--[U-$^{14}$C] gluconate was oxidized with periodate to yield [$^{14}$C] carbon dioxide de rived from C-1. As a check on methods D-[1-$^{14}$C] glucose was submitted to the same procedures.

### *Periodate oxidation of potassium -D-[U-$^{14}$C] gluconate.*

Potassium-D-[U-$^{14}$C] gluconate (76 mg, 0.325 mM) was dissolved in 0.5M sodium phosphate buffer (pH 5.8, 11 ml) and the solution cleared of carbon dioxide by passage of carbon dioxide-free nitrogen for 1 hour. Sodium metaperiodate solution (6.2 ml of 0.3M, in carbon dioxide-free water) was added, the oxidation flask was covered with black paper and the mixture was aerated with carbon dioxide-free nitrogen for 1.5 hours. [$^{14}$C] carbon dioxide released from C-1 was isolated as barium [$^{14}$C] carbonate by passage of the exit gas stream through hot (70° C) saturated barium hydroxide solution.

The barium [$^{14}$C] carbonate was filtered, washed with carbon dioxide-free water and dried in the oven. A simple apparatus for this experiment is shown in Figure 1. The use of pH 5.8 phosphate buffer for this type of oxidation was developed by Bernstein [4].

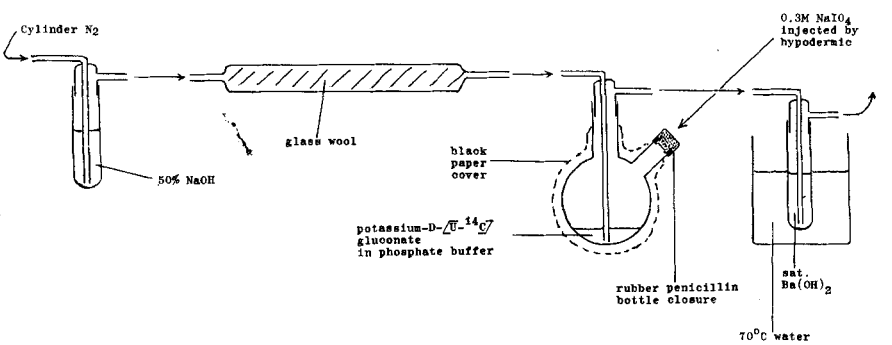


Fig. 1

*Carbon atom 2.*

D-[U-$^{14}$C] glucose from *Canna Indica* and from *Chlorella* was converted into potassium-D-[U-$^{14}$C] arabonate as described above. The potassium-D--[U-$^{14}$C] arabonate was oxidized with periodate in exactly the manner described for potassium-D-[U-$^{14}$C] gluconate in the section on carbon atom 1 above, and the resulting [$^{14}$C] carbon dioxide (from C-1 of arabonate or C-2 of glucose) was similarly isolated as barium [$^{14}$C] carbonate.

*Carbon atom 3.*

D-[U-$^{14}$C] glucose from *Canna Indica* and from *Chlorella* was converted into methyl α-D-[U-$^{14}$C] glucopyranoside as described above. The methyl α-D-[U-$^{14}$C] glucopyranoside was oxidized with periodate and the [$^{14}$C] formic acid released from C-3 was isolated as lead [$^{14}$C] formate as described below.

*Periodate oxidation of methyl α-D-[U-$^{14}$C] glucopyranoside.*

Methyl α-D-[U-$^{14}$C] glucopyranoside (388 mg, 2 mM) was dissolved in sodium metaperiodate solution (0.3M, 20 ml) and stored in a closed vessel, in the dark, overnight at room temperature. Ethylene glycol (0.6 g) was then added, mixed and the solution was allowed to stand for 15 minutes. The solution was then titrated with sodium hydroxide solution (2 N) until just alkaline (B.D.H. "Universal" indicator). Lead nitrate solution (20% w/v) was added slowly with mixing until no further precipitate resulted and a further 1 g in water was then added. The mixture was heated to 80° C, filtered through Whatman No. 1 paper, washed with hot water and the filtrate and washings were combined and freeze-dried. The residue from freeze-drying was dissolved in hot water (5 ml), a little ethanol was added and the solution was stored at 0° C overnight. The resulting crystals of crude lead [$^{14}$C] formate from C-3 were filtered off, washed with several small portions of ice water, then ethanol and ether and dried. The product was recrystallized twice by the same method and dried *in vacuo* over phosphorus pentoxide. Yields of pure lead [$^{14}$C[ formate were about 20%.

*Carbon atom 6.*

The potassium-D-[U-$^{14}$C] gluconate prepared for the carbon atom 1 degradations was also oxidized with periodate to yield [$^{14}$C] formaldehyde from C-6, isolated as [$^{14}$C] formaldehyde-dimedone derivative in the following manner.

Potassium-D-[U-$^{14}$C] gluconate (50 mg, 0.21 mM) in water (4 ml) was oxidized with sodium metaperiodate solution (4 ml) in the dark at room temperature for 1 hour. Hydrochloric acid (N, 6 ml) and sodium arsenite solution (1.2N, 4 ml) were added in turn with thorough mixing. Sodium

acetate solution (M, 4 ml) and dimedone reagent (80 mg of 5,5-dimethyl--1,3-cyclohexanedione/ml of 95% ethanol) (4 ml) were added. The mixed solution was heated at 100° C for 15 minutes, then left at room temperature for 1 hour and at 0° C overnight. The crude crystalline [$^{14}$C] formaldehyde-dimedone derivative from C-6 was washed well with water, dried and recrystallized three times from ethanol/water. The pure product was desiccated *in vacuo* over phosphorus pentoxide. Yields of pure [$^{14}$C] formaldehyde-dimedone derivative ranged from 81% to 85%.

Melting points of all pure compounds obtained were in satisfactory agreement with those reported in the literature.

SPECIFIC ACTIVITY DETERMINATIONS.

All activity measurements were made using Nuclear Chicago Series 720 or Mark I liquid scintillation counters. The scintillant consisted of ethanol (150 ml), dioxane (300 ml), naphthalene (50 g) and 2,5-diphenyloxazole (P.P.O.) (5 g) made up to 1 l with toluene. Emulsion counting of aqueous samples was carried out by the method of Patterson and Greene [5], using toluene containing 2,5-diphenyloxazole (P.P.O.) (0.4% w/v) and 1,4-di-[2-(5-phenyloxazolyl)] benzene (P.O.P.O.P.) (0.01% w/v), mixed with one half-volume of Triton X-100 emulsifying agent; the aqueous phase formed about 5% of the counting mixture. Gel suspension counting of barium [$^{14}$C] carbonate was carried out by the method of Cluley [6] using ~5% Cab-O-Sil [7] in the counting mixture as the thixotropic gelling agent. At least 50,000 counts were accumulated for each measurement of activity. It will be noted that specific activities of compounds measured by gel suspension counting of derived barium [$^{14}$C] carbonate are lower than those of the same compound measured by emulsion counting. This is due to self absorption of β particles in the barium [$^{14}$C] carbonate and the ratio of the two sets of specific activities corresponds to the "*f* factor" of Hayes, Rogers and Langham [8] and of White and Helf [9], although the present work yields a slightly different value for *f*. Emulsion counting as used in the present work does not lead to any self absorption in the aqueous phase (such characteristics of various counting systems will be the subject of a paper to be published shortly [10]). All counting efficiency determinations were made using the Channels Ratio method [11, 12], the quench correction graph of efficiency versus Channels Ratio was plotted using [1-$^{14}$C] hexadecane as reference standard.

*Carbon atom 1.*

D-[U-$^{14}$C] glucose samples were burnt in porcelain boats (which had been previously fired to ensure removal of any combustible inactive carbon) in a stream of carbon dioxide-free oxygen in a silica tube at 800° C.

The [$^{14}$C] carbon dioxide produced was isolated as barium [$^{14}$C] carbonate by passage of the exit gas stream into hot barium hydroxide solution as described earlier.

Potassium-D-[U-$^{14}$C] gluconate samples were burnt in the same way except that to ensure combustion potassium dichromate was added to the boats which were then encased in a roll of carefully cleaned copper foil. All combustion boats were checked for activity, when combustion was completed, by washing with water which was then counted. No activity was found in any instance. Four separate combustions were carried out on each compound giving ~60 mg of barium [$^{14}$C] carbonate which was accurately weighed out as 3 × ~20 mg samples and counted by the gel suspension method; 12 samples were thus obtained from each compound. Four separate periodate oxidations were carried out on each sample of potassium-D-[U-$^{14}$C] gluconate giving ~60 mg of barium [$^{14}$C] carbonate which was counted as above (for barium [$^{14}$C] carbonate obtained from the periodate oxidation of potassium--D-[1-$^{14}$C] gluconate, ~4 mg samples were counted due to the higher specific activity).

*Results.*

Specific activities of compounds were calculated as follows :

specific activity in μc/mM =

$$\frac{\mu c \text{ in } Ba^{14}CO_3 \text{ sample}}{\text{wt. } Ba^{14}CO_3 \text{ sample (mg)}} \times \text{M. wt. } Ba^{14}CO_3 \times \text{No. of C atoms in compound}$$

The resulting 12 values for specific activity were averaged to give the mean value. Deviations from the mean for each value were calculated and used to calculate the standard deviation (S.D.) from the formula

$$\text{S.D.} = \sqrt{\frac{\varepsilon^n d^2}{n-1}} \qquad \text{where } n = \text{number of values}, \quad d = \text{deviation}$$

Where results are quoted as a ratio or multiple of two specific activities A and B with their S.D.'s $a$ and $b$ respectively, the standard deviation of the ratio or multiple was calculated from the formulae :

$$\frac{A \pm a}{B \pm b} = \frac{A}{B} \pm \frac{1}{B^2} \sqrt{B^2a^2 + A^2b^2}$$

and

$$(A \pm a)(B \pm b) = AB \pm \sqrt{a^2B^2 + b^2A^2}$$

*Standard deviations of mean* values are simply derived from the quoted S.D.'s by dividing by $\sqrt{n}$.

| Compound | Specific Activity (μc/mM) | S.D. |
|---|---|---|
| 1. D-[1-$^{14}$C] glucose | 1.768 | 0.047 (2.7%) |
| 2. Potassium-D-[1-$^{14}$C] gluconate | 1.772 | 0.027 (1.5%) |
| 3. $^{14}CO_2$ ex $10_4$ oxidation of potassium-D-[1-$^{14}$C] gluconate | 1.804 | 0.104 (5.7%) |
| 4. *Canna* D-[U-$^{14}$C] glucose | 1.491 | 0.033 (2.2%) |
| 5. *Canna* potassium-D-[U-$^{14}$C] gluconate | 1.548 | 0.026 (1.7%) |
| 6. $^{14}CO_2$ ex $10_4$ oxidation of *Canna* potassium-D-[U-$^{14}$C] gluconate | 0.2485 | 0.0069 (2.8%) |
| 7. *Chlorella* D-[U-$^{14}$C] glucose | 1.559 | 0.017 (1.1%) |
| 8. *Chlorella* potassium-D-[U-$^{14}$C] gluconate | 1.527 | 0.013 (0.87%) |
| 9. $^{14}CO_2$ ex $10_4$ oxidation of *Chlorella* potassium-D-[U-$^{14}$C] gluconate | 0.2560 | 0.0065 (2.5%) |

*A.* % activity in C-1 of *Canna* potassium-D-[U-$^{14}$C] gluconate and ∴ of *Canna* D-[U-$^{14}$C[ glucose =

$$\frac{\text{specific activity 6}}{\text{specific activity 5}} \times 100\% = \frac{0.2485 \pm 0.0069}{1.548 \pm 0.026} \times 100\%$$
$$= \underline{16.05 \text{ S.D. } 0.52\%}$$

*or*, based on original *Canna* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 6}}{\text{specific activity 4}} \times 100\% = \frac{0.2485 \pm 0.0069}{1.491 \pm 0.033} \times 100\%$$
$$= \underline{16.67 \text{ S.D. } 0.59\%}$$

*B.* % activity in C-1 of *Chlorella* potassium-D-[U-$^{14}$C] gluconate and ∴ of *Chlorella* D-[U-$^{14}$C] glucose :

$$\frac{\text{specific activity 9}}{\text{specific activity 8}} \times 100\% = \frac{0.2560 \pm 0.0065}{1.527 \pm 0.013} \times 100\%$$
$$= \underline{16.76 \text{ S.D. } 0.45\%}$$

*or*, based on original *Chlorella* D-[U-$^{14}$C[ glucose =

$$\frac{\text{specific activity 9}}{\text{specific activity 7}} \times 100\% = \frac{0.2560 \pm 0.0065}{1.559 \pm 0.017} \times 100\%$$

$$= \underline{16.42 \text{ S.D. } 0.45\%}$$

*Carbon atom 2.*

Potassium-D-[U-$^{14}$C] arabonate samples were burnt in porcelain boats and the resulting [$^{14}$C] carbon dioxide isolated as barium [$^{14}$C] carbonate as described for potassium-D-[U-$^{14}$C] gluconate in the section on carbon atom 1.

These barium [$^{14}$C] carbonate samples and those isolated from the periodate oxidation of potassium-D-[U-$^{14}$C] arabonate were counted by gel suspension as previously described. Four separate combustions or periodate oxidations were carried out on each compound giving in each case ~60 mg barium [$^{14}$C] carbonate which was accurately weighed out as 3 × ~20 mg samples for counting. A total of 12 barium [$^{14}$C] carbonate samples was thus counted for each specific activity determination.

The specific activity of each sample of D-[U-$^{14}$C] glucose and potassium--D-[U$^{14}$C] arabonate was also measured by emulsion counting. ~200 mg of each compound was dissolved in water (~10 g) and 0.5-0.6 g samples of this solution were removed for counting. Six such samples were counted for each compound. All weighings were accurately made. In this way the percentage activity in C-1 of D-[U-$^{14}$C] glucose could be calculated by difference as a check on the previous results obtained.

*Results.*

Specific activities of compounds based on barium [$^{14}$C] carbonate (Numbers 10, 11, 12 and 13) were calculated as described in the results from carbon atom 1. Specific activities of compounds obtained by emulsion counting (Numbers 14, 15, 16 and 17) were calculated as follows :

specific activity in μc/mM =

$$\frac{\mu\text{c in aqueous sample}}{\text{wt. aqueous sample (g)}} \times \frac{\text{wt. solution (g)}}{\text{wt.compound (mg)}} \times \text{M. wt. compound}$$

Standard deviations were calculated as before.

*A.* % activity in C-1 of *Canna* potassium-D-[U-$^{14}$C[ arabonate =

$$\frac{\text{specific activity 11}}{\text{specific activity 10}} \times 100\% = \frac{0.2315 \pm 0.0081}{1.2213 \pm 0.013} \times 100\%$$

$$= \underline{18.96 \text{ S.D. } 0.69\%}$$

(Theoretical value 20%)

| Compound | Specific Activity (μc/mM) | S.D. |
|---|---|---|
| 10. *Canna* potassium-D-[U-$^{14}$C] arabonate | 1.2213 | 0.013 (1.1%) |
| 11. $^{14}CO_2$ ex $10_4$ oxidation of *Canna* potassium-D-[U-$^{14}$C] arabonate | 0.2315 | 0.0081 (3.5%) |
| 12. *Chlorella* potassium-D-[U-$^{14}$C] arabonate | 1.208 | 0.035 (2.9%) |
| 13. $^{14}CO_2$ ex $10_4$ oxidation of *Chlorella* potassium-D--[U-$^{14}$C] arabonate | 0.2322 | 0.0078 (3.4%) |
| 14. *Canna* potassium-D-[U-$^{14}$C] arabonate | 1.424 | 0.0032 (0.23%) |
| 15. *Canna* D-[U-$^{14}$C] glucose | 1.712 | 0.0054 (0.31%) |
| 16. *Chlorella* potassium-D-[U-$^{14}$C] arabonate | 1.415 | 0.0052 (0.37%) |
| 17. *Chlorella* D-[U-$^{14}$C] glucose | 1.715 | 0.0077 (0.45%) |

$\therefore$ % activity in C-2 of *Canna* D-[U-$^{14}$C] glucose =

$$18.96 \pm 0.69 \times 5/6 = \underline{15.80 \text{ S.D. } 0.58\%}$$

(Theoretical value 16.67 %)

*or*, based on original *Canna*-D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 11}}{\text{specific activity 15} \times \dfrac{\text{specific activity 10}}{\text{specific activity 14}}} \times 100\%$$

$$= \frac{0.2315 \pm 0.0081}{1.712 \pm 0.0054 \times \dfrac{1.2213 \pm 0.013}{1.424 \pm 0.0032}} \times 100\%$$

$$= \underline{15.77 \text{ S.D. } 0.77\%}.$$

*B.* % activity in C-1 of *Chlorella* potassium-D-[U-$^{14}$C] arabonate =

$$\frac{\text{specific activity 13}}{\text{specific activity 12}} \times 100\% = \frac{0.2322 \pm 0.0078}{1.208 \pm 0.035} \times 100\%$$

$$= \underline{19.22 \text{ S.D. } 0.85\%}$$

(Theoretical value 20 %)

$\therefore$ %activity in C-2 of *Chlorella* D-[U-$^{14}$C] glucose =

$$19.22 \pm 0.85 \times 5/6 = 16.02 \text{ S.D. } 0.71\ \%$$

(Theoretical value 16.67%)

*or*, based on original *Chlorella* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 13}}{\text{specific activity 17} \times \dfrac{\text{specific activity 12}}{\text{specific activity 16}}} \times 100\%$$

$$= \frac{0.2322 \pm 0.0078}{1.715 \pm 0.0077 \times \dfrac{1.208 \pm 0.035}{1.415 \pm 0.0052}} \times 100\%$$

$$= \underline{15.86 \text{ S.D. } 0.71\%}$$

*C.* % activity in C-1 of *Canna* D-[U-$^{14}$C] glucose =

$$100 - \left\{ \frac{\text{specific activity 14}}{\text{specific activity 15}} \times 100 \right\} \% =$$

$$100 - \left\{ \frac{1.424 \pm 0.0032}{1.712 \pm 0.0054} \times 100 \right\} \% =$$

$$100 - (83.18 \pm 0.32)\ \% = \underline{16.82 \text{ S.D. } 0.32\%.}$$

*D.* % activity in C-1 of *Chlorella* D-[U-$^{14}$C] glucose =

$$100 - \left\{ \frac{\text{specific activity 16}}{\text{specific activity 17}} \times 100 \right\} \% =$$

$$100 - \left\{ \frac{1.415 \pm 0.0052}{1.715 \pm 0.0077} \times 100 \right\} \% =$$

$$100 - (82.51 \pm 0.48)\ \% = \underline{17.49 \text{ S.D. } 0.48\%.}$$

*Carbon atom 3.*

The specific activity of each sample of methyl α-D-[U-$^{14}$C] glucopyranoside and lead [$^{14}$C] formate was measured by emulsion counting. Each periodate oxidation of methyl α-D-[U-$^{14}$C] glucopyranoside to give lead [$^{14}$C] formate was carried out four times and the resulting four samples of crude lead [$^{14}$C[ formate were combined for purification. Each compound (~100 mg) was dissolved in water (~10 g) and 0.5-0.6 g samples were removed for count-

ing. Nine samples were counted for each compound and all weighings were accurately made.

*Results.*

Specific activities of these compounds assayed by emulsion counting were calculated as previously described. Standard deviations were calculated as before.

| Compound | Specific Activity (μc/mM) | S.D. |
|---|---|---|
| 18. *Canna* methyl α-D-[U-$^{14}$C] glucopyranoside | 1.771 | 0.0072 (0.41%) |
| 19. *Canna* lead [$^{14}$C] formate | 0.5609 | 0.0035 (0.62%) |
| 20. *Chlorella* methyl α-D-[U-$^{14}$C] glucopyranoside | 1.793 | 0.0076 (0.42%) |
| 21. *Chlorella* lead [$^{14}$C] formate | 0.5892 | 0.0023 (0.39%) |

Specific activity of [$^{14}$C] formic acid ex C-3 =

$$\frac{\text{specific activity of lead [}^{14}\text{C] formate}}{2} \quad (Pb(HCOO)_2)$$

*A.* % activity in C-3 of *Canna* methyl α-D-[U-$^{14}$C] glucopyranoside and ∴ of *Canna* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity } 19/2}{\text{specific activity } 18} \times 100\% = \frac{0.2805 \pm 0.0018}{1.771 \pm 0.0072} \times 100\%$$

$$= \underline{15.84 \text{ S.D. } 0.12\%.}$$

*or*, based on original *Canna* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity } 19/2}{\text{specific activity } 15} \times 100\% = \frac{0.2805 \pm 0.0018}{1.712 \pm 0.0054} \times 100\%$$

$$= \underline{16.38 \text{ S.D. } 0.12\%.}$$

*B.* % activity in C-3 of *Chlorella* methyl α-D-[U-$^{14}$C] glucopyranoside and ∴ of *Chlorella* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity } 21/2}{\text{specific activity } 20} \times 100\% = \frac{0.2946 \pm 0.0012}{1.793 \pm 0.0076} \times 100\%$$

$$= \underline{16.43 \text{ S.D. } 0.097\%}.$$

*or*, based on original *Chlorella* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity } 21/2}{\text{specific activity } 17} \times 100\% = \frac{0.2946 \pm 0.0012}{1.715 \pm 0.0077} \times 100\%$$

$$= \underline{17.18 \text{ S.D. } 0.10\%}.$$

*Carbon atom 6.*

Specific activities of potassium-D-[U-$^{14}$C] gluconate were already determined by combustion and gel suspension counting of derived barium [$^{14}$C] carbonate, in the section on carbon atom 1 (specific activities 5 and 8).

The [$^{14}$C] formaldehyde-dimedone derivative was combusted as for [$^{14}$C] glucose and the derived barium [$^{14}$C] carbonate was counted by gel suspension. Three separate periodate oxidations were carried out for each sample of potassium-D-[U-$^{14}$C] gluconate giving three samples of [$^{14}$C] formaldehyde-dimedone derivative in each case. Each of these samples was combusted to yield barium [$^{14}$C] carbonate which was counted in triplicate. Thus nine samples of barium [$^{14}$C] carbonate were counted and in this case 50-60 mg samples were used because of the dilution of the barium [$^{14}$C] carbonate by the inactive carbon atoms of the derivative.

*Results.*

Specific activities of compounds based on barium [$^{14}$C] carbonate were calculated as described previously (section on carbon atom 1).

| Compound | Specific Activity (μc/mM) | S.D. |
|---|---|---|
| 22. [$^{14}$C] formaldehyde-dimedone from $10_4$ oxidation of *Canna* potassium-D-[U-$^{14}$C] gluconate | 0.2528 | 0.0057 (2.3%) |
| 23. [$^{14}$C] formaldehyde-dimedone from $10_4$ oxidation of *Chlorella* potassium-D-[U-$^{14}$C] gluconate | 0.2583 | 0.0039 (1.5%) |

*A.* % activity in C-6 of *Canna* potassium-D-[U-$^{14}$C] gluconate and ∴ of *Canna* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 22}}{\text{specific activity 5}} \times 100\% = \frac{0.2528 \pm 0.0057}{1.548 \pm 0.026} \times 100\%$$
$$= \underline{16.33 \text{ S.D. } 0.46\%}.$$

*or*, based on original *Canna* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 22}}{\text{specific activity 4}} \times 100\% = \frac{0.2528 \pm 0.0057}{1.491 \pm 0.033} \times 100\%$$
$$= \underline{16.96 \text{ S.D. } 0.54\%}.$$

*B.* % activity in C-6 of *Chlorella* potassium-D-[U-$^{14}$C] gluconate and ∴ of *Chlorella* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 23}}{\text{specific activity 8}} \times 100\% = \frac{0.2583 \pm 0.0039}{1.527 \pm 0.013} \times 100\%$$
$$= \underline{16.92 \text{ S.D. } 0.29\%}.$$

*or*, based on original *Chlorella* D-[U-$^{14}$C] glucose =

$$\frac{\text{specific activity 23}}{\text{specific activity 7}} \times 100\% = \frac{0.2583 + 0.0039}{1.559 \pm 0.017} \times 100\%$$
$$= \underline{16.57 \text{ S.D. } 0.31\%}.$$

*Summary of results.*

% activity in Carbon atoms ± S.D.

| Carbon Atom Number | Calculation based on D-[U-$^{14}$C] glucose | | Calculation based on derivative from which actual C-atom isolated | |
|---|---|---|---|---|
| | *Canna* | *Chlorella* | *Canna* | *Chlorella* |
| C-1 (directly) | 16.67 ± 0.59 | 16.42 ± 0.45 | 16.05 ± 0.52 | 16.76 ± 0.45 |
| C-1 (by difference) | 16.82 ± 0.32 | 17.49 ± 0.48 | — | — |
| C-2 | 15.77 ± 0.77 | 15.86 ± 0.71 | 15.80 ± 0.58 | 16.02 ± 0.71 |
| C-3 | 16.38 ± 0.12 | 17.18 ± 0.10 | 15.84 ± 0.12 | 16.43 ± 0.097 |
| C-6 | 16.96 ± 0.54 | 16.57 ± 0.31 | 16.33 ± 0.46 | 16.92 ± 0.29 |

For uniformly labelled D-[$^{14}$C] glucose each carbon atom will contain 16.67% of the total activity.

## Discussion

In biochemical work the need often arises to degrade D-[$^{14}$C] glucose isolated from biochemical systems in order to determine the pattern of labelling produced by various metabolic pathways, and several methods of degradation both chemical and biochemical are available [13-19, 3]. Also, D-[U-$^{14}$C] glucose is frequently used to study the distribution of activity among various of its metabolites. Because of the latter uses it is essential that the worker be confident of the uniformity of labelling in the D-[U-$^{14}$C] glucose with which he starts.

In the case of *Chlorella* there is little doubt that the D-[U-$^{14}$C] glucose isolated from the cell walls is in fact uniformly labelled. At the Radiochemical Centre *Chlorella* is grown from a small inactive innoculum with high specific activity [$^{14}$C] carbon dioxide as the sole source of carbon. Several generations of growth occur before the cells are harvested, ensuring that the original carbon-12 forms only a small percentage of the final carbon content. Excluding isotope effects during the incorporation of carbon into the cell constituents, this small amount of carbon-12 could at worst cause only a very small departure from uniformity in the labelling of the cell constituents. With *Canna Indica* however, the case for uniform labelling of the D-[U-$^{14}$C] glucose, isolated from intact leaves after photosynthesis in an atmosphere of [$^{14}$C] carbon dioxide, might be less strong. The intact leaves are removed from the parent plant and stored in the dark to deplete the carbohydrate reserves before photosynthesis is allowed to begin. There remains of course a considerable quantity of carbon-12 in the leaves and the [$^{14}$C] carbon dioxide absorbed does not equilibrate completely with all the cell constituents, only with those immediately concerned with photosynthesis and carbohydrate metabolism. Structural features would not become labelled to the same extent in the time allowed to photosynthesis (10-12 hours). It could therefore be argued that simply because of the large quantity of carbon-12 involved, the D-[U-$^{14}$C] glucose might be less likely to be uniformly labelled. For these reasons it was thought desirable to degrade commercially available D-[U-$^{14}$C] glucose, which had been biochemically prepared from leaves of *Canna Indica* and from cell walls of *Chlorella*, in order to demonstrate the uniformity or otherwise of the labelling. So that confidence could be placed in the results of the degradation studies it was decided that purely chemical methods of derivative preparation and of degradation should be used, in order that any question of dilution of activity with inactive carbon could be more closely accounted for and avoided. It was also desirable that no question should arise of migration of activity through the various carbon atoms, caused by unwanted metabolic reactions if a biochemical system of degradation was used. In connection with these

points it was brought to our attention recently [20] that anomalous results had been obtained using the standard biochemical method of degradation with *Leuconostoc mesenteroides* [16]. In this case D-[U-$^{14}$C] glucose had been degraded and carbon atoms 1 and 5 apparently contained respectively more and less activity than would be the case for uniform labelling. The workers concluded that the most likely cause of error was that of unlabelled material coming from the strain of *Leuconostoc mesenteroides* cells used.

The chemical methods of derivative preparation and of degradation were mostly adapted from those in the literature. The scale of working for preparation of derivatives was large in most cases, simply for convenience; however, the actual amount of activity *used* for all the work reported in this paper was approximately 50 μc for each of the two samples of D-[U-$^{14}$C] glucose. The specific activity of the diluted D-[U-$^{14}$C] glucose was chosen to be approximately 1.7 μc/mM to afford a level of activity that would be convenient to work with as regards short counting times of derivatives, dilution of solutions, weighings etc. The counting times used in this work were generally only 1-2 minutes and if 10 minutes or longer counting times were used the specific activity of the D-[U-$^{14}$C] glucose could be lowered by a factor of 10 to ~0.2 μc/mM. Automatic sample changing mechanisms and the capacity for large numbers of samples in modern liquid scintillation counters means that many samples can be counted overnight, when longer counting times are acceptable. With the use of highly efficient liquid scintillation counters a larger chemical scale of working (and thus measurement of lower specific activities) is possible, especially with techniques such as gel suspension counting and emulsion counting whereby considerable quantities of sample can be incorporated for counting. This means that chemical methods such as those reported here are now suitable for the determination of the activity contents of carbon atoms 1, 2, 3 and 6 of approximately 5 μc of D-[U-$^{14}$C] glucose.

The inclusion of D-[1-$^{14}$C] glucose in the degradation work for C-1 of D-[U-$^{14}$C] glucose served as a check on methods. It was found that in the periodate oxidation of potassium-D-[1-$^{14}$C] gluconate, the barium [$^{14}$C] carbonate isolated (and therefore [$^{14}$C] carbon dioxide evolved) was identical in specific activity, within the limits of experimental error, with the potassium-D-[1-$^{14}$C] gluconate. This showed that the methods of periodate oxidation and isolation of barium [$^{14}$C] carbonate did not introduce any dilution with inactive carbon. This therefore indicated that the periodate oxidations of potassium-D-[U-$^{14}$C] gluconate and arabonate, and isolations of barium [$^{14}$C] carbonate, which were carried out in exactly the same way were also satisfactory.

It will be noted that in calculating the activity contents of the various carbon atoms the specific activity of the actual derivative that was degraded to give that particular carbon atom was used. It was felt that this was probably sounder than using the specific activity of the original D-[U-$^{14}$C] glucose samples, since any change in specific activity in going from D-[U-$^{14}$C] glucose

to the derivative might be due to an actual purification. For the sake of completeness however, the specific activity of the original D-[U-$^{14}$C] glucose samples was also used to calculate a second set of results for carbon atoms 1, 3 and 6. Since the specific activities of D-[U-$^{14}$C] glucose samples and of derivatives were very similar, the second set of results differ by little from the first set. The results for carbon atom 2 based on D-[U-$^{14}$C] glucose specific activities could not be directly calculated since the specific activity of the D-[U-$^{14}$C] glucose samples and the specific activity of the barium [$^{14}$C] carbonate from C-2 were measured by different techniques, emulsion counting and gel suspension counting respectively. However, the specific activity of potassium-D-[U-$^{14}$C] arabonate had been measured by both techniques and the ratio of the two results could be used as a factor to convert the specific activity of the D-[U-$^{14}$C] glucose to a common basis with that of the barium [$^{14}$C] carbonate from C-2. By this method the results for carbon atom 2 based on D-[U-$^{14}$C] glucose specific activities were calculated and again were found to differ by little from the first set of results.

The methods used in the experimental section together with the purification stages involved have given accurate and reproducible results. In all cases standard deviations of specific activities and of ratios and multiples of specific activities have been calculated and were found to be satisfactory. The results obtained correspond to those expected for uniformity of labelling in the D-[U-$^{14}$C] glucose obtained from *Canna Indica* and from *Chlorella*. The maximum deviation from the expected result was 5.4%.

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