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# DISTRIBUTION OF TRITIUM IN D-GLUCOSE AND STARCH LABELED BY TRITIUM-ATOM BOMBARDMENT*

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ABSTRACT

Samples of D-glucose and starch were labeled by tritium-atom bombardment Up to 51% incorporation into D-glucose as non-labile tritium was achieved for crystalline, anhydrous D-glucose and 41% for the amylose–butyl alcohol complex Distribution of tritium in the carbon skeleton of D-glucose was calculated by comparing the specific molar activity of D-glucose with that of its derivatives. Derivatives prepared were D-gluconic acid, D-*arabino*-hexulose phenylosotriazole, 4-formyl-2-phenyltriazole, 2-phenyltriazole-4-carboxylic acid, D-*arabino*-hexulose phenylflavazole, 3-formyl-1-phenylflavazole, and formaldehyde dimedone The tritium distribution showed definite structural effects Generally, the products from films of D-glucose and the amylose–butyl alcohol complex had nearly uniform distribution of tritium in D-glucose The product from crystalline α-D-glucose monohydrate had zero tritium at C-2 and twice the expected amount of tritium at C-5, and that from starch granules had zero or near zero tritium at C-3 and close to twice the expected amount of tritium at C-2

## INTRODUCTION

Carbohydrates can be labeled by tritium-atom bombardment[1] D-Glucose having 2 6–6 0 μCi/mg was obtained with 3 6–7 3% of tritium in D-glucose when 200-mg samples of D-glucose were exposed for 30 min The distribution of tritium in the carbon skeleton of D-glucose is reported here The distribution was more random than that reported for labeling[2] by the Wilzbach method or for tritium-atom recoil[3] In Wilzbach labeling, up to 95% of the tritium was at C-3 Some of the parameters affecting distribution and yield upon bombardment by tritium atoms are examined

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EXPERIMENTAL

*Labeling of carbohydrates* — Samples of D-glucose or starch (200–300 mg) were labeled in a high-vacuum apparatus[1] where tritium gas at an initial pressure of 1 mtorr was thermally atomized by a tungsten filament Reaction times were approximately 2 min Finely divided, crystalline samples of D-glucose, starch granules, or retrograded amylose were deposited in the reaction vessel by evaporation of their suspensions in acetone under vacuum Films of D-glucose were prepared by evaporation from water and films of starch from dimethyl sulfoxide Drying of films was aided by heating on a steam cone for one h under vacuum

*Amylose–butyl alcohol complex* — An 8% solution of commercial slightly *O*-(2-hydroxyethyl)ated amylose (Stein–Hall Superlose) in dimethyl sulfoxide was prepared by dissolving the material overnight It was diluted to 1% of amylose and excess butyl alcohol was added The precipitate was collected by centrifugation and redissolved in water at 1% concentration It was reprecipitated with the critical amount of butyle alcohol and the precipitate was dehydrated by dispersion in butyl alcohol Deposition in the reaction vessel was achieved by evaporation of the alcohol A sample was also prepared that was dehydrated several times by acetone before labeling Retrograded amylose was prepared by lyophilizing an aqueous solution of amylose This sample and the acetone-washed butyl alcohol complex were deposited in the reaction vessel by evaporation from an acetone suspension

*Purification of labeled carbohydrates* — After labeling, the samples were first washed 5 times in the dry state with toluene, during a 48-h period After the washing treatment, D-glucose samples were dissolved in water and lyophilized Distillates were collected and counted for labile tritium Starch films and granules were dissolved in 4 ml of dimethyl sulfoxide, diluted with water to about 1% starch, and precipitated with 2 volumes of ethanol Starches were completely hydrolyzed by using 4 mg of glucoamylase (Miles Laboratories) Final purification of D-glucose was accomplished on a cellulose column[4] by eluting with water-saturated butyl alcohol

*Measurement of activity* — Solutions (200 $\mu$l) of D-glucose were pipeted into the counting vial that contained 20 ml of counting solution (3 5 g PPO in 700 ml of toluene and 300 ml of ethanol)[5] A 20% counting efficiency was obtained when measured in a Beckman LS-200B scintillation counter D-Glucose and its derivatives were soluble in the counting solution up to 800 $\mu$g, the maximum concentration used D-*arabino*-Hexulose flavazole and the flavazole-aldehyde were counted at 400 $\mu$g, with readings multiplied by 2 38 and 3 57, respectively, to correct for color quenching The correction factors were determined by counting various amounts of the non-radioactive derivatives in the presence of tritium-labeled D-glucose

*Determination of tritium distribution* — Tritium distribution was determined for samples of D-glucose (**1**) collected from the cellulose column, and was calculated by comparing the specific molar activity of D-glucose with a series of its derivatives in which one or more carbon-bound hydrogen atoms had been displaced selectively Generally, 200–300 mg of D-glucose (undiluted with carrier) was sufficient to prepare

a set of derivatives Percentages of tritium at C-1, 2, 3, and 6 were determined as described by Simon[2] (except that periodate oxidation was used as recommended by Hudson[6]) D-*arabino*-Hexulose phenylosotriazole (**3**) was first prepared Periodate oxidation of **3** yielded 4-formyl-2-phenyltriazole (**4**) Permanganate oxidation[7] of **4** yielded 2-phenyltriazole-4-carboxylic acid (**5**) Formaldehyde dimedone (**8**) was prepared from the formaldehyde produced during periodate oxidation Tritium at C-4 and 5 was calculated from the activity of D-*arabino*-hexulose phenylflavazole[8a,b] (**6**) In **6** all hydrogen atoms are displaced from C-1, 2, and 3 Periodate oxidation[9] of **6** yielded crystalline 3-formyl-1-phenylflavazole (**7**) In **7** the hydrogen at C-4 is retained, and the percent of tritium at C-4 was obtained from the specific molar activity of **7**

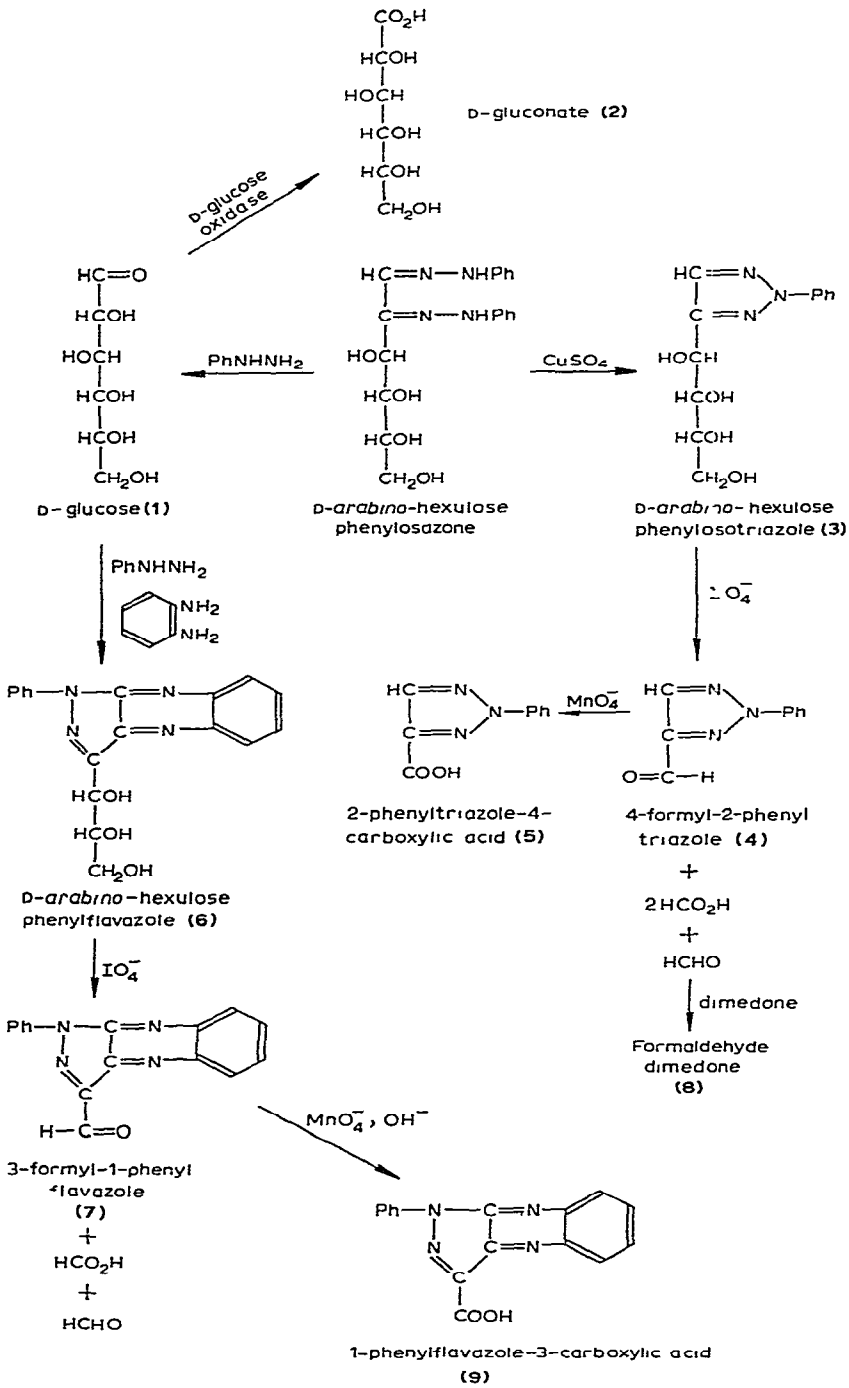
1-Phenylflavazole-3-carboxylic acid (**9**) was also prepared as a control It should be totally inactive because all original hydrogen atoms are displaced The experimental procedures were similar to those for preparing **4**, except that the permanganate was made alkaline with sodium hydroxide The sodium salt of the product was insoluble in cold water but was sparingly soluble in hot water After oxidation with permanganate, the product was filtered off and the precipitate extracted with hot water After acidification of the extract with acetic acid, the derivative was obtained as a yellow precipitate (yield 70%, m p 251–252°, $\lambda_{max}$ 407, 336, 265, 247 nm) The flavazole derivative **9** contained <1% of the tritium originally in D-glucose

The tritium at C-1 was also determined by the reaction of D-glucose oxidase as follows To about 3 mg of tritium-labeled D-glucose dissolved in 3 ml of water there was added 5 units of D-glucose oxidase (Schwartz/Mann) Oxygen was bubbled in for a period of 10 h Paper chromatography in 2 1 1 butyl alcohol–ethanol–water showed no traces of D-glucose remaining and 100% conversion to D-gluconate (**2**) After complete oxidation, the sample was lyophilized twice to remove labile tritium produced from C-1 The sample was redissolved in water, made up to the original volume, and recounted As the number of moles of carbohydrate and volume are unchanged in the reaction, the percent of tritium at C-1 can be calculated from the following equation

$$\frac{\text{disintegrations min}^{-1}\,\text{ml}^{-1}\text{ of D-glucose soln} - \text{disintegrations min}^{-1}\,\text{ml of gluconate soln}}{\text{disintegrations min}^{-1}\,\text{ml}^{-1}\text{ of glucose soln}} \times 100 = \%\text{ tritium on C-1}$$

The remainder of the calculations were accomplished by a comparison of the specific molar activities of the selected derivatives, as follows

C-1 = **5** or **1**−**2**
C-2 = **1**−**3**
C-3 = **4**−**5** or **4**−(**1-2**)
C-4 = **7**
C-5 = **6**−**7**−**8** or **3**−**4**−**7**−**8**
C-6 = **8**


D-gluconate (2)
D-glucose oxidase
D-glucose (1)
PhNHNH2
D-arabino-hexulose phenylosazone
CuSO4
D-arabino-hexulose phenylosotriazole (3)
PhNHNH2
IO4−
MnO4−
2-phenyltriazole-4-carboxylic acid (5)
4-formyl-2-phenyl triazole (4)
+ 2HCO2H + HCHO
dimedone
Formaldehyde dimedone (8)
D-arabino-hexulose phenylflavazole (6)
IO4−
3-formyl-1-phenyl flavazole (7)
+ HCO2H + HCHO
MnO4−, OH−
1-phenylflavazole-3-carboxylic acid (9)

The values obtained here, when expressed as percentages of specific molar activity of D-glucose, are percentages of tritium on the carbon atoms indicated

## RESULTS AND DISCUSSION

*Purification of D-glucose* — An example of the purification of D-glucose that was achieved on the cellulose column is shown in Fig 1 The lower sample of D-glucose shown in Fig 1 was derived from labeled starch and the upper was D-glucose that had been labeled directly Pure D-glucose was obtained in each case The major contaminant was a fast-running peak that was probably identical to that extracted with toluene The D-glucose from starch had an additional slow-running component

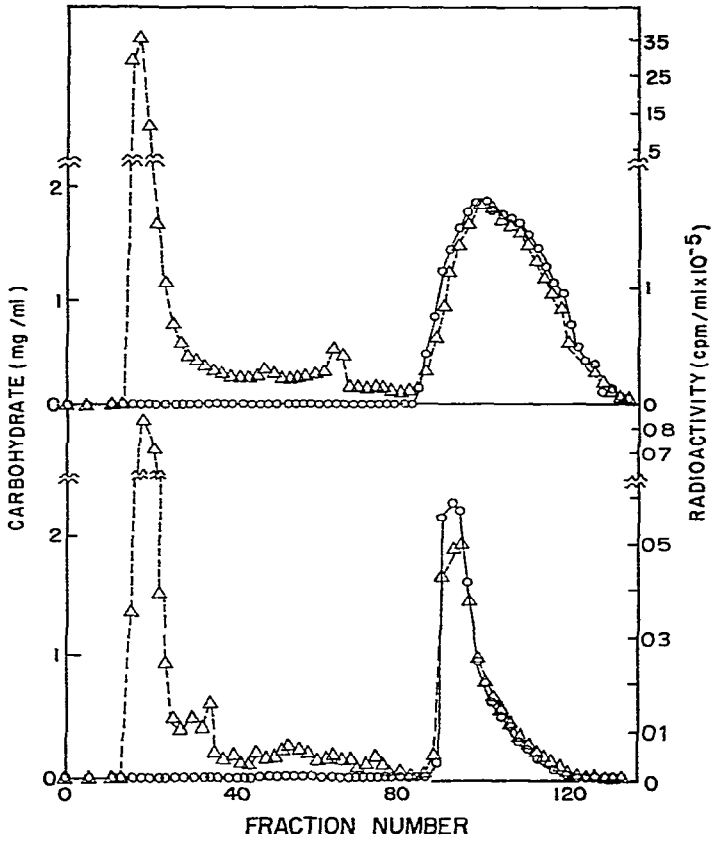



Fig 1 Purification of D-glucose, after extraction by toluene and removal of labile tritium, on a cellulose column (1 5 × 90 cm) The sample was eluted with water-saturated butyl alcohol at room temperature, and 5-ml fractions were collected △, Radioactivity, ○, carbohydrate Upper graph tritiated D-glucose (235 mg, 148 μCi, recovery of carbohydrate ~100% and of radioactivity ~95% Lower graph D-glucose from tritiated waxy-maize starch (93 2 mg, 16 2 μCi, recovery of carbohydrate ~100% and of radioactivity ~80% Specific activity of recovered D-glucose, 0 055 μCi/mg, after first crystallization, 0 052 μCi/mg, and after second crystallization, 0 053 μCi/mg

that was retained on the column with butyl alcohol but was eluted with water Repeated crystallizations of the D-glucose from 95% *p*-dioxane did not lower its specific activity (Fig 1, lower) The reaction of D-glucose oxidase was used also as a test of purity D-Glucose oxidase converted the entire sample into D-gluconic acid and its lactone No activity was found on the paper chromatogram in the area corresponding to D-glucose after the D-glucose oxidase reaction D-Glucose oxidase reacts only to a minor degree with other hexoses[10] Thus one can conclude that epimerization is not a significant reaction during tritiation Isomerization of D- and L-threaric acids has been observed upon Wilzbach labeling[11] The amount of activity in side-reaction products from several samples is shown in Table I As thoroughly dried toluene was not used and the samples were extracted in the dry state, the toluene extract probably contained much of the labile tritium However, the side-reaction products also contained labile tritium[12] Hence a rigorous separation of the two quantities listed in columns 1 and 2 was not attempted The percent yield varied considerably, being a maximum for the anhydrous, crystalline samples Water molecules react preferentially, and thus lower yields are to be expected if traces of water are present Possibly the lower yields obtained for the amorphous films are due to incomplete removal of water One factor judged to influence the results is that of time of exposure to bombardment The shorter exposure time of 2 min in this study *vs* 30 min in an earlier study[1] may be a factor in the better yields obtained here The labeling reactions occur at the surface, and longer exposures could be accompanied by consecutive reactions of H atoms or photolysis to produce a greater fraction of side-reaction products

TABLE I

TRITIUM INCORPORATION IN D-GLUCOSE

| D-*Glucose sample* | *Activity in mCi (and percentage of tritium incorporated)* | | | |
|---|---|---|---|---|
| | *Toluene extract* | *Labile tritium* | *Impurity separated by cellulose column* | *Purified* D-*glucose* |
| 1 Amorphous film | 1 73 (87) | 0 113 (5 6) | 0 095 (4 8) | 0 052 (2 6) |
| 2 Amorphous film | 0 907 (48) | 0 590 (31) | 0 168 (9 0) | 0 227 (12) |
| 3 Crystalline, monohydrate[a] | 0 295 (16) | 0 955 (55) | 0 150 (9 0) | 0 340 (20) |
| 4 Crystalline, monohydrate[b] | 1 25 (54) | 0 136 (6 0) | 0 111 (5 0) | 0 794 (35) |
| 5 Crystalline[c] | 1 09 (75) | 0 0045 (0 5) | 0 050 (3 6) | 0 295 (21) |
| 6 Crystalline, anhydrous[d] | 0 454 (36) | 0 022 (2 0) | 0 138 (11) | 0 685 (51) |

[a]Glucose solution was evaporated in the reaction vessel with a rotary vacuum-drier at room temperature Crystalline α-D-glucose monohydrate is formed on the wall during evaporation [b]Recrystallized α-D-glucose monohydrate was milled to a fine powder before deposition in the reaction vessel [c]Commercial crystalline anhydrous α-D-glucose [d]Anhydrous α-D-glucose was obtained by crystallization in an oven at 65° It was milled to a fine powder and coated inside the reaction vessel The reaction vessel was immersed in a water bath at 65° and evacuated for 4 h at a pressure less than 0 004 torr before tritiation

*Distribution of tritium in* D-*glucose* — Distributions of tritium in the carbon skeleton of several D-glucose samples labeled directly are given in Table II The distribution in the samples from D-glucose films is nearly uniform (14 2% for C-1–5 and 28 4% for C-6) and differs from that for the samples from crystalline α-D-glucose monohydrate The most significant difference is the lack of activity at C-2 and high activity at C-5 in crystals The samples from anhydrous α-D-glucose showed a small amount of activity at C-2 and slightly lower amounts at C-5 Reasonably good checks were obtained between duplicate samples, despite wide variations in yield (see Table I)

TABLE II

DISTRIBUTION OF TRITIUM IN D-GLUCOSE

| D-*Glucose sample*[a] | *Specific activity* (*μCi/mg*) | *Percentage of non-labile tritium*[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *Total* |
| 1 Film | 0 22 | 14,15 | 13 | 13,12 | 11 | 16,16 | 33 | 100 |
| 2 Film | 0 48 | 20,18 | 16 | 8,10 | 8 | 17,18 | 29 | 98 5 |
| 3 Crystalline $H_2O$ | 1 15 | 20,18 | 0 | 8,10 | 9 | 26,29 | 34 | 98 5 |
| 4 Crystalline $H_2O$ | 2 29 | 22,20 | 0 | 13,15 | 11 | 25,25 | 29 | 100 |
| 5 Crystalline | 0 89 | 19,20 | 1 | 13,12 | 14 | 26,29 | 24 | 98 5 |
| 6 Crystalline | 1 46 | —,17 | 5 | —,17 | 17 | 20,21 | 23 | 100 |
| Crystalline D-glucose[c] | 0 003 | 3 | 6 | 29 | 14 | 0 | 48 | 100 |
| D-Glucose[d] | — | 2 1 | 0 | 89 | 0 7 | 0 6 | 6 4 | 98 8 |

[a]Details of preparation are listed in Table I [b]Carbons 1, 3, and 5 are calculated two ways (Experimental) The average is used for the total [c]Tritium recoil-labeled D-glucose, Rowland *et al* [3] [d]D-Glucose labeled by the Wilzbach method, Simon *et al* [2]

The lack of activity at C-2 of samples 3 and 4 is highly significant and is probably not due to steric effects, even though the labeling reactions occur primarily at the surface This conclusion is based upon the following experimental results The two samples of D-glucose monohydrate differed in that in the first case the D-glucose was allowed to crystallize on the wall of the reaction vessel, whereas, in the second, the sample was deposited as a crystalline powder In the latter, all possible surfaces of the crystals should have been exposed, but a uniform distribution was not obtained Both samples have no activity at C-2 Rather than steric effects, it is more likely that the lack of activity at C-2 is related in some manner to the crystal structure These effects would not be present in the amorphous films Also there is the possibility that the position of the water of crystallization[13] between O-2 and O-6 distorts bond angles and energies leading to zero activity at C-2 and increased activity at C-5

Distributions of tritium in D-glucose from several samples of starch are listed in Table III As with D-glucose crystals, distribution of tritium was non-uniform Twice the expected activity at C-2 and zero activity at C-3 was observed for samples of starch granules (sample 7) The effect was decreased somewhat in the amorphous film

sample, but was not eliminated entirely Possibly the complete lack of activity at C-3 is related to the water of crystallization in starch granules, similar to that found for D-glucose monohydrate The amylose–butyl alcohol crystals (sample 9) on the other hand gave a distribution that was more nearly uniform Whereas in amylose–butyl alcohol crystals, 1 molecule of water of crystallization[14] between O-2 and O-5 is retained, it is easily removed by drying If the same reasoning is applied here as with D-glucose crystals, one would conclude that there is very little distortion of bond angles and energies in amylose–butyl alcohol crystals In acetone-washed crystals (sample **10**) one would expect replacement of butyl alcohol molecules by acetone or water[15] Considerable strain should develop, and indeed this is evident in the altered pattern of distribution

TABLE III

DISTRIBUTION OF TRITIUM IN D-GLUCOSE OBTAINED FROM TRITIATED STARCH

| *Starch sample* | *Specific activity (μCi/mg) and yield* | *Percentage of non-labile tritium*[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *Total* |
| 7 Waxy maize starch granule | 0 45 (<10)[c] | 9,11 | 29 | 0,0 | 16 | 14,15 | 31 | 100 5 |
| 8 Gelatinized waxy maize starch | 0 27 (3 6)[c] | 12,11 | 22 | 3,4 | 14 | 16,16 | 33 | 100 |
| 9 Amylose–butyl alcohol complex | 0 92 (41)[c] | 8,8 | 13 | 16,16 | 13 | 19,20 | 30 | 99 5 |
| 10 Amylose–butyl alcohol complex[b] | 1 82 (28)[c] | 11,10 | 9 | 24,25 | 4 | 32,36 | 15 | 97 0 |
| 11 Retrograded amylose | 0 10 (1 5)[c] | —,10 | 34 | —,2 | 13 | 10,8 | 33 | 101 |
| 12 Potato starch granule | | | | | | | | |
| amylopectin | 0 09 (2 4)[c] | —,10 | 18 | —,3 | 16 | 10,13 | 39 | 97 5 |
| amylose | 0 07 (0 7)[c] | —,10 | 25 | —,3 | 15 | 13,10 | 38 | 102 5 |

[a]Carbons 1, 3, and 5 are calculated two ways (Experimental) The average is used for the total [b]Amylose–butyl alcohol complex was washed with acetone (Experimental) [c]Values in parentheses are percent of total activity found in purified D-glucose

*Method for determining distribution of tritium* — The flavazole derivative appears not to have been used for determining tritium distribution It offers some advantages when combined with the osotriazole derivative Tritium at C-4 can be determined in two steps Periodate oxidation proceeds smoothly and is practically quantitative The insoluble aldehyde derivative that results is highly crystalline and is easily obtained pure without recrystallization The primary step of flavazole formation, although not quantitative, proceeds in good yield with D-glucose and the product was obtained pure after two recrystallizations For micro work, paper chromatography could be used, although it was not essential in our case The color quenching that was observed during scintillation counting for both the flavazole and

the flavazole-aldehyde could be a limitation if low activities were encountered The reliability of the flavazole procedure is shown in Table IV, where the sums of tritium percentages at C-1, C-2, and C-3 are compared with these values from the osotriazole method

TABLE IV

A COMPARISON OF THE SUMS OF PERCENTAGES OF C-1, C-2, AND C-3 OF D-GLUCOSE DERIVED FROM THE FLAVAZOLE AND OSOTRIAZOLE DERIVATIVES

| *Sample*[a] | *Derivatives*[b] | |
|---|---|---|
| | *Flavazole* (1–6) | *Osotriazole* (1−3+4) |
| 1 | 40 | 40 |
| 2 | 45 | 44 |
| 3 | 31 | 28 |
| 4 | 35 | 35 |
| 5 | 36 | 33 |
| 6 | 41 | 39 |
| 7 | 39 | 38 |
| 8 | 37 | 37 |
| 9 | 38 | 37 |
| 10 | 49 | 44 |
| 11 | 44 | 46 |
| 12 | 35 | 32 |
| | 34 | 37 |

[a]Details of samples are listed in Tables I and III [b]See Experimental

The D-glucose oxidase method for C-1 offers the advantage of simplicity, only two measurements are required The sample weight can be merely estimated, as only the disintegrations $min^{-1}$ before and after oxidation are involved in the calculation Unlike the rest of the procedure with isolated derivatives, care must be taken to ensure that the reaction goes to completion

The overall scheme also allows for considerable cross checking The sum of percent tritium at C-1, C-2, and C-3 is available in two ways, and the values for the individual carbon atoms C-1, C-3, and C-5 can be calculated in two ways In addition, the sum of percent tritium for all carbons must total 100% (Table II) This type of cross checking ensures reliability

*Tritium labeling* — Some factors contributing to good yields, such as crystallinity and low moisture, have been shown to be important by this research The maximum yield of 51% that was observed for crystalline anhydrous D-glucose and 41% for the amylose–butyl alcohol complex should be noted In previous communications it has been observed that damage to the sample is negligible These properties of high yield and low damage suggest possible application of tritium-atom bombardment as a means of labeling sensitive biological compounds

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