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Bacterial Biosynthesis of Cellulose from D-Glucose or Glycerol Precursors Labelled with Deuterium

B. F. Chumpitazi-Hermoza, D. Gagnaire & F. R. Taravel

Centre de Recherches sur les Macromolécules Végétales,
C.N.R.S.,* 53 X, 38041 Grenoble Cédex, France

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SUMMARY

D-Glucose and glycerol precursors randomly labelled with deuterium were prepared and used for the biosynthesis of bacterial cellulose by Acetobacter xylinum. *The materials obtained were converted into triacetate derivatives and analysed by 250 MHz nuclear magnetic resonance.*

Labelling percentages on each position are reported. The weighted addition of combinations of different ^2H or ^1H sites for mixtures of multiple labelled compounds was performed by means of an N.M.R. spectrum simulation program according to different hypotheses. The non-random nature of the results showed the importance of exchange phenomena and of the biosynthetic pathways which take place during cellulose biosynthesis.

While showing less favourable properties than ^{13}C enrichment, deuterium labelling can nevertheless lead to significant results (in particular if one is dealing with labelled fragments of precursors incorporated partly or totally into a final molecule), particularly in view of the easy preparation of deuterated compounds by catalytic exchange.

1. INTRODUCTION

The biosynthesis of cellulose by *Acetobacter xylinum*, as reported by Hestrin (1963), has been extensively studied in our laboratory in recent

* Laboratoire Propre C.N.R.S., Associé à l'Université Scientifique et Médicale de Grenoble.

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years using as precursors: deuterated D-glucose and/or deuterated water (Barnoud *et al.*, 1971), D-glucose specifically deuterated at C-6 (Gagnaire & Taravel, 1975), glycerol specifically deuterated at C-1, C-2 or C-3 (Chumpitazi-Hermoza *et al.*, 1978), and a mixture of randomly enriched ^{13}C and unlabelled D-glucose (Gagnaire & Taravel, 1979, 1980). The material obtained after acetylation was analysed by 250 MHz N.M.R., or converted into monosaccharide derivatives prior to N.M.R. analysis. Our first report (Barnoud *et al.*, 1971) showed that about 90% of the protons linked to C-1 and C-6 came from the D-glucose used in the nutritive medium, whereas 10% were exchanged with other sources of protons. Also, 40% of the protons linked to C-2, C-3, C-4 and C-5 arose from the water in the nutritive medium.

Using selectively labelled D-glucose (Gagnaire & Taravel, 1975) and selectively deuterated glycerol (Chumpitazi-Hermoza *et al.*, 1978) as precursors we were able to define the role of such different enzymes as glucose-phosphate isomerase, dehydrogenase, triose-phosphate isomerase and aldolase involved during biosynthesis and thereby explain the observed reaction products and intermediates. In particular, with glycerol, a 'head to head' mechanism of triose fragment condensation was clearly demonstrated. In all cases the effect of labelling with deuterium was evident in terms of changes in the inhibition period during growth and the ultimate reaction equilibrium.

Experiments carried out with a 50 : 50 mixture of unlabelled D-glucose and D-glucose uniformly enriched with ^{13}C gave complementary biosynthetic and structural information (Gagnaire & Taravel, 1979, 1980). Such experiments are very interesting, because the products resulting from the molecular rearrangements occurring during biosynthesis can be analysed using a mathematical treatment of the N.M.R. data. This treatment permitted the determination of the probability of two neighbouring sites being formed from the same precursor molecule. The overall statistical calculations also provided structural information since the probability of occurrence of any sequence of the atoms in the skeletal molecule could be determined. This then allowed a prediction of the results for a precursor selectively enriched at a particular position and evaluation in terms of the main pathways proposed so far (White & Wang, 1964a, b): direct incorporation of the molecule precursor, the pentose phosphate cycle, the Entner–Doudoroff pathway and triose recombination.

We report now a series of experiments carried out with D-glucose or glycerol precursors, randomly labelled with deuterium according to the method of Koch *et al.* for deuterium labelling of carbohydrates by catalytic exchange (Koch & Stuart, 1977, 1978). Using deuterated or light water as aqueous medium, the extent of exchange taking place during biosynthesis was determined by N.M.R. spectroscopy. In addition, the existence of any correlation between the labelling percentage on each position and the varying contribution of the different isotopic species present at each site, was investigated in order to determine to what extent the labelling obtained could be considered random or obeyed strict biochemical laws.

2. EXPERIMENTAL

2.1 Precursors

D-[2,3,4,6*R*,6*S*-$^2H_5$] glucose (1) and *sn*-[1*R*,1*S*,2,3*R*,3*S*-$^2H_5$] glycerol (2) were prepared from methyl α-D-glucopyranoside and glycerol respectively according to the method of Koch & Stuart (1977, 1978) using deuterated Raney nickel in refluxing deuterium oxide. After acid hydrolysis, compound 1 was obtained and analysed at 250 MHz by means of its acetylated derivative ^1H spectrum; the average deuteration obtained was 90% at the five mentioned sites. In the case of compound 2, which was analysed identically by means of its acetylated derivative, an 80% deuteration was found.

2.2 Cell culture and cellulose biosynthesis

Cellulose-forming *Acetobacter xylinum* strains were supplied from the American Type Tissue Culture Collection and grown as described by Hestrin (1963). The culture medium had the following composition: bactopeptone 0·5%, yeast extract 0·5%, precursor 2%, potassium monohydrogen phosphate (anhydrous) 0·1%; pH 7·0. The solution was sterilised by autoclaving for 20 min at atmospheric pressure. Cultures were incubated at 30°C.

Three experiments were carried out:

experiment A:
using D-[2,3,4,6*R*,6*S*-$^2H_5$] glucose and deuterated water
experiment B:
using *sn*-[1*R*,1*S*,2,3*R*,3*S*-$^2H_5$] glycerol and deuterated water
experiment C:
using *sn*-[1*R*,1*S*,2,3*R*,3*S*-$^2H_5$] glycerol and water.

With deuterated water, the growing period in the case of experiments A and B was 9 weeks, while it was only 6 weeks for experiment C (H_2O). The cellulose pellicles were collected and washed as previously described (Barnoud *et al.*, 1971). The yield was approximately 40 mg of deuterated bacterial cellulose per g of precursor.

2.3 Biosynthesised cellulose analysis

Samples **3**, **4** and **5** were obtained after acetylation by the usual method (Barnoud *et al.*, 1971) from the products of experiments A, B and C respectively. They were analysed by ^1H N.M.R. at 250 MHz to determine the percentage of deuterium bonded to each of the six carbon atoms of the monomer unit. A small fraction of each sample was also hydrolysed and converted into glucitol hexaacetate (compounds **6**, **7** and **8** respectively). Analysis of these derivatives was made by ^1H N.M.R. and mass spectrometry to corroborate the data obtained for the polysaccharide samples.

2.4 Proton nuclear magnetic resonance

The ^1H N.M.R. spectra were recorded at 250 MHz in the C.W. mode using a CAMECA spectrometer with deuterated chloroform as the sample solvent and a probe temperature of 55°C in the case of cellulose triacetate. Assignments are well known (Friebolin *et al.*, 1969; Angyal *et al.*, 1972; Gagnaire *et al.*, 1973; Gagnaire & Taravel, 1975).

2.5 Mass spectroscopy

Mass spectra were obtained on an A.E.I. MS 30 spectrometer with the following operating conditions for electron impact: ionisation energy, 70 eV; ionisation current, 100 μA; ion source temperature, 150°C.

2.6 N.M.R. spectrum simulation

Calculations of the theoretical spectrum were made with the aid of a BNC 12 ITRCAL program. The weighted addition of several spectra arising from different isotopomers of the same molecule was performed either by using a BRUKER FT NMR program or a LAOCD2 program available in our laboratory.

3. RESULTS AND DISCUSSION

Table 1 shows the deuterium labelling attained in the case of precursors **1** and **2**. This has been determined by N.M.R. analysis of their acetylated derivative. After biosynthesis, according to experiments A, B and C, the situation is changed as illustrated in Fig. 1 and Table 2. Clearly the best labelling obtained is from experiment A starting from D-[2,3,4,6*R*,6*S*-$^2H_5$]glucose and deuterated water. Direct incorporation of the precursor is not the only pathway to consider as deuterium is found also at positions 1 and 5. This means that other mechanisms occur producing rearranged molecules.

TABLE 1
Deuterium Labelling on each Hydrogen Site for Precursors **1** and **2**

	1	*2*	*3*	*4*	*5*	*6 pro-R*	*6 pro-S*
1	0	95	85	95	0	90	80
	1 pro-R	*1 pro-S*	*2*	*3 pro-R*	*3 pro-S*		
2	70	80	95	70	80		

By examining the results obtained for experiments B and C, one can see how significant are the exchange phenomena with the medium. In fact, using deuterated water (experiment B) instead of water (experiment C) more label is introduced at positions 2, 3, 4 and 5 of the biosynthesised cellulose, while at positions 1 and 6 the deuterium labelling remains approximately the same in both experiments. This result cor-

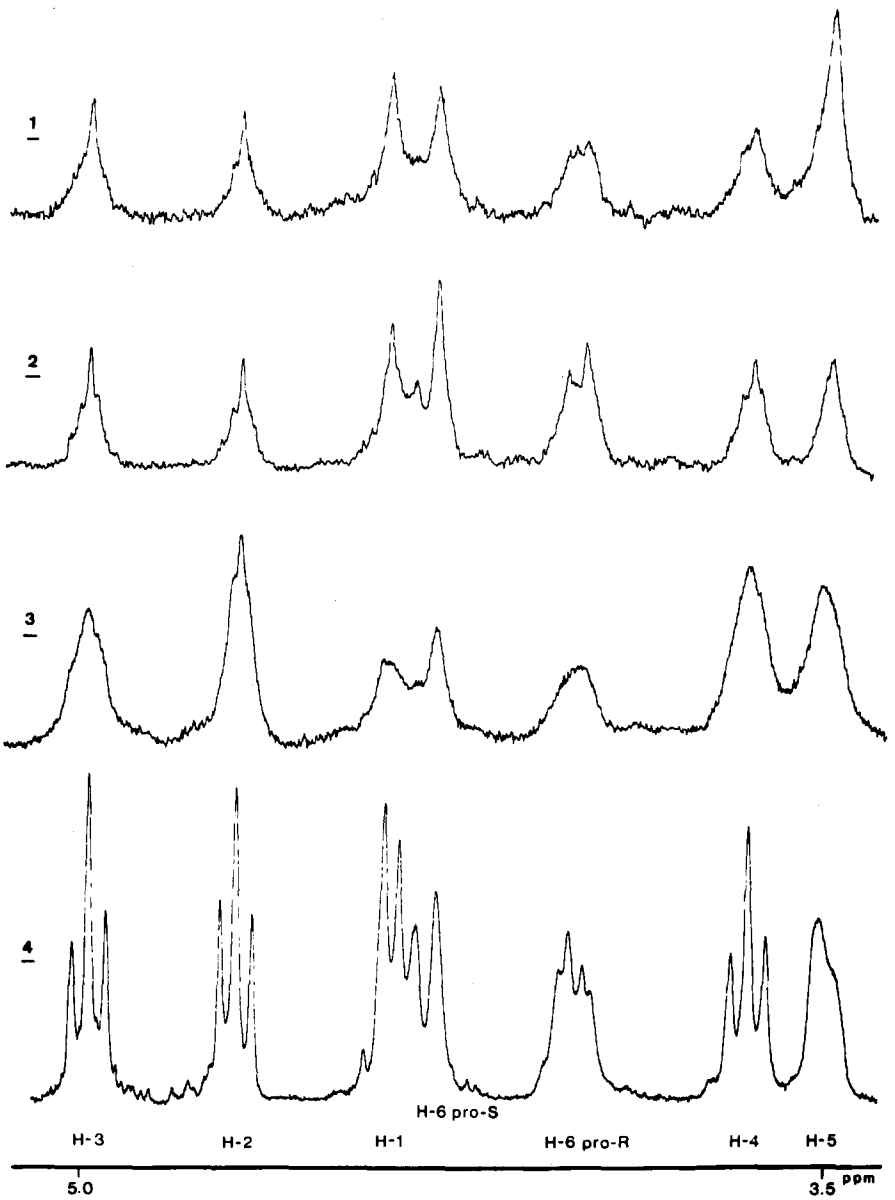

Fig. 1. ^{1}H N.M.R. Spectra (250 MHz) of cellulose triacetate (1% solution) in $CDCl_3$ at 328 K, corresponding to bacterial cellulose samples according to: (1) experiment A using D-[2,3,4,6*R*,6*S*-$^2H_5$]glucose and D_2O; (2) experiment B using *sn*-[1*R*,1*S*,2,3*R*,3*S*-$^2H_5$]glycerol and D_2O; (3) experiment C using the same glycerol precursor but with H_2O; (4) undeuterated glucose and H_2O.

TABLE 2
Deuterium Labelling (%) on each Hydrogen Site of the Glucose Unit in the Biosynthesised Cellulose

Experiment	*1*	*2*	*3*	*4*	*5*	*6 pro-R*	*6 pro-S*	*Average labelling per site*
A	79	85	81	83	60	76	70	76
B	64	73	70	70	67	56	52	65
C	63	34	42	26	30	61	61	45

roborates previous findings (Chumpitazi-Hermoza *et al.*, 1978) obtained with glycerol selectively deuterated at C-1, C-2 or C-3, and is in agreement with the particular action of the two enzymes, triosephosphate isomerase and aldolase, involved in the head-to-head condensation of triose fragments during the biosynthesis. Triosephosphate isomerase which converts dihydroxyacetone phosphate into glyceraldehyde-3-phosphate is stereoselective for the mobilisation of the *pro-R* atom at C-3 (Arigoni & Eliel, 1969). In contrast, aldolase catalyses the condensation of dihydroxyacetone phosphate with glyceraldehyde-3-phosphate to give fructose-1,6-diphosphate mobilising the *pro-S* atom at C-3 of the starting molecule. This is also in agreement with the finding that the exchange in fact occurs in the free (and not in the phosphate-esterified) CH_2OH group, i.e. at positions 3 and 4 in both the aldolase and isomerase catalysed exchanges (Rieder & Rose, 1956, 1959).

Other interesting features are shown in Table 3, where the results obtained by mass spectrometry of hexa-*O*-acetyl-D-glucitol (**6**), (**7**) and (**8**) are recorded. These results are obtained by analysis of the region corresponding to the heaviest ion of such molecules, i.e. at m/e 375 (M-59) and above, because of deuterated species. This fragmentation affects only an acetyl group (Kochetkov & Chizhov, 1966). Calculations, performed with the necessary precautions in order to correct for the observed intensities of the different peaks, give the percentages of the various deuterated species. Table 3 shows fairly well the predominance of species with more than three deuterium atoms in the case of experiment A, for which the best average labelling per site is obtained. This phenomenon corresponds to an N.M.R. spectrum with sharp peaks for compound **3** (see Fig. 1), i.e. showing that the occurrence of species

TABLE 3
Deuterated Species (%) Determined by Mass Spectrometry Analysis of hexa-*O*-acetyl-D-glucitol (**6**), (**7**) and (**8**)

Compound	*Experiment*	d_0	d_1	d_2	d_3	d_4	d_5	d_6	d_7	*Average labelling per site*
6	A	0	0	0	6	14	29	32	19	78
7	B	7	3	6	11	2	20	25	25	69
8	C	8	8	22	28	23	11	0	0	40

with two vicinal hydrogen atoms is weak. This feature is reversed in the case of experiment C as shown by mass spectrometry and N.M.R. analysis (Table 3 and Fig. 1). As expected from the average labelling per site (45%), many hydrogen–deuterium couplings in addition to hydrogen–hydrogen couplings contribute to a broadening of the lines as observed in the N.M.R. spectrum of compound **5**. Experiment B shows results which are intermediate in character between the other experiments as species containing two vicinal hydrogen atoms are found more and more predominantly. This is clear from the N.M.R. spectrum of compound **4** since an increasing occurrence of species corresponding to doublets on each hydrogen signal may be observed. N.M.R. analysis of such labelled polysaccharides containing several isotopomers, i.e. a mixture of multiple labelled compounds or combinations of different ^2H or ^1H sites, is very interesting as information relating to three neighbouring atoms (for H-2, H-3, H-4, H-6 *pro-R* and H-6 *pro-S*) or two neighbouring atoms (for H-1) and four (for H-5) can be extracted. For example, the H-2 N.M.R. signal appears as the addition of three signals: (a) a triplet corresponding to species with only hydrogen atoms: H-1/H-2/H-3; (b) two doublets corresponding to the two possibilities of having one deuterium atom on one neighbouring site or the other: ^{2}H-1/H-2/H-3 or H-1/H-2/^{2}H-3; (c) a singlet corresponding to species with two deuterated neighbours: ^{2}H-1/H-2/^{2}H-3. In general the two doublets and the singlet are shifted upfield relative to the chemical shift signal of the unlabelled species (Chumpitazi-Hermoza *et al.*, 1978). The knowledge of the contribution of the different isotopic species for each site as well as the determination of the labelling percentage on

each specific position open up the possibility of correlating the various results in order to estimate the occurrence of all the possible isotopomers found for the whole molecule. This kind of analysis constitutes a good approach to the complete elucidation of reaction mechanisms in synthetic and biosynthetic studies.

In order to proceed towards this goal, theoretical spectrum calculations were performed with the aid of a BNC 12 ITRCAL program, for each hydrogen site and for all the possible species (deuterated and undeuterated) at this particular position. A weighted addition of these different spectra according to (a) a random hypothesis, or (b) estimates taking into account the various recorded observations, including the extent of water exchange and the predominance of certain species, was made and compared with the experimental results. A typical example of this procedure is shown (Fig. 2) in the case of experiment B for H-1, H-6 *pro-R* and H-6 *pro-S*. This simulation included five sites (1, 2, 5, 6 *pro-R* and 6 *pro-S*) and necessitated the weighted participation of 32 species according to the possibilities of having a hydrogen atom or a deuterium atom at each position. In the random hypothesis the contributions were calculated directly from the labelling percentage on each specific position, while the best solution gave results from experimental data determined on each signal (see Table 4). The simulation of spectra took into account the isotopic effect (seen above) but neglected the hydrogen–deuterium couplings.

The fit between the best solution given and the experimental spectrum is good. The random hypothesis is completely unrealistic as the biosynthesis follows precise mechanisms (White & Wang, 1964a, b; Gagnaire & Taravel, 1980) which includes also the exchange phenomena. Definitive calculations corresponding to the whole molecules for the different experiments were not systematically achieved because of a lack of experimental data which would permit good correlations from the N.M.R. information. However, site by site, and for the three experiments described, some general conclusions can be drawn. Incorporation of unlabelled materials is greater than expected and could be originating from exchange phenomena or from preferential biosynthesis. Contamination from a sugar source contained in our bacterial strain may also be possible. In the same way incorporation of unmodified parts of molecules seems to be preferential, labelled or unlabelled and this is particularly true in the case of groups including sites 4, 5 and 6 of the biosynthesised molecules. This observation agrees well with our

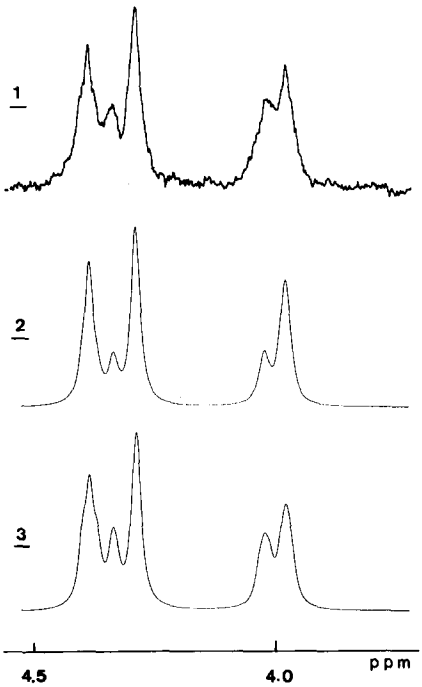

Fig. 2. Weighted addition of spectra in the case of bacterial cellulose triacetate obtained according to experiment B. (1) Partial experimental spectrum arising from H-1, H-6 *pro-R* and H-6 *pro-S*. (2) Theoretical spectrum obtained from the random hypothesis: the individual contributions are calculated directly from the labelling percentage determined on each specific position (see Table 3). (3) Theoretical spectrum obtained from experimental observations taking into account the shape of the peaks and the predominance of certain species (see Table 3). The simulation includes the isotopic effect (see text) but neglects the hydrogen deuterium couplings.

preceding results (Gagnaire & Taravel, 1980) obtained with the ^{13}C label and for which we could indicate the following values for the different pathways involved during the biosynthesis: (1) 23% of the glucose molecules in cellulose come from an unmodified molecule of glucose; (2) 26% have been involved at least once in the pentose phosphate cycle, and 10% at least twice; (3) 30% of the glucose molecules come from triose recombination. All these pathways with the exchange phenomena as limits, incorporate parts of molecules and also whole molecules without further modifications.

TABLE 4

Occurrence (‰) of the Various Isotopomers Defined for Sites 6*R*, 6*S*, 5, 1 and 2. According to: [a] the Random Hypothesis, and [b] Experimental Data, in the Case of Experiment B

	*Fragments**							
6R 6S 5 \ *1 2*	*111*	*011*	*101*	*001*	*110*	*010*	*100*	*000*
11	7[a] (34)[b]	9 (9)	7 (9)	9 (7)	14 (23)	18 (20)	15 (13)	19 (64)
01	12 (17)	15 (5)	13 (5)	17 (4)	25 (12)	31 (10)	27 (6)	34 (32)
10	18 (34)	23 (9)	20 (9)	25 (7)	37 (23)	47 (20)	40 (13)	51 (64)
00	33 (104)	41 (28)	35 (28)	45 (22)	66 (71)	84 (61)	72 (39)	91 (198)

* For each site, a hydrogen atom is symbolised by 1 and a deuterium atom by 0.

4. CONCLUSION

Although promising our results using deuterated precursors to study the bacterial biosynthesis of cellulose are limited by the exchange phenomena taking place in the medium during growth and also by a lack of N.M.R. resolution which does not permit good correlations between all the information concerning the possible isotopic species; we believe that this kind of study involving a polymer can only be solved by N.M.R. To attain that end more detailed N.M.R. spectral information is needed. The use of two-dimensional spectroscopy is being seriously considered as its improved resolution should allow a differentiation of the possible isotopomers on the basis of their isotopic effect (Gagnaire *et al.*, 1982).

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