Phytochemistry, 1969, Vol. 8, pp. 737 to 741. Pergamon Press. Printed in England

SOLUBLE NUCLEOTIDES IN DEVELOPING COTTON HAIR

GERHARD FRANZ

Botanisches Institut, Universität Fribourg, Schweiz

(*Received* 22 *October* 1968)

Abstract—Soluble nucleotides were studied during primary and secondary wall formation of growing cotton hairs. UDPG was the major component at both stages of growth. No guanine nucleotides could be detected. The possible role of the sugar nucleotides in the biosynthesis of polysaccharides is discussed.

INTRODUCTION

IT IS known that the biosynthetic pathway to cellulose proceeds via sugar nucleotides. Glaser[1] demonstrated the *in vitro* synthesis of cellulose with enzyme preparations from *Acetobacter xylinum* and UDPG as glucosyl donor. Barber *et al.*[2] obtained cellulose with particulate enzyme systems from different higher plants using GDPG as substrate. Other workers,[3–5] using enzyme systems from various higher plants, were able to show that cellulose biosynthesis can also proceed *in vitro* via UDPG.

In a recent study it was found that UDPG is an excellent substrate for the *in vivo* synthesis of cellulose when administered to living cotton hairs.[6] It was therefore of interest to study the naturally occurring nucleotides and sugar nucleotides in these cells.

RESULTS AND DISCUSSION

Cotton hairs can be harvested during two clearly distinguishable growth stages; that of primary wall and that of secondary wall formation. Up to 25 days after flowering, the seed-coat forms hairs having primary walls only. 25–35 days after flowering the formation of the secondary wall takes place in the hairs. The different stages can be distinguished with the aid of a polarizing microscope.

The nucleotides and sugar nucleotides present in the cotton hair cells at both stages of cell wall formation were extracted with aqueous ethanol and purified as described in the Experimental section. A typical chromatographic elution sequence of the nucleotides from a PEI (polyethyleneimine)-cellulose column is shown in Fig. 1 (A and B). The peaks were pooled and further fractionated by paper chromatography. U.v. light absorbing bands were cut out and eluted from the paper. Analytical results of the different fractions are shown in Table 1. Sugar nucleotides were not separated from one another by this method. A separation of UDPG and UDPGal as borate complexes could be obtained on PEI-cellulose thin-layer plates.[7] The mixture of sugar nucleotides was subjected to mild acid hydrolysis to give free

[1] L. GLASER, *J. Biol. Chem.* **232**, 627 (1958).
[2] G. A. BARBER, A. D. ELBEIN and W. Z. HASSID, *J. Biol. Chem.* **239**, 4056 (1964).
[3] D. A. BRUMMOND and A. P. GIBBONS, *Biochem. Z.* **342**, 308 (1965).
[4] C. L. VILLEMEZ, G. FRANZ and W. Z. HASSID, *Plant Physiol.* **42**, 1219 (1967).
[5] M. ORDIN and M. A. HALL, *Plant Physiol.* **43**, 473 (1968).
[6] G. FRANZ and H. MEIER, *Phytochem.*, in press.
[7] K. RANDERATH and E. RANDERATH, *Anal. Biochem.* **13**, 575 (1965).

sugars and free nucleotides. The ratio of the sugars was determined by quantitative paper chromatography. The only free nucleotides detected were UDP and to a lesser extent UMP. From both primary and secondary wall cotton hairs the mono-, di-, and triphosphates of uridine and adenosine could be isolated. There was a remarkable difference in the sugar nucleotide composition between the two stages. In the primary wall fraction UDPG was the major component of the sugar nucleotide mixture. Much smaller amounts of UDPGal,

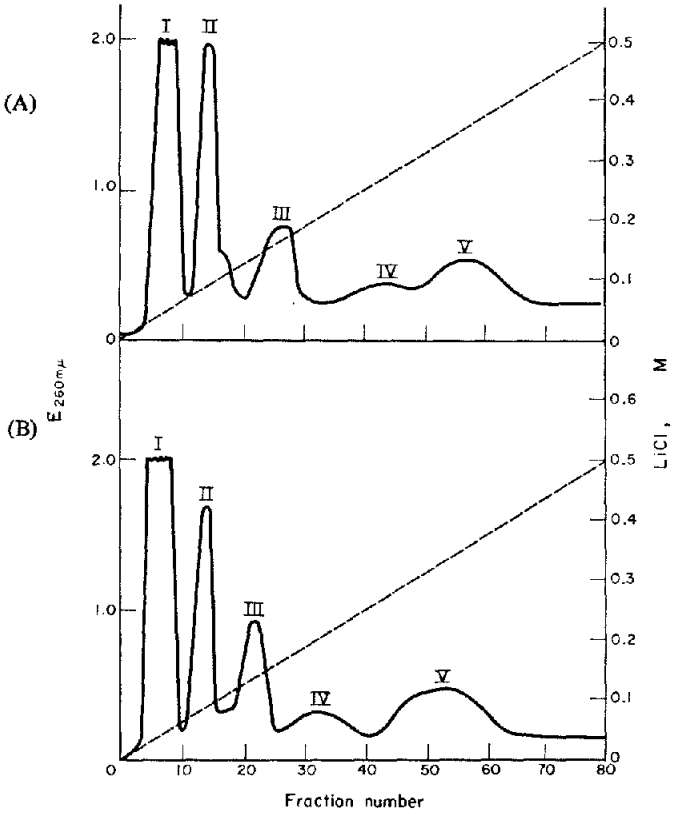

FIG. 1. ELUTION CURVE OF NUCLEOTIDES EXTRACTED FROM COTTON HAIRS. THE NUCLEOTIDES WERE FRACTIONATED ON A PEI-CELLULOSE COLUMN (1·0 × 40 cm, CHLORIDE FORM) AND 10 ml FRACTIONS WERE COLLECTED.

A: Elution sequence of nucleotides from cells having primary walls only. B: Elution sequence of nucleotides from cells having primary and secondary walls. (See Table 1 for identity of fractions.)

UDPAra and UDPXyl were present. UDPGal A was identified in a separate fraction. In the secondary wall fraction only UDPG and UDPGal could be identified, the former predominating.

These results agree with the findings of Elnaghy and Nordin[8] and Grégoire *et al.*[9] who isolated similar nucleotides from *Avena sativa* and *Phaseolus aureus* seedlings. They also found other nucleotides, which is understandable, since the plant material they used was of a

[8] M. A. ELNAGY and P. NORDIN, *Arch. Biochem. Biophys.* **113**, 72 (1966).
[9] J. GRÉGOIRE, J. GRÉGOIRE, N. LIMOZIN and L. V. VAN, *Bull. Soc. Chim. Biol.* **47**, 195 (1965).

TABLE 1. ANALYTICAL DATA OF NUCLEOTIDES ISOLATED FROM GROWING COTTON HAIRS

Fraction*	Spectral type	Total P	Moles P/mole base† labile P	Sugars detected after mild acid hydrolysis	Compound identified	Amount isolated‡ (μm)
A. Primary wall fraction						
Ia	Adenosine	1·05	—		AMP	2·4
b	Adenosine	1·85	—		NAD	3·7
c	Uridine	1·10	—		UMP	5·3
d	Uridine	2·01	1·07	Galactose, Glucose, Xylose, Arabinose (3·1:14·1:1·0:1·2)	UDPGal, UDPG UDPXyl, UDPAra	12·0§
IIa	Adenosine	1·90	0·85		ADP	4·7
b	Uridine	2·03	Not determined	Galacturonic acid	UDPGal A	1·1
III	Uridine	1·91	0·94		UDP	3·4
IV	Adenosine	Not determined			NADP	0·5
Va	Adenosine	2·50	1·53		ATP	1·2
b	Uridine	2·70	1·8		UTP	2·8
B. Secondary wall fraction						
Ia	Adenosine	0·95	—		AMP	3·8
b	Adenosine	1·78	—		NAD	5·2
c	Uridine	1·01	—		UMP	5·1
d	Uridine	2·05	0·81	Galactose, Glucose (1·0:8·6)	UDPG, UDPGal	16·7
II	Adenosine	1·90	1·10		ADP	5·6
III	Uridine	1·78	0·80		UDP	2·4
IV	Adenosine	Not determined			NADP	0·4
Va	Adenosine	2·83	Not determined		ATP	0·6
b	Uridine	2·55	1·75		UTP	3·1

* Corresponding to peak numbers in Fig. 1.
† Calculated from molar absorbancy of adenosine and uridine.[21]
‡ From sixty cotton bolls.
§ Calculated for total UDP-hexoses.

more complex composition than cotton hairs and contained cells which were probably forming both primary and secondary walls. All studies on growing plant tissues have shown that UDPG is always the most abundant sugar nucleotide. Other sugar nucleotides may be present but in much smaller amounts. Grégoire *et al.*[9] found GDPmannose and GDP in *P. aureus* seedlings. Elbein *et al.*[10] showed that GDPG can serve as glucosyl donor for the *in vitro* biosynthesis of cellulose. So far, however, GDPG has only been found in the tissues of strawberry leaves.[10a] Therefore it might be questioned whether the sugar nucleotide GDPG serves as the normal glucosyl donor for the biosynthesis of cellulose *in vivo*.

UDPG is not only used as substrate for polysaccharide biosynthesis; it has also been shown to serve as glucosyl donor for the biosynthesis of sucrose.[11] This sugar was identified in the free sugar mixture of cotton hairs.[12] The presence of sugar nucleotides other than UDPG in the cotton hairs is due to the formation of non-cellulosic cell-wall polysaccharides which are found in quite large amounts, especially in the primary walls. UDPGal A was shown by Villemez and Hassid[13] to serve as substrate for the *in vitro* biosynthesis of pectin. UDPXyl and UDPAra were used by Bailey and Hassid[14] for the *in vitro* biosynthesis of a xylan and an arabinoxylan respectively, using particulate enzyme preparations from young corn cobs. McNab *et al.*,[15] using particulate enzyme preparations of *P. aureus* seedlings, showed that UDPGal could act as galactosyl donor for the *in vitro* biosynthesis of a galactan. Pridham and Hassid[16] used UDPGal for the biosynthesis of raffinose. Sensler and Kandler[17] on the other hand demonstrated that galactinol served as galactose donor for stachyose biosynthesis. It has been shown, that galactinol is formed from UDPGal and myoinositol.[18] Both raffinose and galactinol are present in the soluble sugar mixture of cotton hairs. Stachyose however could not be detected.[12]

To summarize, it appears very probable that the *in vivo* biosynthesis of cellulose in growing cotton hairs proceeds via the sugar nucleotide UDPG as glucosyl donor. There is no evidence for the presence of GDPG which has been shown to act as a glucosyl donor for the *in vitro* biosynthesis of cellulose.

EXPERIMENTAL

Material

The cotton (*Gossypium arboreum*) was grown in a greenhouse under constant temperature (28°). The nucleotides used as reference substances were purchased from Boehringer Company, Mannheim, Germany. UDPGal A was obtained by courtesy of Dr. H. Kauss, München, and UPDGal from Dr. P. Mühlradt, Freiburg.

Paper chromatography and paper electrophoresis was carried out on acid-washed Schleicher-Schüll 2043b paper. PEI-cellulose powder and plates were purchased from Machery and Nagel, Düren, Germany. The granular charcoal was a gift of Dr. R. H. Egli, Nestlé Forschungslaboratorien, Vevey, Switzerland. U.v. measurements were completed using a Beckman DB spectrophotometer.

Methods

The freshly harvested cotton bolls were opened with a sharp knife and a small aliquot of hairs was examined under the polarization microscope. The remainder of the hairs was immediately detached from the

[10] A. D. ELBEIN, G. A. BARBER and W. Z. HASSID, *J. Am. Chem. Soc.* **86**, 309 (1963).
[10a] F. A. ISHERWOOD, *Biochem. J.* **105**, 723 (1967).
[11] L. F. LELOIR and C. E. CARDINI, *J. Biol. Chem.* **214**, 157 (1955).
[12] G. FRANZ, unpublished.
[13] C. L. VILLEMEZ, T. Y. LIN and W. Z. HASSID, *Proc. Natl Acad. Sci. U.S.* **54**, 1626 (1965).
[14] R. W. BAILEY and W. Z. HASSID, *Proc. Natl Acad. Sci. U.S.* **56**, 1568 (1966).
[15] J. M. MCNAB, C. L. VILLEMEZ and P. ALBERSHEIM, *Biochem. J.* **106**, 355 (1968).
[16] J. B. PRIDHAM and W. Z. HASSID, *Plant Physiol.* **40**, 984 (1965).
[17] T. SENSLER and M. O. KANDLER, *Z. Pflanzenphysiol.* **57**, 376 (1967).
[18] R. B. FRYDMAN and E. F. NEUFELD, *Biochim. Biophys. Res. Commun.* **12**, 121 (1963).

seeds and placed directly in liquid N_2 for 60 min. The deep-frozen material was stored at −30° for 3 days to inactivate phosphatases.[19] The frozen hairs were then crushed in a cooled mortar and transferred to cold 40% ethanol. The extraction was carried out at 2–4° for 60 min with constant stirring. The extract was filtered through sintered glass and the hairs were reextracted with the above solvent. The extracts were combined and concentrated *in vacuo* at 30° to a third of the original volume. This solution was extracted 2× with an equal volume of ether and the ether layer discarded. The remaining solution was concentrated to small volume and applied to the top of a Dowex 1 × 4 (200–400 mesh; Cl^- form) column (1·5 × 20 cm). Nucleotides were adsorbed on the resin, and sugars and other neutral compounds were eluted with water until the effluent gave no further positive anthrone reactions.[20] Subsequently the total nucleotides were eluted with 1 N NaCl/0·03 N HCl until the effluent showed no further absorption at 260 nm. The effluent was concentrated to a few millilitres and purified further on a column of granulated charcoal.[21] Salts and other non-nucleotide compounds were eluted with water followed by 30% ethanol until the effluent gave no further positive Cl reaction. The total nucleotides were then eluted from the column with 50% ethanol/0·1% NH_4OH until the effluent gave no further absorption at 260 nm. This solution was concentrated to a third of the original volume and shaken twice with an equal volume of *n*-BuOH. The *n*-BuOH layers were discarded and the remaining solution concentrated to small volume.

The primary separation of the nucleotides was carried out on a PEI-cellulose column (1·0 × 40 cm; Cl^- form). The nucleotides were separated using a gradient of LiCl 0·0–0·5 M (800 ml). Fractions of 10 ml were collected. Each fraction was analysed for absorbance at 260 nm. The different peaks were pooled and the nucleotides freed from salts by the charcoal method described above. Further fractionation was achieved by paper chromatography in 96% EtOH:1 M ammonium acetate, pH 3·8; u.v. absorbing bands were cut out and eluted with H_2O. The different fractions were concentrated and identified by the following criteria: (1) u.v. spectra in both 0·1 N HCl and 0·1 N NaOH; (2) paper chromatography using the solvent system described above; (3) TLC on PEI-cellulose with the solvent systems described by Randerath;[7] (4) electrophoresis on paper in ammonium formate buffer, 0·2 M, pH 3·7; (5) total and labile phosphate content determined after the method of Fiske and Subbarow;[22] labile phosphate being determined after hydrolysis for 10 min in 1 N HCl at 100°; (6) identification of the products after mild acid hydrolysis with 0·01 N HCl at 100° for 15 min. The hydrolysate was dried *in vacuo* (KOH), dissolved in 2·0 ml of H_2O and transferred to a charcoal column (0·5 × 4·0 cm). The sugar moiety was eluted with water and the nucleotide moiety with aqueous ammoniacal ethanol solution. The sugars were further analysed by paper chromatography using the solvent systems ethyl acetate–pyridine–water, 8:2:1 (v/v) and ethyl acetate–acetic acid–water, 3:1:3 (v/v; upper phase). Uronic acid was analysed in the solvent system acetone–ethanol–iso propanol–0·05 M borate buffer, pH 10, 3:1:1:2 (v/v). Sugars were located by the $AgNO_3$ method.[23] The ratio of the sugars was calculated after paper chromatography using the triphenyltetrazolium chloride method described by Fischer and Dörfel.[24]

Acknowledgements—The author thanks Prof. Dr. H. Meier and Dr. J. S. G. Reid for their suggestions on the preparation of the manuscript and for their valuable discussions.

[19] C. V. Cole and C. Ross, *Anal. Biochem.* **17**, 526 (1966).
[20] D. L. Morris, *Science* **107**, 254 (1948).
[21] F. Kiefer and R. H. Egli, *Z. Anal. Chem.* **221**, 416 (1966).
[22] C. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375 (1925).
[23] W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature* **166**, 444 (1950).
[24] F. G. Fischer and H. Dörfel, *Z. Physiol. Chem.* **297**, 164 (1954).