Reactions of uridine diphosphate sugars catalyzed by enzymes of French-bean leaves

Many compounds containing uridine diphosphate linked to sugars have now been isolated from plants, and these sugar nucleotides have been shown to undergo a variety of conversions and to act as glycosyl donors in the presence of plant enzymes[1]. Whereas D-glucuronic acid occurs in plants chiefly as a constituent of complex polysaccharides such as hemicelluloses and gums, in a few instances simple conjugates with flavones, of similar structure to the phenolic β-D-glucopyranosiduronic acids excreted in mammalian urine, have been isolated[2]. It was thus of interest to study the reactions undergone by UDPGA in the presence of plant extracts, and to determine whether an enzyme analogous to the UDP-glucuronyl transferase in certain mammalian tissues[3] was present.

All the experiments were carried out with radioactive UDP-sugars labelled uniformly with ^{14}C in the terminal sugar moiety and of identical specific activity (37 μC/μmole)[4]. The techniques of incubation in sealed capillary tubes, electrophoresis of the reaction products on strips of acid-washed Whatman No. 1 filter paper, and location of radioactive spots by autoradiography were those of HASSID *et al.*[5]. Reaction products after elution were generally identified by coelectrophoresis, and in specific cases by cochromatography on paper, with authentic compounds.

Soluble enzyme preparations were prepared from French beans (*Phaseolus vulgaris*, variety unspecified), the young greenhouse plants being gathered just prior to flowering*. The washed leaves were homogenized in 0.01 *M* phosphate buffer, pH 7, containing 0.01 *M* mercaptoethanol. After being centrifuged at 20,000 × *g* for 30 min, the fraction of the supernatant which precipitated between 30–70% ammonium sulphate saturation was redissolved in the minimum quantity of 0.1 *M* tris(hydroxymethyl)aminomethane buffer, pH 7.0, containing 0.1 *M* mercaptoethanol and dialysed against the same buffer for 4 h. All operations were carried out at 0°, and such preparations contained about 30 mg protein/ml[6].

[^{14}C]UDPGA ($1.6 \cdot 10^{-3}$ μmoles, 5000 counts/min) was incubated with the enzyme solution (20 μl) in a total volume of 40 μl containing 0.005 *M* $MgCl_2$ for 2 h at 37°, and the products separated by electrophoresis at pH 3.6 in 0.1 *M* ammonium formate buffer. There was complete disappearance of UDPGA, with formation of GA, GA-1-P, xylose and arabinose. Inclusion of UTP (0.5 μmole) in the incubation mixture decreased the rate of UDPGA decomposition, 25% being retained; UDP or ATP had similar but less pronounced effects. Probably UDPGA is destroyed by nucleotide pyrophosphatases, and other nucleotides protect by competition. However, UTP may have an additional effect, for when [^{14}C]GA-1-P ($0.8 \cdot 10^{-3}$ μmole) was substituted for UDPGA in the system containing UTP together with addition of a conc. inorganic pyrophosphatase preparation, after 4 h incubation at 37° UDPGA was synthesised in 60% yield. Hence it appears that a UDPGA pyrophosphorylase is present in the bean preparation, causing reformation of UDPGA in the experiments above; this enzyme has previously been reported in plants[7].

Abbreviations: UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate; UDPGA, uridine diphosphate glucuronic acid; UDPGalA, uridine diphosphate galacturonic acid; GA, glucuronic acid; GA-1-P, α-glucuronic acid 1-phosphate.

* Supplied by the Department of Botany, University of California (Davis), to whom the author is indebted.

In some experiments where UTP was included, further radioactive spots of mobility 1.0–1.2 with respect to picrate were noted after electrophoresis. These were eluted and tentatively identified as UDP-pentose and pentose 1-phosphate. Hydrolysis of these compounds with 1 *N* HCl at 100° for 30 min yielded a mixture of xylose and arabinose, but the parent UDP-pentoses could not be separated by paper chromatography.

Quercetin (10 μl satd. solution, pH 7.0, containing 0.1 μmole) was added to the mixture containing enzyme, [^{14}C]UDPGA, Mg^{++} and UTP and the period of incubation increased to 4 h. On electrophoresis, 42 % of the total radioactivity had zero mobility, and was partly adsorbed on to or incorporated into insoluble material. Elution of this with water removed only pentose (7 % of the original radioactivity); elution of the residue with 0.05 *M* ammonium carbonate, pH 9.0, at 0° for 6 h removed 8 % of the original radioactivity. This latter fraction was then found to be identical with authentic quercetin β-D-glucosiduronic acid[2] in electrophoretic mobility at pH 5.8 (mobility, 0.28) and pH 9.0 (mobility, 0.71) and by ascending chromatography in *n*-butanol–acetic acid–water (4:1:5) (R_F, 0.66). It was completely hydrolysed by a partially purified β-glucuronidase preparation from female-rat preputial gland[8] whereas in the presence of 0.001 *M* boiled D-glucosaccharic acid, a specific inhibitor of β-glucuronidase[9], 85 % of the radioactivity retained its original electrophoretic mobility. It thus seems probable that there had been transfer of glucuronic acid to quercetin to form a β-glucuronide identical with that previously isolated from the French bean[2]. The same compound was obtained when [^{14}C]GA-1-P together with UTP, but not with ATP or alone, was substituted for UDPGA. The nature of the residual non-mobile and insoluble radioactive material, in incubates containing quercetin, is unknown, but a water-insoluble product containing xylose and presumably lipid material has been reported in transfer reactions involving UDP-xylose[10].

The UDP-glucuronyl transferase system in mammalian tissues has wide acceptor specificity, but when phenol, *l*-menthol, benzoic acid, GA, GA-1-P, D-xylose or D-xylose 1-phosphate were substituted for quercetin in the above experiments, no evidence of the transfer of glucuronic acid could be obtained; YAMAHA AND CARDINI[11,12] were unable to demonstrate transfer of GA from UDPGA to hydroquinone or phenyl β-glucoside, using a wheat-germ extract.

When [^{14}C]UDPGalA was incubated with the bean-enzyme preparation in the presence of Mg^{++}, galacturonic acid was the sole reaction product. When UTP was included, galacturonic acid 1-phosphate (90 % yield) was produced after 3 h incubation at 37°; the further addition of quercetin had no effect and gave no evidence of glucuronic acid conjugation with the flavonol. As pentoses were not formed in these experiments, it appeared that no UDPGalA 4-epimerase or UDPGalA decarboxylase was present. The occurrence of arabinose as a decomposition product of UDPGA thus probably arises from the formation of UDP-arabinose by action of a UDP-pentose 4-epimerase on UDP-xylose which results from UDPGA decarboxylation[13].

It is of interest that, despite the existence of the five free hydroxyl groups in the quercetin molecule, only the mono-β-D-glucuronide appears to be formed; in other compounds of this type the C-7 position of the flavone nucleus seems to be the site of conjugation[14].

The author is greatly indebted to Drs. W. Z. HASSID, E. F. NEUFELD and

D. S. FEINGOLD for their advice and encouragement, and for the supply of radioactive nucleotide sugars. This investigation was carried out during the tenure of a W.K. Kellogg, Foundation Fellowship, and was supported in part by a research grant (No. A-1418) from the U.S. Public Health Service, National Institutes of Health, and by a research contract with the U.S. Atomic Energy Commission.

Department of Biochemistry, University of California, Berkeley, Calif. (U.S.A.) C. A. MARSH*

1 W. Z. HASSID, E. F. NEUFELD AND D. S. FEINGOLD, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 905.
2 C. A. MARSH, *Nature*, 176 (1955) 176.
3 G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 57 (1954) 275.
4 E. F. NEUFELD, D. S. FEINGOLD AND W. Z. HASSID, *J. Am. Chem. Soc.*, 80 (1958) 4430.
5 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 233 (1958) 783.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *Arch. Biochem. Biophys.*, 78 (1958) 401.
8 G. A. LEVVY, A. MCALLAN AND C. A. MARSH, *Biochem. J.*, 69 (1958) 22.
9 G. A. LEVVY, *Biochem. J.*, 52 (1952) 464.
10 D. S. FEINFOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 234 (1959) 488.
11 T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 127.
12 T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 133.
13 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 235 (1960) 910.
14 K. SHIBATA AND S. HATTORI, *Acta Phytochim. (Japan)*, 5 (1930) 117.

* Present address: Rowett Research Institute, Bucksburn, Aberdeen (Scotland).

Received June 16th, 1960

Biochim. Biophys. Acta, 44 (1960) 359–361

The binding of Fe^{+++} to native and chemically modified human serum albumin in the presence of sodium citrate

CAMON[1] studied the binding of Fe^{+++} to bovine serum albumin over the pH range 1.5 to 3.5. His ultrafiltration experiments showed that a maximum binding of 38 atoms/mole occurred at pH 3.5 in a medium with a free-Fe^{+++} concentration of $1.7 \cdot 10^{-3}$ *M*. Ferric hydroxide precipitation at a more alkaline pH effectively limited his study. WARNER AND WEBER[2] studied Fe^{+++} binding to conalbumin in the pH range 3 to 11 and overcame precipitation by complexing the iron with citrate. They used citrate in their dialysis systems but did not find it as part of the conalbumin–iron complex. For purposes of calculation, however, they assumed a citrate–iron complex of composition 1:1 as their source of iron. BOBTELSKY AND GOLDSCHMIDT[3] studied simple complex formation between Fe^{+++} and citrate ions. Their results suggested that in the pH range 3 to 5 the complex had the composition 2 Fe^{+++}: 3 citrate ions with a net negative charge of three.

We have studied Fe^{+++} binding at near physiological pH. In view of the evidence quoted we have attempted to measure both the iron and citrate of the protein complex. Our experimental method was equilibrium dialysis at room temperature. The pH range used was 3.0 to 7.3 and the Fe^{+++} concentration was $1.8 \cdot 10^{-4}$ *M*. The buffer systems used were: pH 3.0 to 3.8, 0.05 *M* potassium hydrogen phthalate–HCl; pH 3.8 to 5.8, 0.05 *M* sodium acetate–acetic acid; pH 5.7 to 7.3, 0.01 *M* sodium cacodylate.