BIOSYNTHESIS OF POLYPRENOL PHOSPHATE SUGARS BY *CERATITIS CAPITATA* EXTRACTS

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1. Introduction

Polyprenol-phosphate-sugars have been found to occur in bacteria [1], fungi [2], plants [3] and vertebrate tissues [3–5].

In a previous paper we described a lipid phosphate which we called IGAL (insect glycosyl acceptor lipid) extracted from insect tissues [6]. This compound, in the presence of rat liver microsomes, accepts sugars from sugar nucleotides. It behaves as a prenol-phosphate with an α-saturated isoprene unit [6]. This makes it similar to rat liver DolMP* [4]. We now want to describe an insect enzyme which catalyzes similar reactions. UDP-Glc, GDP-Man and UDP-GlcNac were used as sugar donors and IGAL and DolMP as sugar acceptors.

2. Materials and methods

The extraction of IGAL has been described previously [6]. Liver DolMP was a gift from Dr N. H. Behrens and Dr E. Tábora. FMP and FMP [^{14}C]Gal were a gift from Dr P. Romero. Fruit fly *Ceratitis capitata* pupae were kindly provided by Dr A. Turica of the Instituto Nacional de Tecnología Agropecuaria, Argentina.

2.1. *Enzyme preparation*

Pupae of *Ceratitis capitata* (100 g) were rinsed with water and dried between filter papers. They were then homogenized in a Sorvall Omnimixer for 40 sec at maximum speed in 50 ml of: 40 mM Tris-HCl buffer (pH 7.5), 4 mM $MgCl_2$, 1 mM EDTA-Na, 4 mM 2-mercaptoethanol and 0.25 M sucrose. The resulting extract was centrifuged for 10 min at 2000 *g* and the pellet was again homogenized and centrifuged as previously. Both supernatants were pooled and centrifuged for 20 min at 25 000 *g*. The resulting supernatant fraction was centrifuged for 90 min at 140 000 *g*. The resulting pellet, 'insect microsomes' [7], was resuspended in the above-mentioned homogenizing buffer. The protein concentration was 180 mg/ml as measured by the method of Lowry et al. [8]. (Dilutions were made with homogenizing buffer when needed.)

Abbreviations: IGAL, insect glycosyl acceptor; DolMP, dolichol monophosphate; FMP, ficaprenol monophosphate.

2.2. *Enzyme assay*

The standard incubation mixture contained the following reagents in a final volume of 50 μl: 0.1 M Tris-HCl buffer at pH 7.8, 0.1 M 2-mercaptoethanol, 0.25% Triton X-100 and either 6 μM UDP [^{14}C]Glc (about 100 000 cpm), 8 μM UDP [^{14}C]Gal (about 100 000 cpm), 9 μM GDP [^{14}C]Man (about 75 000 cpm) or 40 μM UDP [^{14}C]GlcNAc (about 10^5 cpm). Triton X-100 was replaced by 0.5% deoxycholate when UDP-GlcNAc was the substrate. The solutions were added to a sample of IGAL or DolMP which had been dried in vacuo together with 0.5 μmol EDTA-Na and 1.5 μmol $MgCl_2$. The mixture was shaken and 0.5 mg of enzyme was added. The incubation was carried out at 25°C for 20 min. The reaction mixture was partitioned and counted as described by Behrens et al. [9].

2.3. *Chromatography and electrophoresis*

Paper chromatography was carried out on Schleicher & Schüll 2043 paper. Solvent A was

2-propanol : acetic acid : water (27 : 4 : 9). Solvent B was *n*-butanol : pyridine : water (6 : 4 : 3). Thin layer chromatography on silica gel G plates was carried out in solvent C: chloroform : 2-propanol : 96% ethanol : 1 N acetic acid (2 : 2 : 3 : 1). Paper electrophoresis was performed using 70 mM acetic acid: pyridine buffer, pH 6.5, at 25 V/cm for 90 min.

3. Results

3.1. *Glycosyl transfer reactions*

As shown in table 1 the insect enzyme catalyzes the transfer of glucose, mannose and *N*-acetylglucosamine to a chloroform–methanol soluble material. The transfer is greatly stimulated by the addition of IGAL or DolMP. There is a very low level of transfer of galactose which was not investigated further. Magnesium ions are required, and excess of Na-EDTA inhibits the reactions. In the case of glucose transfer, a low level of transfer remained.

Fig.1 shows how increasing concentrations of added exogenous acceptor lipids stimulate transfer. For reasons that are not clear, deoxycholate was more efficient than Triton X-100 for *N*-acetylglucosamine transfer. The optimum detergent concentration varied between 0.05 and 0.5% and depended on the enzyme preparation used.

Optimal transfer temperature was found to be 25°C. Maximal activity with Tris-HCl buffer was found at pH 7.7 (data not shown).

Similar transfer activities were found in enzyme preparations of *Triatoma infestans* and *Periplaneta americana*.

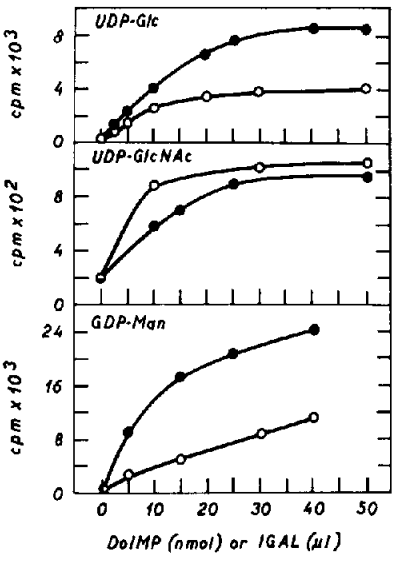

Fig.1. Transfer of glycosyl moiety from sugar-nucleotides to added acceptor lipids. The standard conditions of 'Enzyme assay' were used except for the amount of acceptor lipid. One µl of IGAL added corresponds to 2 g of insect. (○) IGAL; (●) DolMP.

3.2. *Properties of the lipid–phosphate sugar formed*

3.2.1. DEAE- cellulose chromatography

Samples of glycosylated IGAL obtained from incubations such as those described in table 1 were submitted to DEAE–cellulose chromatography. A

Table 1
Incorporation of radioactivity into lipid fraction from labelled sugar nucleotides

Additions	Sugar nucleotide			
	UDP-Glc	GDP-Man	UDP-GlcNAc	UDP-Gal
	(cpm incorporated)			
None	576	798	297	49
IGAL[a]	4849	4906	1900	167
DolMP[b]	8270	9436	1794	192
DolMP[b], minus Mg^{2+}, plus EDTA, 20 mM	538	156	99	48

[a]IGAL extracted from 40 mg of living insect was added to each tube.
[b]DolMP containing 24 nmol of phosphate.
Incubations were performed as described under 'Enzyme assay'.

column of 1.2 X 40 cm equilibrated with 99% methanol was used, as described by García et al. [10]. Most of radioactivity remained bound to the column. In a linear ammonium acetate gradient, IGAL-Glc and IGAL-Man were eluted with 50 mM acetate, while IGAL-GlcNAc required 100 mM acetate. Similar results were obtained with the corresponding DolMP derivatives.

Chromatography was also carried out in chloroform : methanol : water (10 : 10 : 3) as solvent in 0.6 X 6.0 cm columns. Under these conditions DolMP derivatives are not retained by the DEAE–cellulose while the diphosphate derivatives are [9]. Accordingly IGAL-Glc and IGAL-Man were not retained whereas IGAL-GlcNAc eluted only when 5 mM ammonium formate in chloroform : methanol : water (10 : 10 : 3) was passed through the column.

3.2.2. Acid hydrolysis

Treatment of IGAL-Glc, IGAL-Man and IGAL-GlcNAc for 20 min at 100°C in 0.01 N aqueous HCl resulted in 82, 85 and 75% hydrolysis, respectively. The water-soluble compounds liberated by this treatment were chromatographed in solvent A. The radioactive peaks appeared in the same place as spots of internal standards of the corresponding sugars. Chromatography on 0.2 M $Na_2B_4O_7$ impregnated paper with solvent B was carried out to distinguish between liberated GlcNAc and GalNAc [11].

3.2.3. Alkaline hydrolysis

The IGAL-derivatives of Glc, Man and GlcNAc are stable to a treatment with 0.1 N NaOH in chloroform : methanol : water (6 : 4 : 1) for 10 min at 37°C. However they are partially hydrolyzed by treatment in 0.1 N NaOH in n-propanol : water (1 : 1) for 90 min at 68°C. The compounds prepared with liver microsomes behaved in a similar manner [6].

Alkaline treatment of IGAL-Glc liberated a water-soluble substance which migrated like 1,6-anhydro-glucosan as shown in fig.2A. This product is obtained in a similar way from DolMP-Glc, where the glucose is bound as the β-anomer [12]. Hydrolysis of this alkaline product with 0.5 N H_2SO_4 gave free glucose (fig.2B).

Alkaline treatment of IGAL-GlcNAc gave two negatively charged substances. This is the same result as that obtained with the IGAL-GlcNAc synthesized by liver microsomes [6].

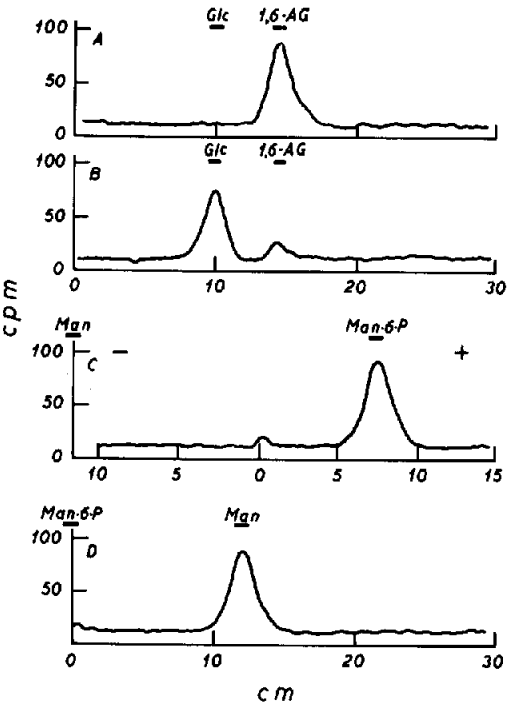

Fig.2. Alkaline treatment of glycolipids. The conditions are described in the text. (A) Neutralized alkaline-treated IGAL [^{14}C]Glc submitted to paper chromatography in solvent B. (B) Radioactive zone from A was eluted from the paper, treated with 0.05 N H_2SO_4 at 100°C for 2 h, neutralized and chromatographed again. (C) Neutralized alkaline-treated IGAL [^{14}C]Man was submitted to paper electrophoresis. (D) Radioactive zone detected by scanning the preceding electropherogram was eluted from the paper and incubated with alkaline phosphatase. The incubation mixture was submitted to paper chromatography in solvent B. 1,6-AG: 1,6-anhydro-glucosan.

Alkaline treatment of IGAL-Man gave one negatively charged peak which migrated on paper electrophoresis like mannose 6-phosphate (fig.2C). The compound was eluted from the paper and remains unchanged when treated with 0.1 N HCl for 7 min at 100°C. This latter treatment would have hydrolyzed the phosphate had it been bound to C_1. However alkaline phosphatase breaks the compound down to free mannose as is shown in fig.2D. The relative acid stability of the mannose phosphate could be explained if the liberated compound was mannose 2-phosphate.

Table 2
Phenol treatment of glycosylated lipids

	Before treatment	After treatment	
	(cpm)	Aqueous layer (cpm)	Phenol layer (cpm)
Endogenous mannosylated lipid	1298	69	1204
IGAL-Glc	1877	186	1754
IGAL-Man	2402	192	1920
DolMP-Glc	3190	210	3059
DolMP-Glc (liver)	3000	154	2877
FMP-Gal (*Acetobacter xylinum*)	2473	2221	370

Samples of glycosylated lipids labelled in the sugar moiety were heated in phenol for 3 h as described by García et al. [10] and the distribution of the radioactivity between aqueous and phenol phases was measured. DolMP-Glc (liver) refers to the compound obtained with rat liver microsomes.

This sugar is much more stable to acid treatment than mannose 1-phosphate [13]. Mannose 2-phosphate would have originated from the cyclic diester mannose 1,2-diphosphate; this degradation pattern is to be expected if IGAL-Man is the β-amoner [14].

3.2.4. Hydrolysis with phenol

Allylic prenol-phosphate-sugar derivatives are hydrolyzed by phenol treatment [10]. Table 2 shows the results obtained with IGAL, dolichol and ficaprenol derivatives. Only those of the latter were broken down by phenol treatment. This seems to indicate that the derivatives of IGAL formed by insect enzymes have a saturated isoprene unit.

3.2.5. Thin-layer chromatography

Silica gel G thin-layer chromatography with solvent C was carried out as described [6]. IGAL-Glc and IGAL-Man chromatographed in the same position as the corresponding DolMP derivatives prepared either with liver or insect enzymes. They migrated (R_{Glc} = 1.84–2.0) somewhat faster than FMP-Man prepared with insect microsomes and FMP-Gal prepared with an *Acetobacter* enzyme (R_{Glc} = 1.50–1.60).

The glucose- and mannose-lipids formed with endogenous acceptor behave like the IGAL or DolMP derivatives (R_{Glc} = 1.90).

4. Discussion

A particulate enzyme preparation from insect transferred glucose, mannose and *N*-acetylglucosamine to an endogenous lipid acceptor. In the presence of a partially purified lipid extract from the insect, sugar transfers are stimulated. Similar stimulations were observed when liver DolMP was added to the insect enzyme. This allowed us to prepare larger amounts of the labelled sugar derivatives for their identification. Their behaviour was similar to that of the compounds obtained with liver microsomes [6], as shown by the various tests to which they were submitted.

The presence in insects of compounds of the polyprenol-phosphate-sugar type and, as has been shown here, of the necessary enzymes for their synthesis, support the hypothesis that this system plays a role in insect glycoprotein synthesis. This has been shown to be the case in vertebrate tissues [5]. We will now try to confirm this hypothesis.

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