Studies on Uridine-Diphosphate-Glucose

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A previous paper (Caputto, Leloir, Cardini & Paladini, 1950) reported the isolation of the coenzyme of the galactose-1-phosphate→glucose-1-phosphate transformation, and presented a tentative structure for the substance. This paper deals with: (*a*) studies by paper chromatography of purified preparations of uridine-diphosphate-glucose (UDPG); (*b*) the identification of uridine-5′-phosphate as a product of hydrolysis; (*c*) studies on the alkaline degradation of UDPG, and (*d*) a substance similar to UDPG which will be referred to as UDPX.

UDPG preparations studied by chromatography. Paper chromatography with appropriate solvents has shown that some of the purest preparations of UDPG which had been obtained previously contain two other compounds, uridinemonophosphate (UMP) and a substance which appears to have the same constitution as UDPG except that it contains an unidentified component instead of glucose. This substance will be provisionally referred to as UDPX (Fig. 1*a*).

The three components have been tested for coenzymic activity in the galactose-1-phosphate→glucose-1-phosphate transformation, and it has been confirmed that UDPG is the active substance. For each mole of uridine of UDPG in a sample extracted from the paper the total phosphate was 2·04, the labile phosphate (15 min. in N-acid at 100°) 1·04, and the reducing power (calc. as glucose) after hydrolysis (10 min. in 0·01 N-acid at 100°) 1·03 moles.

When UDPG is hydrolysed at pH 2 during 10 min. at 100° glucose is liberated and, as shown in Fig. 1*b*, the UDPG and UDPX peaks are replaced by a slow-moving component which is uridinediphosphate.

Fig. 1*c* shows the results obtained after inactivating UDPG with alkali. Besides uridine phosphate a fast- and/or a slow-moving sugar ester are formed.

Identification of uridine-5′-phosphate. The product obtained by hydrolysing off with acid the glucose and one phosphate group from UDPG was previously (Caputto *et al.* 1950) postulated to be uridine-5′-monophosphate. However, the hydrolysis curves of this compound resembled more those given by Gulland & Smith (1947) for uridine-2′-phosphate. Since then Brown, Haynes & Todd (1950) have found that the substance supposed to be uridine-2′-phosphate was uridine-5′-phosphate. The hydrolysis product of UDPG has now been compared with a synthetic specimen of uridine-5′-phosphate. Both substances were found to be identical as judged by chromatographic behaviour (Fig. 1) and by the rate

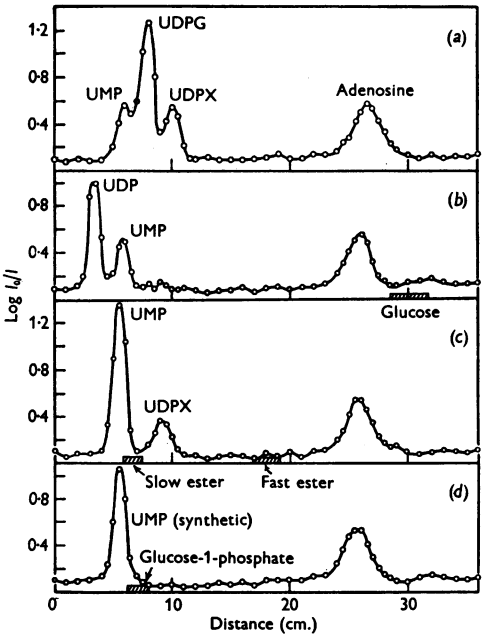

Fig. 1. Chromatograms of UDPG preparations. Samples run simultaneously at 30°. Solvent: ethanol-M-ammonium acetate, pH 7·5. Adenosine was added as reference substance. The log I_0/I values were measured at 260 mμ. *a*, partially purified UDPG; *b*, same after heating 15 min. at 100° at pH 2; *c*, heated 5 min. at 100° with excess NH_4OH; *d*, synthetic uridine-5′-phosphate plus glucose-1-phosphate. Glucose and its esters were located after removing the paraffin by ether extraction followed by spraying with aniline phthalate.

of acid hydrolysis (Table 1). The crystalline barium salts of the two substances were prepared, and after recrystallization from water it was found that the microscopic aspect of both samples was the same. The X-ray diffraction patterns obtained by Prof. Galloni were identical for both samples.

Table 1. *Acid hydrolysis of uridine phosphates*

(Samples heated at 100° in 0·1 N-H_2SO_4.)

Time (hr.)	P hydrolysed (%) Synthetic uridine-5′-phosphate	UMP from UDPG by acid hydrolysis	UMP from UDPG by alkaline hydrolysis
8·2	12·5	13·7	13·7
20·4	26·5	28·2	29·2
36·5	44·4	46·0	43·9
59·5	57·0	59·7	58·7

The alkaline degradation of uridine-diphosphate-glucose. It has been reported previously (Caputto *et al.* 1950) that UDPG loses its catalytic activity after a mild treatment with alkali. It was found that this inactivation was accompanied by a stabilization of the glucose residue and by the liberation of a secondary acid group of phosphoric acid. Further work on this point has shown that mild alkaline treatment of UDPG leads to the formation of UMP and a glucose ester in which the phosphate is doubly esterified. This substance ('Fast Ester') moves faster than any of the known glucose esters during paper chromatography. With a more drastic alkaline treatment or with acid the 'Fast Ester' is transformed into another substance or substances which move more slowly. These are referred to as 'Slow Ester(s)'.

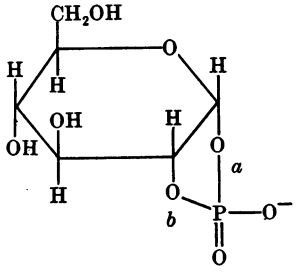

Fig. 2.

The experiments which will be described can be interpreted by assigning to the 'Fast Ester' the structure of a 1:2-monophosphoric ester of glucose (Fig. 2). Further treatment with alkali would yield a mixture of glucose-2- and glucose-1-phosphate by hydrolysis of the links marked *a* and *b* respectively. Treatment with acid would yield the same products, but since glucose-1-phosphate is hydrolysed immediately only glucose-2-phosphate would remain. Thus the 'Slow Ester' prepared with alkali should be a mixture of glucose-1- and glucose-2-phosphates, while that prepared with acid should be glucose-2-phosphate.

The exact conditions under which UDPG is degraded with alkali have not been determined. It is decomposed rapidly during chromatography with the ethanol-ammonia solvent. Under these conditions the formation of the 'Fast Ester' apparently occurs in less than 20 min., since the latter appears as a well defined spot with practically no tailing. At pH 8 at 18° UDPG remained unchanged during 18 hr. At pH 8·5 in 2 min. at 100° a mixture of UMP, and 'Fast' and 'Slow' ester was formed. In concentrated ammonia at 0° during 30 min. UMP and 'Fast Ester' were formed.

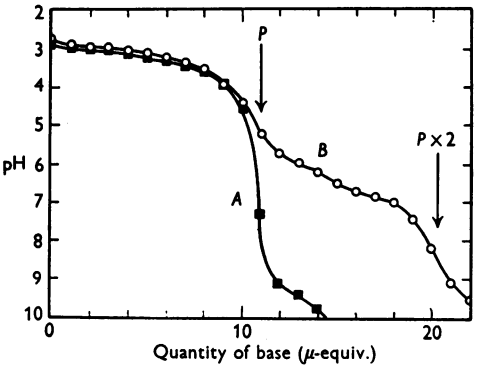

Fig. 3. Titration curve of the 'Fast Ester'. The substance was passed through a column of cation exchange resin in the hydrogen form. One sample was titrated directly (curve *A*) and another sample (curve *B*) was heated 15 min. to 100° before titration. After this treatment 10% of inorganic phosphate and 10% of the glucose were liberated. The arrow marked *P* shows the μmoles of phosphate in the sample.

Properties of the 'Fast Ester'. Table 2 shows the R_F values of the 'Fast Ester' compared with glucose and glucose-1-phosphate. With the solvents which were used the R_F values are grossly inversely proportional to the number of acid groups in the molecule: thus hexosediphosphates move slower than the monophosphates. For the 'Fast Ester' the values

Table 2. *Paper chromatography of the 'Fast' and 'Slow' esters*

(Whatman no. 1 paper.)

Solvent	R_F values 'Fast Ester'	'Slow Ester'	Glucose-1-phosphate	Glucose
Ethanol (77% v/v)	0·29	—	0·10	0·42
Ethanol ammonia	0·53	0·17	0·14	0·58
Ethanol ammonium acetate, pH 7·5	0·55	0·22	0·20	0·71

are nearly as high as those of free glucose. This fact was the first indication that the substance contains fewer acid groups than any of the known hexose-monophosphates. This was confirmed by electrometric titration (Fig. 3) which shows the presence of a primary but no secondary acid group. Acid hydrolysis of the 'Fast Ester' yielded a sugar which was identified as glucose by paper chromatography in several solvents. The same result was obtained after hydrolysis with alkaline phosphatase.

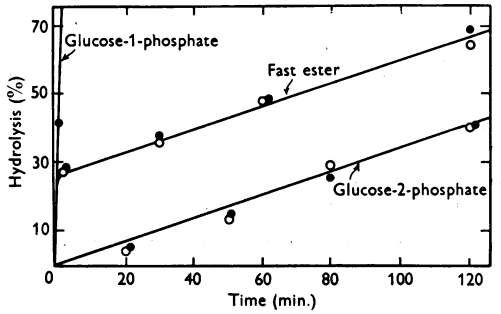

Fig. 4. Acid hydrolysis of the 'Fast Ester'. Samples heated at 100° in 0·1 N-H_2SO_4. Reducing power measured with Somogyi's (Somogyi, 1945) copper reagent followed by arsenomolybdic acid (Nelson, 1944). ●—●, phosphate; ○—○, reducing power as glucose.

The curve of hydrolysis of the 'Fast Ester' in 0·1 N-acid is shown in Fig. 4. The curve shows a break at about 26% hydrolysis as if it were the curve of a mixture of 26% glucose-1-phosphate and 74% glucose-2-phosphate. The curve of liberation of reducing power is parallel to that of phosphate liberation. With the reagent used (Somogyi, 1945) glucose-2-phosphate does not give a detectable reduction. On heating the 'Fast Ester' in 0·1 N-alkali (Fig. 5) 26% glucose-1-phosphate is formed in less than 5 min. and may be detected with the specific phosphoglucomutase test. The hydrolysis curve of the remaining 74% of organic phosphate is similar to that of glucose-2-phosphate.

Heating the 'Fast Ester' in dilute acid for a few minutes leads to the liberation of a secondary acid group of phosphoric acid (Fig. 3). The change can also be detected by paper chromatography, since the 'Fast Ester' is transformed into esters having about the same R_F as the normal hexosephosphates (Table 2).

Osazone formation from 'Slow Ester'. The phosphate liberated during osazone formation was estimated on a sample of 'Slow Ester' obtained from the 'Fast Ester' by heating at 100° for 5 min. in 0·1 N-acid. The inorganic phosphate formed by the acid treatment was measured and subtracted from the value obtained after phenylhydrazine treatment. For comparison glucose-1-phosphate and glucose-2-phosphate were also tested. The results were as follows (% liberation of P): glucose-1-phosphate, 0; glucose-2-phosphate, 94%; 'Slow Ester', 100%.

Liberation of phosphate during osazone formation would appear in theory to be specific for sugars with a free carbonyl containing phosphate in the 1 or 2 positions. However, it has been observed that glucose-3-phosphate (Raymond & Levene, 1929) and fructose-3-phosphate (Levene, Raymond & Walti, 1929) also lose phosphate during osazone formation.

The structure proposed in Fig. 2 for the 'Fast Ester' is consistent with experiments described in a previous paper (Leloir, 1951), in which a 'Fast

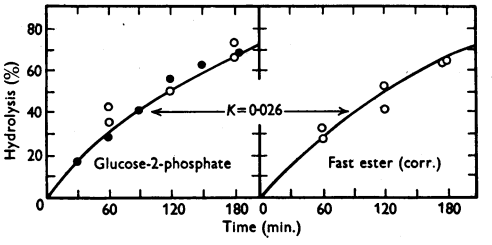

Fig. 5. Alkaline hydrolysis of the 'Fast Ester'. Inorganic phosphate was estimated after the samples (1·1 μM) were heated in 2 ml. of 0·1 N-NaOH to 100° in stoppered bronze tubes. In a parallel experiment glucose-1-phosphate was estimated with yeast phosphoglucomutase (Cardini, Paladini, Caputto, Leloir & Trucco, 1949) activated with glucose diphosphate. Glucose-1-phosphate standards were run at the same time. The samples of the 'Fast Ester' gave 26% of glucose-1-phosphate after heating 5 min. in 0·1 N-NaOH, and the values remained constant after heating 10 or 20 min. Glucose-1-phosphate was not affected by phosphoglucomutase. The corrected values for the percentage hydrolysis of the 'Fast Ester' were calculated by considering the total phosphate minus the glucose-1-phosphate as equal to 100. ●, Farrar's data; ○, this paper.

Ester' containing galactose was detected besides that containing glucose. Evidence for the α structure of glucose in UDPG has been obtained from preliminary polarimetric observations. It was found that an acid treatment which hydrolysed off the glucose produced a decrease in dextrorotation: $\Delta[M] = 183°$. This value is similar to that for the conversion of α-glucose-1-phosphate to αβ-glucose ($\Delta[M] = 218°$). For an α-glucose ester the likely positions for the formation of a cyclic phosphate would be 1:2 or 1:4. But since 1:4 would be very unlikely for an α-galactose ester it was concluded that both the glucose and the galactose esters were probably esterified at the 1 and 2 positions.

It may be mentioned that Forrest & Todd (1950) have described the formation of a cyclic phosphate of riboflavin by alkaline treatment of flavin-adenine-dinucleotide. Periodate oxidation used 1 mole of oxidant and gave no formic acid so that it was concluded that the phosphate was esterified at positions 4 and 5 of the ribityl residue.

UDPX. The substance giving the small peak which runs faster than UDPG (Fig. 1*a*) has been isolated in small amounts by paper chromatography. Analysis showed that the ultraviolet spectrum at different pH values and after bromine treatment was that of uridine, and that for each mole of uridine in UDPX the total phosphate was 2·04, labile phosphate (15 min. in N-acid at 100°) 1·0, and reducing power after hydrolysis (10 min. in 0·01 N-acid at 100°, calc. as glucose) 0·5 mole.

UDPX was found to remain unaffected by a treatment with alkali sufficient to decompose UDPG (Fig. 1*c*).

The unknown component of UDPX has been studied by chromatography in various solvents, and it has been found to be different from the following substances: aldohexoses, pentoses, fructose, tagatose, sorbose, glucosamine, uronic acids, fucose, rhamnose, xylulose, ribulose, deoxyribose, adonose, erythrulose, 1- and 3-methyl fructose, 2- and 3-methyl glucose, glyceraldehyde and dihydroxyacetone.

UDPX was found to be clearly different from UDP galactose (Leloir, 1951) and from the compound found by Park & Johnson (1949) and Park (1950) in *Staphylococcus aureus*. The R_F values, both of the intact substances and of the sugars obtained by hydrolysis, were different.

The substance X was found to be unfermentable by baker's yeast and to give negative results in the following tests: resorcinol for ketoses (Roe, 1934); Elson & Morgan (1933) for amino sugars, orcinol for pentoses (Mejbaum, 1939), and the test for methyl pentoses (Nicolet & Shinn, 1941). With the aniline-phthalate reagent it gave a brownish-yellow colour which only appeared after prolonged heating.

EXPERIMENTAL

Methods. Analytical methods and preparations were as described in previous papers (Caputto *et al.* 1950). Glucose-2-phosphate was prepared from diphenyl 1:3:4:6-tetra-acetyl-β-D-glucose-2-phosphate (Farrar, 1949) kindly supplied by Mrs K. R. Farrar. A sample of synthetic uridine-5′-phosphate was obtained from Prof. A. R. Todd.

The 'Slow Ester' was usually prepared by paper chromatography of UDPG with ethanol-ammonia as solvent. The position of the substance was revealed in a small part of the paper with aniline phthalate and the ester was subsequently extracted with water.

Paper chromatography. Descending chromatography was usually employed, except when the experiments were carried out in a thermostat. In these cases a more compact chamber similar to that described by Block (1950) was used in which the solvent travels first upwards and then downwards.

Whatman paper no. 1 was employed throughout. For nucleotides and phosphoric esters it was found convenient to wash the paper with 2 N-acetic acid (Hanes & Isherwood, 1949). Usually a pad of blotting paper was stapled at the end of the strips, and the position of the substances was referred to appropriate substances such as glucose for sugars and adenosine for nucleotides. The solvents used were: (*a*) 7·5 vol. of 95% ethanol plus 3 vol. of M-ammonium acetate (pH ~ 7·5); (*b*) same as (*a*) but with M-ammonium acetate buffer of pH 3·8; and (*c*) 7·5 vol. of 95% ethanol plus 3 vol. of concentrated ammonia. With solvent (*a*) the nucleotides give values of $R_{adenosine}$ below 0·7, while the nucleosides give higher values (Table 3). The R_F values of nucleotides vary with the pH of the solvent (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) and with temperature. As shown in Fig. 6, the changes with temperature are not parallel for all the substances.

Table 3. $R_{adenosine}$ *values, at* 20 ± 1·5° *of some purines, pyrimidines and derivatives*

Substance	Solvent: Ethanol-ammonium acetate, pH 7·5	Solvent: Ethanol-ammonium acetate, pH 3·8
Thymine	—	1·25
Uracil	1·13	1·16
Uridine	1·13	1·16
Cytidine	—	1·10
Adenine	1·06	1·00
Hypoxanthine	—	1·00
Adenosine	1·00	1·00
Uridine-3′-phosphate	0·46	0·94
Guanosine	—	0·88
Cytidylic acid	0·34	0·85
Uridine-5′-phosphate	0·35	0·84
Adenosine-3′-phosphate	0·29	0·76
Adenosine-5′-phosphate	—	0·73
UDPX	0·55	0·73
UDPG	0·43	0·65
Guanylic acid	0·22	0·63
Uridine diphosphate	0·14	0·59
Diphosphopyridinenucleotide	0·21	0·32
Adenosinetriphosphate	0·07	—
Xanthine	0·0	0·0
Guanine	0·0	0·0

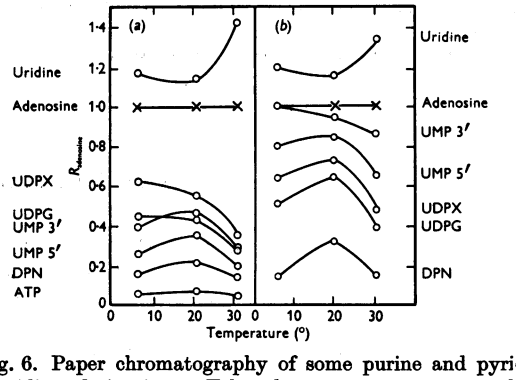

Fig. 6. Paper chromatography of some purine and pyrimidine derivatives. Ethanol-ammonium acetate solvents. *a*, of pH 7·5, and *b*, of pH 3·8, as described in text.

The position of ultraviolet-absorbing substances was ascertained by measuring the extinction at 260 mμ. after impregnation of the paper with liquid paraffin. A standard Beckmann spectrophotometer was used with an accessory

which allowed strips of paper to be run along the photocell entrance (Leloir & Paladini, 1951).

Sugars and their esters were revealed with aniline phthalate reagent (Partridge, 1949).

Liberation of phosphate with phenylhydrazine. This procedure was described by Deuticke & Hollmann (1939) for the estimation of fructosediphosphate. The analytical procedure has been modified by Dr Cardini as follows:

Reagents: (*a*) 6% (w/v) phenylhydrazine hydrochloride in water (decolorized with charcoal if necessary); (*b*) saturated solution of sodium acetate; (*c*) saturated Na_2SO_3.

The samples and phosphate standards in 0·5 ml. of water plus 0·1 ml. of (*a*), 0·05 ml. of (*b*) and 0·1 ml. of (*c*) were heated 30 min. in a boiling-water bath. After cooling 0·75 ml. of 5N-H_2SO_4, 0·75 ml. of 2·5% ammonium molybdate and water to a total vol. 7·5 ml. were added. After 10 min. the optical density was measured at 660 mμ. Controls heated without phenylhydrazine were run at the same time.

SUMMARY

1. Purified preparations of uridine-diphosphate-glucose (UDPG) were studied by paper chromatography and found to be contaminated with uridylic acid and a substance UDPX.

2. The uridylic acid obtained by degradation of UDPG has been identified as uridine-5′-phosphate.

3. The alkaline degradation products of UDPG are uridine-5′-phosphate and a cyclic phosphate ester of glucose, probably esterified at positions 1 and 2 of the glucose. This ester decomposes with acid or alkali giving glucose-1-phosphate (25%) and glucose-2-phosphate (75%).

4. The contaminating substance UDPX appears to have the same structure as UDPG except that it contains an unidentified component instead of glucose.

The studies with synthetic uridine-5′-phosphate and with many samples of rare sugars were possible owing to the kindness of Prof. A. R. Todd, F.R.S., and the identification of glucose-2-phosphate by the generosity of Mrs K. R. Farrar. We wish to express our thanks to them as well as to Prof. E. E. Galloni for the X-ray diffraction studies, to Dr C. E. Cardini for his co-operation with the phenylhydrazine method and to Dr J. T. Park for a sample of the *Staphylococcus aureus* compound.

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The Structure of Urorosein

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Urorosein is a red pigment obtained by the action of mineral acids on the tryptophan oxidation product indole-3-aldehyde (Ellinger & Flamand, 1909). Fearon & Boggust (1950) have reviewed earlier work on the pigment and assigned to it the structure dehydro-indolo-3′:2′:2:3-carbazole. This is the correct systematic name for the structure as shown by Fearon & Boggust and described by them as indolo-3′:2′:2:3-carbazole. It is, however, difficult to see how such a structure containing only a *p*-quinone-diimine chromophore could be so intensely coloured and, moreover, the absorption spectrum as quoted shows a close correspondence to those of the di-3-indolylmethenes described by König (1925). It