CXXXII. SYNTHETIC GALACTOSE-1-PHOSPHORIC ACID

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In previous papers [Kosterlitz, 1937; 1938] evidence was given which indicated the presence of galactose-1-phosphate in the livers of rabbits assimilating galactose. Attempts to isolate this ester were unsuccessful because its properties were not known in sufficient detail and the yields obtained were small. Therefore it was found necessary to prepare the synthetic ester before continuing the investigations on the naturally occurring ester.

It was impossible to obtain α- and β-tetraacetyl galactose-1-phosphoric acids by phosphorylation of the corresponding 2:3:4:6-tetraacetyl galactoses with $POCl_3$ in dry pyridine. Cori *et al.* [1937] reported a similar failure with glucose. These workers synthesized barium glucose-1-phosphate by the action of silver phosphate on acetobromoglucose in dry benzene, hydrolysis of tri-(tetraacetyl glucose)-1-phosphate in methyl alcohol containing 4% of aqueous 5*N* HCl for 16 hr. at 25° and neutralization of the acid with $Ba(OH)_2$.

By using this method it was found possible to synthesize galactose-1-phosphoric acid [Colowick, 1938; Kosterlitz, 1938] which is non-reducing and very readily hydrolysed by acid, as is the glucose ester. The preparations obtained by both authors, however, still contained impurities: only 92% (Kosterlitz) and 94·5% (Colowick) of the total P were easily hydrolysable and the specific rotations were +81·3° and +91° respectively. A possible explanation of the difference in the specific rotations of the two preparations will be offered in the experimental part.

The present paper deals mainly with the final stages of the purification of the ester. The method of preparing the crude Ba salt was essentially the same as that used by Cori *et al.* [1937] for the synthesis of glucose-1-phosphoric acid. Barium galactose-1-phosphate could not be completely purified by the repeated precipitation of its aqueous solution with ethyl alcohol. Repeated recrystallization of the dibrucine salt from 40 to 85% methyl alcohol increased the percentage of labile P from 86 to 96%. Crystallization as the dipotassium salt, either starting with the crude Ba salt or better with the dibrucine salt, yielded the pure product.[2]

The dipotassium salt is very soluble in water and only sparingly soluble in aqueous ethyl alcohol from which it crystallizes with $2H_2O$. This water of crystallization is easily given off *in vacuo* over P_2O_5 at 100° and less readily at room temperature. The anhydrous salt has $[\alpha]_D^{18°}$ +108·2° and the free acid +148·5°. That the dipotassium salt is homogeneous is shown by the fact that fractional crystallization effects no separation into fractions of different specific rotations. Barium galactose-1-phosphate prepared from the dipotassium salt, is readily soluble in water and insoluble in 50% ethyl alcohol; the anhydrous salt has $[\alpha]_D^{18°}$ +92·7°.

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[2] Dipotassium glucose-1-phosphate was described by Kiessling [1938].

Galactose-1-phosphoric acid is non-reducing. It is completely hydrolysed by 0·09 *N* H_2SO_4 at 100° in 2 min. The *k* of hydrolysis in 0·25 *N* HCl is $0·89 \times 10^{-3}$ at 25° and $5·9 \times 10^{-3}$ at 37°; it is therefore more rapidly hydrolysed than glucose-1-phosphoric acid [Cori *et al.* 1937]. The ester is completely resistant to hydrolysis in 0·2 *N* NaOH at 100° for 30 min. The hexose component can be identified as galactose by the fermentation and methylphenylhydrazone tests.

The method of preparation of the ester and its specific rotation strongly suggest that it is α-galactopyranose-1-phosphoric acid. Acetobromogalactose has a pyranose structure, and the molecular rotation of the ester is of the same order of magnitude as are the molecular rotations of the known α-galactosides of acyclic alcohols (Table I). All known and otherwise unsubstituted β-galactosides are laevorotatory. A similar relationship may be shown to exist between α-glucosides and the glucose-1-phosphoric acid of Cori *et al.* [1937].

Table I*

Substance	$[\alpha]_D$	$[M]$	Reference for $[\alpha]_D$
α-Methylgalactopyranoside	+196·6°	+38200	Riiber *et al.* [1929]
α-Ethyl galactoside	+186·8°	+38900	Pascu & Ticharich [1929]
α-Propyl galactoside	+179·0°	+39800	Bourquelot & Aubry [1916]
α-Allyl galactoside	+171·7°	+37800	Pascu & Ticharich [1929]
α-Ethyleneglycol monogalactoside	+169·9°	+38100	Bourquelot [1917]
α(?)-Galactose-1-phosphoric acid	+148·5°	+38600	Present synthesis

* The two known α-galactosides of cyclic alcohols have molecular rotations which are higher than those of the acyclic alcohols, viz. phenyl-α-galactoside 55300 and *o*-cresyl-α-galactoside 51000.

Experimental

Tri-(tetraacetyl galactose)-1-phosphate

A solution of 15·5 g. acetobromogalactose [Fischer & Fischer, 1910] in 75 ml. dry benzene was refluxed with 7·5 g. freshly prepared silver phosphate, a calcium chloride tube being attached to the condenser. The reaction was complete in 1 hr. The filtrate from the silver salts was boiled with charcoal and left overnight over $CaCl_2$ at 0° to remove the last traces of colloidal silver salts. The filtered solution was then evaporated under reduced pressure at 30° until a thick syrup was obtained. Owing to excessive frothing the final evaporation had to be done in a desiccator. The product (yield 11·5 g. = 87·3 % of theoretical) consisted of an easily pulverizable glass, which could not be crystallized. (Found: C, 44·88, 44·65; H, 5·48, 5·37; CH_3CO, 45·2, 45·6; P, 2·60, 2·58 %. $C_{42}H_{57}O_{31}P$ requires C, 46·29; H, 5·28; CH_3CO, 47·4; P, 2·85 %.) P was determined by Lohmann & Jendrassik's [1926] modification of the method of Fiske & Subbarow [1925]. The compound had $[\alpha]_D^{17°} = +119·9°$ ($c = 2·25$ in methyl alcohol).

It was possible to prepare this substance in greater quantities than those stated above. The yield was satisfactory, viz. 80–90 % when 60–140 g. of acetobromogalactose were used, but the product was less pure. This, however, did not interfere with the further steps of the synthesis.

Barium galactose-1-phosphate (crude product)

107 g. tri-(tetraacetyl galactose)-1-phosphate were dissolved in 2140 ml. methyl alcohol to which 88 ml. 5 *N* aqueous HCl had been added. The mixture was incubated for 8 hr. at 25° and, after cooling, brought to *p*H 8·4 with $Ba(OH)_2$ (phenolphthalein). After the precipitate had been allowed to settle overnight at 0°, it was centrifuged and extracted exhaustively with water. The Ba salt

was precipitated by adding to its solution an equal volume of alcohol. This process was repeated three times. Finally, the Ba salt was washed with 80% alcohol and dried *in vacuo* over P_2O_5 at room temperature. Yield 14·1 g. = 34·7% of theoretical. 85·6% of the total P was hydrolysed by 0·09*N* H_2SO_4 at 100° in 10 min. $[\alpha]_D$ (for anhydrous salt): +81·8°, $[\alpha]_D$ (calculated for labile P): +95·5°.

Dibrucine galactose-1-phosphate

12 g. Ba salt were dissolved in 30 ml. warm H_2O; after cooling, 58 ml. *N* H_2SO_4 were added, the $BaSO_4$ centrifuged off and 23 g. brucine in 30 ml. warm methyl alcohol added. The solution (120 ml.) was decolored with charcoal and, after addition of acetone (360 ml.), allowed to crystallize at 0°. Yield 26·2 g. = 86% of theoretical. A second crop of 1·6 g. was obtained from the mother liquor. The dibrucine salt was subjected to repeated recrystallizations from 40 to 83% methyl alcohol. The analytical values of the Ba salts prepared from the different fractions are given in Table II.

Table II

Procedure	Yield %	Labile P in % of total	$[\alpha]_D$ of anhydrous Ba salt	$[\alpha]_D$ calculated from labile P
Crude Ba salt	—	85·6	+81·8°	+ 95·5°
1st recrystallization, from 83% methyl alcohol:				
(*a*) 1st crop	68	91	+86·9°	+ 95·5°
(*b*) 2nd crop from mother liquor after concentration and addition of acetone	10	59·6	+59·8°	+100°
2nd recrystallization, from 83% methyl alcohol (slow)	85	92·2	+87·5°	+ 94·9°
3rd recrystallization, from 50% methyl alcohol (rapid)	87·5	96	+89·5°	+ 93·2°
4th recrystallization, from 40% methyl alcohol (rapid)	93·5	96·5	+90·4°	+ 93·6°
Pure Ba salt	—	100	+92·7°	+ 92·7°

From the values given in this table it may be concluded that the repeated recrystallizations of the dibrucine salts purified the crude product to a considerable degree. The last traces of the impurity, however, were not removed by this method. The specific rotation of the impurity was very small in this particular instance.

The low $[\alpha]_D$ of the Ba salt described earlier [Kosterlitz, 1938], viz. +81·3° of the anhydrous salt and +88° when calculated from labile P, is most probably explained by the presence of a laevorotatory impurity. In that instance, tri-(tetraacetyl galactose)-1-phosphate was hydrolysed by a method different from that employed in the present synthesis: it was refluxed for 2¼ hr. with dry methyl alcohol without addition of acid. The crude Ba salt (yield 41·5%) was partially purified by two crystallizations as the dibrucine salt. In Colowick's preparation [1938] the impurity appears to have been dextrorotatory. Acid hydrolysis liberated 94·5% of the theoretical quantity of galactose; the $[\alpha]_D$ of the anhydrous Ba salt was +91°, and +96·4° if calculated from labile galactose.

Dipotassium galactose-1-phosphate

To a solution of 11 g. dibrucine salt with 96·5% of the theoretical labile P in 55 ml. water, 10% KOH was added to *p*H 8·4 (about 12 ml.); the brucine was filtered off and washed. Ethyl alcohol was added to the combined filtrate and washings until the solution became just turbid. From the mixture, which was kept at 0° overnight, a considerable quantity of the dipotassium salt

crystallized out. The solution was again rendered just turbid with alcohol, this process being repeated at 2-hourly intervals until no further crystallization occurred. Yield 3·25 g. = 83 % of theoretical. For analysis, the substance was recrystallized twice from a 5 % aqueous solution by addition of alcohol. Dipotassium galactose-1-phosphate crystallizes with $2H_2O$, which are readily given up on drying *in vacuo* over P_2O_5 at 100° and less readily at room temperature. The anhydrous salt is hygroscopic. The dipotassium salt may also be prepared from the crude Ba salt by removal of the Ba with K_2SO_4, e.g. 1·5 g. crude Ba salt with 85·6 % labile P gave 0·58 dipotassium salt. Yield 50 % of theoretical. (Found for anhydrous salt: C, 22·06, 21·84; H, 3·45, 3·56; P (total), 9·16, 9·06; P (labile), 9·16, 9·12; P (inorganic: Lohmann [1928]), 0; galactose after hydrolysis, 53·4, 53·9 %. $C_6H_{11}O_9PK_2$ requires C, 21·41; H, 3·30; P (total = labile), 9·22; P (inorganic), 0; galactose, 53·5 %. Found for hydrated salt: P (total), 8·31, 8·32; P (labile), 8·30, 8·35; P (inorganic), 0; galactose, 48·9 %. $C_6H_{11}O_9PK_2, 2H_2O$ requires P (total = labile), 8·33; P (inorganic), 0; galactose, 48·4 %.) The anhydrous salt had $[\alpha]_D^{18°} + 108·0°$ and $[\alpha]_{5461}^{18°} + 127·5°$ ($c = 2·557$ in water); the hydrated salt had $[\alpha]_D^{17°} + 98·0°$ and $[\alpha]_{5461}^{18°} + 116·0°$ ($c = 2·375$ in water). The free acid (solution of the dipotassium salt in 0·2 *N* HCl) had $[\alpha]_D^{18°} + 148·5°$ ($c = 1·678$).

Fractional crystallization of the dipotassium salt. To a solution of 2·6 g. of this salt in 52 ml. water an equal quantity of ethyl alcohol was added and the mixture kept at 0° for 36 hr. Yield 1·24 g. A second crop of 0·96 g. was obtained after further additions of alcohol. $[\alpha]_D^{19°}$ of original salt + 97·7° ($c = 1·684$); of 1st crop + 97·5° ($c = 2·668$); of 2nd crop + 98·1° ($c = 2·754$).

Barium galactose-1-phosphate prepared from the dipotassium salt

0·8 g. barium acetate in 5 ml. H_2O was added to 0·8 g. dipotassium salt in 5 ml. H_2O. The barium galactose-*l*-phosphate was then precipitated by the addition of 10 ml. alcohol and a few drops of a saturated solution of $Ba(OH)_2$ in order to bring the *p*H to 8·4. The Ba salt was precipitated five times, washed with 80 % alcohol and dried *in vacuo*. Since this salt retained varying quantities of H_2O after drying *in vacuo* over $CaCl_2$ or P_2O_5 at room temperature, the following method was used for the determination of its specific rotation. The P and galactose liberated by acid hydrolysis were estimated and the

Table III

Method of drying and approximate H_2O content	Total P %	Labile P %	$[\alpha]_D$ of anhydrous salt calculated from P content	Galactose after hydrolysis %	$[\alpha]_D$ of anhydrous salt calculated from galactose content	$\frac{\text{Galactose}}{\text{P}}$	Ba [King, 1932] %
(1) Dried incompletely over P_2O_5; $2\frac{1}{2}H_2O$	6·99	6·95	+ 93·1° ($c = 2·22$)	41·5	+ 91·0°	5·95	—
(2) Redried over P_2O_5; $\frac{1}{2}H_2O$	7·55	7·55	+ 94·6° ($c = 2·53$)	44·9	+ 92·4°	5·95	—
(3) No. (2) was redissolved, precipitated again, and dried over $CaCl_2$; $1\frac{1}{2}H_2O$	7·40	7·37	+ 92·4° ($c = 2·34$)	42·9	+ 92·4°	5·82	31·6 31·7

Average rotation = $[\alpha]_D^{18°}$ (anhydrous Ba salt): + 92·7 ± 0·5°.

Average $\frac{\text{galactose}}{\text{P}}$: 5·91; calculated 5·81.

$\frac{\text{Ba}}{\text{P}}$: found 4·29; calculated 4·43.

measured specific rotations corrected by the factors $\frac{\text{P content of anhydrous salt}}{\text{P content found}}$ and $\frac{\text{galactose content of anhydrous salt}}{\text{galactose content found}}$ (Table III).

Hydrolysis of galactose-1-phosphoric acid

(1) *In 0·09*N H_2SO_4 *at 100°.* 21·91 mg. dipotassium galactose-1-phosphate were dissolved in 25 ml. H_2O with addition of 1·18 ml. 0·1*N* H_2SO_4 in order to liberate the free acid. To 20 ml. of this solution 2 ml. *N* H_2SO_4 were added. Test tubes containing about 5 ml. of the ester-H_2SO_4 mixture were immersed in a boiling water bath for 1, 2 and 3 min., and after cooling, galactose and inorganic P were determined; 1 ml. contained 0·796 mg. dipotassium salt with 0·0663 mg. P and 0·386 mg. galactose (Table IV).

Table IV

Time min.	P formed mg./ml.	Galactose formed mg./ml.
1	0·050	0·295
2	0·066	0·396
3	0·066	0·396

The ester was 76 % hydrolysed in 1 min. and completely hydrolysed in 2 min.

(2) *In 0·25*N *HCl at 25° and 37°.* Dipotassium galactose-1-phosphate was used for these experiments. The calculated amount of H_2SO_4 was added to liberate the free galactosephosphoric acid. Then equal quantities of ester solution and 0·5*N* HCl were mixed and incubated in a water bath. The galactose formed was estimated by the method of Hagedorn and Jensen. Since the reducing power of galactose is less than that of glucose, a calibration curve was used which was a straight line when the heating period was extended from 15 to 30 min. Care was taken to neutralize the HCl before the estimation. The inorganic P formed was estimated by the colorimetric method of Fiske & Subbarow [1925]. Since a measurable amount of ester was split by the H_2SO_4 present in the reagent, a correction had to be applied. The time which elapsed between the addition of the molybdic-sulphuric acids and the first reading was

Table V

Time (min.)	Hydrolysis at 37·0°. Concentration of ester 2·84 m.-molar. Galactose formed %	$k \times 10^3$	Hydrolysis at 25·0°. Concentration of ester 2·78 m.-molar. Galactose formed %	$k \times 10^3$
15	17·6	5·6	—	—
30	32·7	5·85	6·2	0·93
45	45·1	5·9	—	—
60	55·4	6·0	11·3	0·81
90	69·6	5·55	16·7	0·91
120	80·4	6·35	—	—
	Average	5·9		0·88

Hydrolysis at 25·0°. Concentration of ester 4·08 m.-molar.

Time min.	Galactose formed %	$k \times 10^3$	Inorganic P formed %	$k \times 10^3$
50	10·0	0·915	9·9	0·905
110	20·6	0·905	20·3	0·885
180	31·2	0·89	30·6	0·86
255	41·2	0·91	40·0	0·845
	Average	0·905		0·875

noted, and two further readings were taken at intervals of 5 min. From the three values the initial value was extrapolated. The k of hydrolysis was calculated from the equation:

$$k = \frac{1}{t_2 - t_1} \log \frac{(a - x_1)}{(a - x_2)} \text{ (Table V).}$$

(3) *In 0·2N NaOH at 100°.* 4 ml. of a 2·672 % solution of dipotassium galactose-1-phosphate and 1 ml. N NaOH in a sealed test tube were immersed in a boiling water bath for 30 min. After cooling, 1 ml. N H_2SO_4 was added and the rotation determined. $[\alpha]_D^{20°} = +3·49°$ ($l = 2$) calc. $+3·49°$. 1 ml. of this solution was diluted to 19·80 ml., and reducing power and inorganic P were estimated in the usual way before and after hydrolysis in 0·09N H_2SO_4 at 100° for 5 min. The quantities of galactose (0·0072 mg. in 2 ml.) and inorganic P (0·0006 mg. in 2 ml.) found before acid hydrolysis were negligible and within the ranges of error of the methods employed. After acid hydrolysis, the values found per ml. were 0·449 mg. galactose (calc. 0·435 mg.) and 0·0754 mg. P (calc. 0·075 mg.). Therefore, the ester was completely resistant to hydrolysis in 0·2N NaOH at 100° for 30 min.

Examination of the hexose liberated by acid hydrolysis

(1) *Identification of galactose as methylphenylhydrazone.* 47·7 mg. hydrated dipotassium galactose-1-phosphate were dissolved in 0·5 ml. water in a small centrifuge tube; 1·02 ml. N H_2SO_4 were added to liberate the free ester and make the solution 0·5N with regard to H_2SO_4. The ester was then hydrolysed for 10 min. at 100°. After neutralization with 1·02 ml. N NaOH, 0·05 ml. glacial acetic acid and 0·03 ml. methylphenylhydrazine were added and the mixture left overnight at 0°. The crystals of the hydrazone were centrifuged off, washed in the centrifuge tube with about 2 ml. ice-cold water and then recrystallized from about 2 ml. ethyl alcohol. The final product was washed with 2 ml. ice-cold alcohol. Yield 34·6 mg. galactose methylphenylhydrazone (95 % of theoretical). M.P. 189 (corr.) with decomposition; mixed M.P. 188. Votoček [1921] gave M.P. 190°.

(2) *Identification of galactose by fermentation analysis.* The method employed was described in an earlier paper [Kosterlitz, 1937]. For the analysis of the unhydrolysed ester a solution containing 0·874 mg. hydrated dipotassium galactose-1-phosphate with 0·423 mg. galactose per 2 ml. was used while for the ester hydrolysed by 0·09N H_2SO_4 at 100° for 10 min. a solution containing 0·728 mg. dipotassium salt with 0·352 mg. galactose per 2 ml. was employed (Table VI).

Table VI

Treatment	Reducing power of 2 ml. unhydrolysed ester in mg. galactose		Reducing power of 2 ml. hydrolysed ester in mg. galactose	
	Calc.	Found	Calc.	Found
Before fermentation	0	0	0·352	0·358
After fermentation with *S. Ludwigii* (not fermenting galactose)	0·423	0·420	0·352	0·351
After fermentation with galactose-adapted *S. cerevisiae* Frohberg	0	0	0	0

These experiments prove that the hexose component of the ester is galactose; the increase in reducing power of the unhydrolysed ester after treatment with *S. Ludwigii* is due to the liberation of galactose by phosphatase.

SUMMARY

The synthesis and properties of α(?)-galactose-1-phosphoric acid are described.

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