CCLXXII. THE PRESENCE OF A GALACTOSE-PHOSPHATE IN THE LIVERS OF RABBITS ASSIMILATING GALACTOSE

By HANS WALTER KOSTERLITZ[1]

From the Physiological Laboratory, Marischal College, University of Aberdeen

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In the present paper, an attempt is made to elucidate the mechanism by which galactose is transformed into glucose in the animal body. That such a transformation takes place has been established beyond doubt by observations on galactose assimilation (1) in the diabetic patient [Voit, 1892; Brasch, 1908; Roe & Schwartzmann, 1932; Kosterlitz & Wedler, 1933, 1], and (2) in animals with phlorhidzin and pancreatic diabetes [Sandmeyer, 1895; Brasch, 1908; Kosterlitz & Wedler, 1933, 2; Bollman & Mann, 1934]. Further, the liver glycogen formed by non-diabetic animals after galactose ingestion is composed of glucose units only [Cramer, 1902; Jewel, 1932; Harding *et al.* 1934; Bell, 1936]. There is not sufficient experimental evidence available to decide whether the transformation of galactose into glucose takes place directly or with glycogen as an intermediate.

With regard to the site of this transformation, the experiments of Draudt [1913] on the dog with an Eck's fistula, and particularly those of Bollman *et al.* [1935] on the hepatectomized dog, have proved that the liver is at least an important, if not the only organ in which galactose can be transformed into glucose.

Nothing is known about the intermediate stages of this process. Robinson [1927] brought forward a tentative suggestion that galactose may be phosphorylated in position 4, and that a Walden inversion may take place on subsequent hydrolysis of this ester. Oldham & Robertson [1935] succeeded in transforming glucose into galactose by chemical means. On alkaline hydrolysis and subsequent acetylation of 4-*p*-toluenesulphonyl-2:3-dibenzoyl-6-trityl-α-methylglucoside they obtained 2-acetyl-3:4-anhydro-6-trityl-α-methylhexoside which, treated with dry hydrogen chloride in acetone solution, yielded a mixture containing the monoacetone derivatives of α-methylgalactoside and α-methylguloside. In modification of Robinson's suggestion they postulated therefore that anhydro-formation may be a necessary precursor to a Walden inversion by which glucose is transformed into galactose. In a similar manner, Müller [1935] succeeded in transforming 3:4-anhydro-β-methylgalactoside into derivatives of glucose and gulose.

It would be of interest to know whether or not a galactosephosphate is formed during alcoholic fermentation of galactose. Nilsson [1930] was able to isolate from the products of fermentation of galactose by dried galactose-adapted yeast a hexosediphosphate which resembled that formed during fermentation of glucose, fructose and mannose. The hexosemonophosphate also isolated by him was not subjected to an analysis with regard to the hexose component.

[1] Carnegie Teaching Fellow.

Grant [1935] also investigated the phosphoric esters occurring during fermentation of galactose by adapted yeast preparations and was unable to detect a galactosephosphate. The synthetic galactose-6-phosphate was not fermentable.

That galactose may undergo phosphorylation during intestinal absorption was suggested by Laszt & Süllman [1935] and by Verzár & Süllman [1937] who found that such absorption was accompanied by an increase of organic phosphate in the mucous membrane of the intestine.

In the present research the acid-soluble organic phosphorus of the liver is examined with a view to determining whether or not a galactosephosphate occurs in this organ during assimilation of galactose.

Experimental

Methods

(1) *Isolation of the phosphoric esters.* Robison's method [1922] was followed with slight modifications. Rabbits, fasted for 24 hr. previous to the experiment, were fed with a solution of either sodium chloride, glucose or galactose by stomach tube and were killed 1–2 hr. later by stunning. The livers were excised as quickly as possible, passed through a cold mincer, weighed and extracted with 4·2 vol. of 6% trichloroacetic acid. After filtration, 96% alcohol was added to give a final concentration of 60%. After 1–2 hr. standing at 0°, the material was centrifuged and the supernatant fluid decanted from the glycogen and brought to *p*H 8·4 (phenolphthalein) with baryta. A precipitate containing the total phosphates was thus obtained. On extracting this precipitate with 10 times its weight of water, part of it dissolved and was again precipitated with alcohol. The water-insoluble part was dissolved in 0·25 *N* hydrochloric acid, the sulphate centrifuged off, and the resulting bile-stained fluid again brought to *p*H 8·4 and centrifuged; the process was repeated twice. In this way part of the insoluble fraction became soluble in water from which it was finally precipitated with alcohol. This precipitate was added to that obtained from the originally water-soluble fraction, and the combined precipitates were dried. The material was then dissolved in 50 times its weight of water to which was added sufficient dilute acetic acid to give a clear solution. This solution was treated with mercuric acetate and the precipitate filtered off and discarded. After removal of the excess of mercury in the usual way, the barium salt was again formed. The treatment with mercuric acetate was repeated, since nitrogen was still present in most instances. Finally, the barium salt was further purified by 6 successive precipitations with alcohol from water.

(2) *Estimation of the galactose and glucose contents of the phosphoric esters and of their hydrolysis products.* The phosphoric esters were prepared for analysis by dissolving 10–20 mg. of the barium salts in water and removing the barium by addition of an equivalent quantity of 0·02 *N* sulphuric acid; to ensure the complete removal of barium 1 drop of a saturated solution of sodium sulphate was added.

The hydrolysis products were obtained in the following manner: 10–20 mg. of the barium salts were dissolved in 2–3 ml. of water and the barium removed by 0·02 *N* sulphuric acid; sufficient water to bring the solution to a volume of 9 ml. and 1 ml. of *N* sulphuric acid were then added. The hydrolysis was carried out at 100° for 63 hr. (Exp. 5) and 69 hr. (Exps. 7 and 9). Baryta and 1½ vol. of alcohol were then added to remove sulphuric acid and inorganic and unhydrolysed organic phosphate. After centrifuging, the aqueous alcoholic solution of the free sugars was removed and evaporated under reduced pressure to a volume of

about 5 ml. This aqueous solution was neutralized with dilute sulphuric acid and, after being freed from barium by the addition of 2 drops of a saturated solution of sodium sulphate, was made up to 25 ml.

The reducing powers of the ester solution and the hydrolysis products were determined in quantities of 2 ml. by the Hagedorn-Jensen method, the period of boiling being extended to 30 min. The "fermentable hexoses" (glucose, fructose and mannose) were then removed from each by means of a pure strain of *S. Ludwigii* which had been subcultured on a yeast extract containing 6% of sucrose. To 10 ml. of ester and of the free sugar solution 1 ml. of a 22% suspension of the yeast was added giving a 2% concentration of yeast. This mixture was incubated at 37° for 90 min., the tubes being shaken every 15 min. After removal of the yeast by centrifuging, the change in reducing powers of the esters and hydrolysis products caused by treatment with *S. Ludwigii* was determined. For this purpose 2 ml. of each solution were used and allowance made for the increased dilution. Finally, the solutions which had been fermented by *S. Ludwigii* were submitted to a second fermentation designed, in this instance, to remove galactose. The residual reduction was then determined. To 5 ml. of each solution was added 1 ml. of a 12% suspension in water of *S. cerevisiae* Frohberg which had been subcultured on a yeast extract containing 5% of galactose. This mixture was incubated at 28° for 90 min., the tubes being shaken every 15 min. After centrifuging, the residual reductions were determined in quantities of 2 ml., allowance for dilution being again made.

Under the conditions described, *S. Ludwigii* completely removes glucose in a concentration of 0·05%, while galactose is not attacked [see also Kosterlitz, 1932].[1] This yeast strain was chosen because it does not become adapted to ferment galactose by being grown on hydrolysed lactose [Slator, 1908]. The fact that the fermentation rate of mannose is approximately half that of glucose and fructose is of no significance in the present work as has been shown by experiment: if mannose is present at all, its concentration is so small that after incubation for 90 min. fermentation is sufficiently complete; continuation of incubation for a further 90 min. has not resulted in a further decrease in reducing power (Exp. 3, fermentation of phosphoric esters; Exps. 7–9, fermentation of the free sugars after acid hydrolysis). The galactose-adapted strain of *S. cerevisiae* Frohberg completely removes galactose in a concentration of 0·035%. Care was taken throughout that the concentrations of sugars did not exceed those of the control experiments.

Harding & Grant [1931] proved that it is possible, by the use of two yeast strains as described, to estimate galactose and glucose in aqueous solutions and in Folin-Wu blood filtrates. Further, Harding *et al.* [1934] have shown that the same holds for aqueous extracts of tissues (muscle, liver and the whole carcass of the rat), after treatment with zinc sulphate and NaOH and Lloyd's reagent. Harding & Nicholson [1933] state that, in analysis of sugars by removal by micro-organisms, it is important to remove as far as possible the non-sugar reducing substances. The method used to effect this in the present experiments has already been described. Besides hexoses, the preparations obtained may contain pentoses: these are not fermentable by either yeast strain and will therefore increase the values of the residual reduction. Harding *et al.* [1932–331 drew attention to the fact that maltose may be removed to a small extent by a galactose-adapted yeast. This possible source of error is ruled out in the present

[1] As commercial galactose always contains some glucose, the galactose used in these control experiments was subjected to a fermentation with *S. Ludwigii* and then recrystallized several times from 70% ethyl alcohol.

instance by the experiments in which glucose and galactose have been estimated after acid hydrolysis.

Our knowledge of the fermentation analysis of glucose and galactose as measured by changes in reduction has invariably been based on experiments with the free hexoses. The experiments here described constitute the first attempt to apply the method to the analysis of a mixture of glucose and galactose phosphoric esters. The results obtained with the esters, however, have been corroborated by applying the same method of analysis to the hexoses liberated from these esters by acid hydrolysis (Exps. 5, 7–9).

(3) *Estimation of phosphorus.* Phosphorus was estimated by the method of Lohmann & Jendrassik [1926] using as a standard 0·052 or 0·078 mg. P in 21 ml. To test the phosphoric esters for an admixture of inorganic phosphorus, 0·052 mg. P in form of KH_2PO_4 was added to 2 ml. of the barium-free ester solution containing the equivalent of about 2 mg. of the barium salt. The inorganic phosphorus was then precipitated by an ammonia-magnesium citrate mixture and the phosphorus content of the precipitate estimated colorimetrically [Lohmann, 1928].

Results

Table I. *Fermentation analysis of unhydrolysed phosphoric esters*

(All results are given in mg. of "glucose" per 1 mg. Ba salt)

No. of exp.	Treatment of animals	Initial reduction	Change in reduction: After fermentation with *S. Ludwigii*	Change in reduction: After fermentation with *S. cerevisiae* Frohberg	Residual reduction	Remarks
			I. Control experiments			
1	Not fed	0·086	$-0·041_5$	−0·003	$0·041_5$	—
7	Fed with 25 ml. 0·9 % NaCl per kg.	0·134	−0·076	$-0·018_5$	$0·039_5$	—
6	Fed with 10 ml. 50 % glucose per kg.	0·128	−0·052	−0·019	0·057	Yield 32 mg. per 100 g. liver
8	Fed with 25 ml. 13·5 % glucose per kg.	$0·085_5$	−0·049	$-0·011_5$	0·025	Yield 67 mg. per 100 g. liver. 0·25 g. galactose per 100 g. liver were added to the CCl_3COOH extract
Average:						
	(1) Not fed or fed with saline	0·110	−0·059	−0·011	0·040	—
	(2) Fed with glucose	0·107	−0·051	−0·015	0·041	—
			II. Animals fed with galactose			
2	Fed with 10 ml. 50 % galactose per kg.	0·126	−0·043	−0·059	0·024	Fasted for 60 hr.
3	,,	$0·080_5$	$+0·073_5$*	−0·114	0·040	78 mg. Ba salt per 100 g. liver. P=7·13 %
4*a*	,,	$0·091_5$	$+0·060_5$	$-0·117_5$	$0·034_5$	Soluble in 10 % precipitated by 30 % alcohol. Yield 38 mg. per 100 g. liver. P=5·85 %
4*b*	,,	0·079	+0·009	−0·062	0·026	Soluble in 30 % precipitated by 60 % alcohol. Yield 55 mg. per 100 g. liver. P=6·12 %
5	,,	$0·073_5$	$+0·047_5$	$-0·088_5$	$0·032_5$	42 mg. per 100 g. liver. P=7·94 %
9	Fed with 25 ml. 13·5 % galactose per kg.	0·134	−0·006	$-0·093_5$	$0·034_5$	—
	Average:	$0·097_5$	$+0·023_5$	−0·089	0·032	—

* There was no change in reduction after a further 90 min. fermentation with *S. Ludwigii*.

Table II. *Fermentation analysis of hydrolysed phosphoric esters*

No. of exp.	Treatment of animals	Initial reduction	Change in reduction: After fermentation with *S. Ludwigii*	Change in reduction: After fermentation with *S. cerevisiae* Frohberg	Residual reduction
		I. Control experiments			
7	Saline	0·118	−0·085*	−0·010	0·023
8	Glucose	0·086	$-0{\cdot}056_5$*	−0·009	$0{\cdot}020_5$
		II. Animals fed with galactose			
5	Galactose	0·142	−0·045	$-0{\cdot}079_5$	$0{\cdot}017_5$
9	Galactose	$0{\cdot}183_5$	−0·084*	$-0{\cdot}078_5$	0·021

* After a further 90 min. fermentation with *S. Ludwigii* there was an insignificant increase in reduction (0·006).

Discussion

(1) *Initial reduction.* The initial reduction of the unhydrolysed phosphoric esters varies but little with the treatment to which the animals have been subjected. This is shown by the reducing values, stated in mg. of glucose per 100 mg. of barium salts, viz. 11 for the animals which have received no sugar, 10·7 for those given glucose and 9·75 for those given galactose. The initial reduction of all the ester preparations obtained is too low for any of the known hexosemonophosphates although the phosphorus content (5·85–7·94 %) agrees best with a monophosphate. There is probably an admixture of non-reducing esters in all preparations.

Acid hydrolysis for 69 hr. does not change very much the reducing value of the esters obtained from the control animals whether or not they have been fed with glucose: from 13·4 to 11·8 % in Exp. 7, and from 8·55 to 8·6 % in Exp. 8. It is doubtful whether the hydrolysis was complete, since the reducing power of a hexosephosphate is smaller than that of the corresponding free sugar. But, on the other hand, similar treatment of the esters obtained from animals fed with galactose caused a marked increase in reducing power: from 7·35 to 14·2 % in Exp. 5 (hydrolysed for 63 hr. only), and from 13·4 to 18·35 % in Exp. 9. This appears to suggest that acid hydrolysis liberates a reducing substance from the esters present in the livers of rabbits assimilating galactose, but not from the esters found in control animals.

(2) S. Ludwigii—*fraction (glucose, fructose and mannose).* On fermentation by *S. Ludwigii* there is a definite decrease in the reducing power of the unhydrolysed esters of the control animals: 5·9 % when no sugar was given, and 5·1 % when glucose was given. Acid hydrolysis appears to increase slightly this loss in reduction: from 7·6 to 8·5 % in Exp. 7, and from 4·9 to 5·65 % in Exp. 8. On the other hand, *S. Ludwigii* fermentation of the unhydrolysed esters obtained from galactose-fed animals caused in four out of six experiments a marked increase in reduction, a decrease was found only in two instances (Exps. 2 and 9). *S. Ludwigii* fermentation of the hydrolysed esters of the galactose-fed animals, however, invariably caused a decrease in reduction similar in magnitude to that of the esters, unhydrolysed and hydrolysed, of the control animals. This observation, together with the increased initial reduction after acid hydrolysis of the esters from the galactose animals, suggests that fermentation with *S. Ludwigii* of the unhydrolysed esters liberates a reducing substance which

itself cannot be fermented by this yeast, but which is fermented by the galactose-adapted *S. cerevisiae* Frohberg as will be seen in the next paragraph. A possible explanation for the decrease in reduction in Exps. 2 and 9 will be given later.

(3) S. cerevisiae *Frohberg (galactose-adapted) fraction.* There is only a slight decrease in reduction during *S. cerevisiae* fermentation of the unhydrolysed and hydrolysed esters obtained from the control animals, viz. 0·3–1·9%. The unhydrolysed esters found in the livers of the galactose animals, on the other hand, show a very marked decrease in reduction, viz. 5·9–11·75%, and it appears that the reducing substance which has been liberated during the fermentation with *S. Ludwigii* has now been fermented by the galactose-adapted *S. cerevisiae* Frohberg. The loss in reduction of the hydrolysed esters, obtained from the galactose-fed animals, is slightly but probably not significantly lower than that of the corresponding unhydrolysed esters (Exp. 5, 8·85 and 7·95%; and Exp. 9, 9·35 and 7·85%).

(4) *Residual reduction.* There is no significant difference in residual reduction between the esters from the control animals and those from the galactose-fed ones, only slightly higher values being obtained with the former.

The fact that only the esters obtained from the galactose animals show a definite decrease during fermentation with *S. cerevisiae* Frohberg strongly suggests the presence of a galactose compound in the organic phosphorus fraction of the livers of these animals. One possible objection, however, has to be considered. The livers of animals assimilating galactose always contain considerable quantities of free galactose which might conceivably be adsorbed on the organic phosphorus fraction. In control experiment 8, however, galactose was added to the trichloroacetic acid extract of the livers, and no galactose was found in either the unhydrolysed or the hydrolysed esters. Hence this objection can fairly safely be ruled out.

If this galactose compound is a galactosephosphate, the rates of increase in inorganic phosphorus and in reducing power during acid hydrolysis should be in a definite relationship. From the curves in Fig. 1 it may be seen that in the first $7\frac{1}{2}$ min. of hydrolysis with 0·1 *N* sulphuric acid the esters from Exp. 9 (galactose-fed) show a liberation of 1·84 mg. P per 100 mg. barium salt, and an increase of reduction equivalent to 9·3 mg. of "glucose", while the esters from Exp. 8 (glucose-fed) show only a very small and possibly insignificant change in both values. The further course of the curves is irrelevant as far as the present investigation is concerned. The increase in reduction of the esters from Exp. 9 is equal to the loss in reduction during fermentation with *S. cerevisiae* of the unhydrolysed esters of the same experiment (Table I). If one considers that, under the experimental conditions of this investigation, the reducing power of galactose is 75% of that of glucose, the ratio of galactose to phosphorus is 6·7 : 1. The theoretical ratio calculated for galactosemonophosphate is 5·8 : 1.

From the figures obtained the author feels justified in accepting not only that a galactosephosphate occurs, but also that it is either non-reducing or has a very small reducing power. The evidence is twofold: (*a*) the increase in reduction caused by hydrolysis of the esters obtained from the galactose-fed animals is much greater than that of the esters from the control animals (difference between initial and residual reductions for esters of control and galactose-fed animals before hydrolysis is 6·8 and 6·55% respectively, and after hydrolysis 8·0 and 14·4%), and (*b*) fermentation by *S. Ludwigii* of the unhydrolysed esters from the galactose-fed animals liberates a reducing substance fermentable by

S. cerevisiae Frohberg. Whether this liberation of galactose during *S. Ludwigii* fermentation will cause an increase or a decrease in reduction will depend on the ratio of galactosephosphate to glucosephosphate.

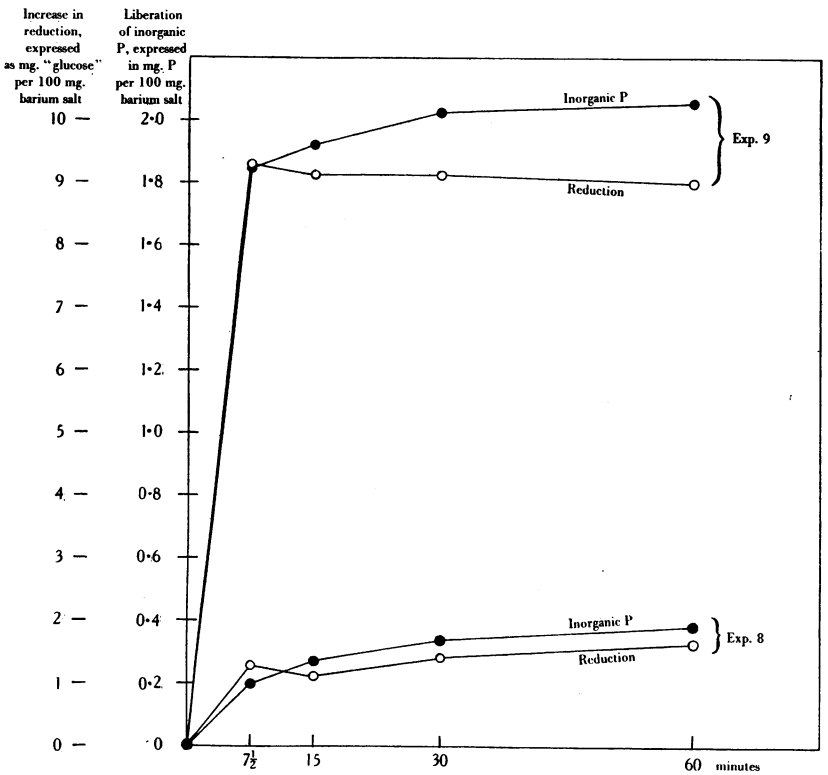

Fig. 1. Hydrolysis in 0·1 *N* H_2SO_4 at 100°. Concentration of esters: Exp. 8, 1·33 m.molar, Exp. 9, 1·26 m.molar. The unhydrolysed esters contained no inorganic P.

If a non-reducing galactose ester occurs, the galactose of which is liberated by the action of *S. Ludwigii*, it is evident that the algebraic sum of the changes in reduction during fermentation of the unhydrolysed esters should be approximately equal to the loss in reduction during *S. Ludwigii* fermentation of the hydrolysed esters.

The figures obtained are not incompatible with this view:

Exp. 5: $+0{\cdot}047_5 - 0{\cdot}088_5 = -0{\cdot}041$; found: $-0{\cdot}045$.
Exp. 9: $-0{\cdot}006 - 0{\cdot}093_5 = -0{\cdot}099_5$; found: $-0{\cdot}084$.

Further investigations on the nature of the galactosephosphate have still to be carried out, particular attention being paid to the possibility that it is a (non-reducing) galactose-1-phosphate analogous to the glucose-1-phosphate of Cori & Cori [1936]. Determination of the *k* of phosphorus and galactose liberation by acid hydrolysis should give further information on this point. The preliminary examination of the hydrolysis of the galactosephosphate already referred to supports this view: it is readily hydrolysed by 0·1 *N* sulphuric acid; this suggests that the phosphoric acid is linked to the reducing group of the galactose molecule.

SUMMARY

By means of fermentation analysis with two strains of yeast, one of which ferments glucose, fructose and mannose, and the other which also ferments galactose, evidence has been brought forward that a galactosephosphate, which is probably non-reducing, occurs in the livers of rabbits assimilating galactose.

I wish to record my thanks to the Diabetic Association for a grant which defrayed part of the expenses of this research during 1935 (Exps. 1–6) and to the Medical Research Council for a grant which partly defrayed the expenses during 1937 (Exps. 7–9). I am also indebted to Dr H. B. Hutchinson of the Distillers Company, Ltd., who generously supplied the strains of yeast.

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