CXCIX. THE METABOLISM OF GALACTOSE.

I. PHOSPHORYLATION DURING GALACTOSE FERMENTATION AND ITS RELATION TO THE INTERCONVERSION OF HEXOSES.

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Whilst the mode of metabolism of glucose by the animal cell has been the subject of numerous investigations during the past fifty years, little has been contributed which would elucidate the path of metabolism of galactose, also a normal constituent of the mammalian organism.

The investigations of Draudt [1913] and of Fischler [1925] suggested that the liver was the site of the preliminary alterations concerned in the metabolism of galactose. Draudt found that in animals with Eck-fistulas, 79 % of orally administered galactose was excreted in the urine as compared with 4–10 % before the operation. Mann and Magath [1924] reported that the resuscitating action of this sugar when injected into dehepatised animals was very slight. Bollmann *et al.* [1935] find that though the utilisation of galactose is definitely impaired by hepatectomy, appreciable amounts appear to be metabolised even in the absence of the liver. In amounts of 0·5 g. per kg. body weight, the proportions are 30 % excreted in the urine, 30 % converted into glycogen in the liver, while the remaining 40 % seems to be metabolised by the extra-hepatic tissues.

Galactose is only converted into glycogen at a slow rate in the animal organism [Cori, 1926], and this glycogen upon hydrolysis yields not galactose but glucose [Harding *et al.*, 1934]. The hexose possesses a limited rate of utilisation which is but little influenced by the type of diet, by fasting, or by such hormones as insulin, thyroxine or adrenaline, factors powerful in affecting the assimilation of glucose and fructose [Wierzuchowski *et al.*, 1931; Harding and Grant, 1932–33]. It is of little value in preventing the onset of insulin convulsions [Macleod and Noble, 1923; Voegtlin *et al.*, 1924–25; Roe and Schwartzmann, 1932], unless fed for some time previously to the administration of the insulin [Moschini, 1924]. Even in the latter case its effect is very limited. In the normal individual [Kosterlitz, 1933; Harding and Grant, 1932–33] and in the diabetic [Roe and Schwartzmann, 1932; Kosterlitz and Wedler, 1933], an increase in the blood glucose may follow the ingestion of galactose. Pollak and Selinger [1933] claim that insulin increases the assimilation of galactose, and Pollak and Fehér [1933] suggest in explanation that a concomitant glucose metabolism improves galactose assimilation. Recently Roe and Cowgill [1935] obtained evidence of increased glucose in the blood flow from the liver of animals which were metabolising galactose. Definite increases in blood glucose were only observed after an hour from the time of galactose administration, a sufficiently long interval for

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glycogen formation to be in active progress. Moreover, in those experiments with well-fed animals, increases in blood glucose may easily be attributed to glycogenolysis of preformed glycogen. These investigators also found little evidence of the metabolism of galactose by the brain, as judged by the absence of a well-marked arterial-venous difference during the galactaemia. Ashford [1933] has obtained evidence of the production of lactic acid from galactose by brain tissue. It is known that galactose is rapidly changed into its intermediary metabolites since, when orally administered in moderate amounts, it disappears from the tissues, as free sugar, within three hours from the time of its ingestion [Harding *et al.*, 1934]. Small rises take place in the glucose of the tissues during galactose metabolism but these are too small to be interpreted, by themselves, as evidence of a direct galactose → glucose conversion.

Galactose is apparently metabolised in two ways by the animal organism, a portion being slowly oxidised by the tissue cells generally and the remainder converted into glycogen in the liver and subsequently utilised as glucose. No investigations yet reported offer definite evidence of an immediate direct conversion of galactose → glucose in the living tissues, under conditions which would preclude previous formation of glycogen as the precursor of the glucose.

The metabolism of galactose by the yeast cell.

The yeast cell was chosen as perhaps offering the simplest method of approach to the problem of the mode of metabolism of galactose. The striking correlation between the carbohydrate metabolism of muscle and yeast is too well known to require further comment. Numerous researches have emphasised the importance of phosphorylation in the intermediary metabolism of glucose, fructose and mannose by the yeast cell, but the question of its relation to galactose metabolism has received comparatively little attention.

Previous researches have established the fact that yeasts which normally possess only slight ability to ferment galactose can be adapted, by repeated culturing, to ferment this hexose with greatly increased velocity [see Lippmann, 1884; Dienert, 1900; Armstrong, 1905; Slator, 1908]. It has been suggested that the process of adaptation only occurs during active production of new cells [Söhngen and Coolhaas, 1925; Euler and Nilsson, 1925].

Harden and Norris [1910] found that a fermenting mixture of yeast-juice (from an adapted yeast) and galactose reacted with added phosphate in a similar manner to ordinary yeast-juice and glucose, though a much longer time was necessary. The rate of CO_2 formation was accelerated, an extra amount of CO_2 equivalent to the phosphate added was evolved and the rate then again became normal. The phosphate was converted into an organic form not precipitable by magnesium citrate mixture. Later Nilsson [1930] returned to the problem and was able to isolate from the products of the fermentation of galactose by a sample of dried adapted yeast, a diphosphoric ester which in its elementary analysis and specific rotation closely resembled the hexosediphosphate formed during the fermentation of glucose, fructose and mannose. He also obtained a monophosphate fraction which in its impure state had a specific rotation much higher than that of the Robison ester formed in glucose fermentations. Moreover, an attempt to purify it further by crystallisation of its brucine salt was unsuccessful. Whether the ester was a derivative of glucose or of galactose was not investigated.

In the present investigation, additional evidence has been produced that the diphosphate formed during the fermentation of galactose by various preparations of a yeast adapted to ferment this sugar, is the 1:6-diphosphofructofuranose.

From the monophosphate fraction trehalosemonophosphate has been isolated. There was no evidence of the occurrence of a galactosephosphoric ester. The polysaccharides built up during the metabolism of galactose yield upon hydrolysis, not galactose but almost entirely the normally fermentable sugars, glucose, fructose and mannose.

Synthetic galactose-6-phosphate was not fermented by an active juice from the adapted yeast.

EXPERIMENTAL.

Adaptation of yeasts to ferment galactose.

A re-investigation of conditions favourable for the adaptation of certain yeasts to ferment galactose with increased velocity has been made. It was found impossible to obtain an active preparation with English brewer's top yeast using Nilsson's method [1930] of bulk adaptation. Aerobic growth on galactose-, glucose-, or hydrolysed lactose-agar flats was found unsatisfactory. *S. marxianus* was tried, but was found to be a slow grower and poor fermenter, even after several months' adaptation.

Repeated subculturing for several months of either English brewer's top yeast, or Frohberg bottom yeast, in a medium of 3 % galactose* in yeast extract +0·6 % KH_2PO_4 proved satisfactory. Such adapted yeasts fermented galactose at 80–100 % the rate for glucose. The most active preparations were obtained when the crop was removed 3–4 days from the time of inoculation. The yield of yeast varied from 8 to 10 g. per litre of medium. Varying the p_H between 4 and 6 had no effect upon the growth or fermentative powers of the yeast.

Fermentation of galactose by preparations of the adapted yeast.

For the investigation of the phosphorylated products formed during the fermentation of galactose, three preparations were employed, (*a*) fresh yeast in the presence of toluene, (*b*) yeast-juice, (*c*) dried yeast. At the beginning of the fermentation, or at appropriate intervals during its course, inorganic phosphate was added (0·5 *M* K_2HPO_4 or Na_2HPO_4† in *M* galactose solution) and the rate of esterification was followed by estimating the inorganic phosphate at intervals; the rate of formation of CO_2 was also measured.

The general method of Robison and Morgan [1930] was used for the isolation and separation of the neutral barium salts of the phosphoric esters formed. The crude diphosphate, precipitated by adjusting the p_H to 8·4 with hot concentrated baryta, was purified by repeated precipitations of its acid salt with four volumes of 96 % alcohol.

The basic lead salt of the crude monophosphate was decomposed with sulphuric acid and converted into the neutral barium salt. The small amounts of diphosphate still remaining were separated by extracting this monophosphate fraction with 10 parts of 10 % alcohol, filtering and precipitating the monophosphate (soluble B) with 2·5 volumes of 96 % alcohol.

Table I shows: the type of preparation used, the period, total CO_2 production and the total amount of phosphate and sugar added in the different fermentations.

* Kerfoot's second quality galactose, which contained some glucose was used.

† Analytical reagent, since commercial grades may contain sufficient fluoride to inhibit fermentation.

Table I. *Fermentation and phosphorylation of galactose by preparations of adapted yeast.*

Exp.	Yeast preparation	Time hours	Total additions: Galactose millimols.	Total additions: Phosphate millimols.	Phosphate esterified millimols.	% of total* ester P in the: di-phosphate fraction	% of total* ester P in the: mono-phosphate fraction	Total CO_2 millimols.
	Fresh yeast+toluene:							
1	Frohberg (115 g.)	8	300	51	48	66	34	250
2	Frohberg (131 g.)	16	300	82	51	83	17	—
3	English mild ale (137 g.)	8	320	69	65	78	22	220
	Yeast-juice: (English mild ale top yeast)							
4	26 ml. from 210 g.	4	65	17	12	70	30	25
5	135 ml. from 460 g.	4·5	267	50	28	84	16	73
6	85 ml. from 310 g.	3·5	289	25	22	58	42	27
7	80 ml. from 250 g.	3·5	289	33	32	81	19	32
8	175 ml. from 460 g.	3·5	278	83	80	88	12	60
9	*a–d* 40 ml. each:							
a	Autofermentation	5	0	0	2·3†	90	10	7·9
b	Autofermentation +inorganic phosphate	5	0	14·7	5·2	84	16	11·3
c	Galactose fermentation	5	100	19·6	20·1	89	11	28·1
d	Glucose fermentation	5	133‡	33·9	37·9	68	32	49·3
10	*a–d* 8 ml. each:							
a	Autofermentation	3	0	0	0·9	77	23	2·0
b	Autofermentation +inorganic phosphate	3	0	3·0	1·8	82	18	2·9
c	Galactose fermentation	3	8	3·0	3·8	75	25	5·8
d	Galactose-6-phosphate (8 millimols.) fermentation	3	0	3·0	8·8	21§	79	2·9
11	*a–d* Dried yeast+toluene 10 g. each:							
a	Autofermentation	5·5	0	0	3·4†	85	15	14·9
b	Autofermentation +inorganic phosphate	5·5	0	7·4	2·7	85	15	16·9
c	Galactose fermentation 25°	5·5	55	9·5	12·2	94	6	31·8
,,	,, ,, 37°	3·3	44	7·1	6·6	80	20	44·0
d	Glucose fermentation	5·5	84‡	25·2	34·3	83	17	51·0

* Esterified phosphorus present at the beginning of the fermentation: Exp. 9, 4·0 millimols.; Exp. 11, 7·4 millimols.

† Diphosphate fraction includes the organic P precipitated as insoluble barium salt in 10 % alcohol at p_H 8·4. It may contain phosphoglycerates. The monophosphate fraction represents the organic P remaining in the filtrate from the above separation. It includes slight amounts of diphosphate, as well as organic P not precipitable by basic lead acetate.

‡ Glucose millimols.

§ 8 millimols. ester P added in this fermentation. The actual amount of diphosphate (1·8 millimols.) is the same as that found in the control fermentation (10, *b*); 87·5 % of the added galactose-6-phosphate was recovered from the protein-free filtrate of the fermentation mixture.

In the earlier experiments (1–8) attention was chiefly directed towards obtaining the maximum amount of fermenting complex and in certain instances (Exps. 4–8) resort was had to a second pressing of the yeast, after regrinding the yeast marc with a small quantity of water. This procedure had the disadvantage of introducing extra amounts of yeast polysaccharides into the juice. For this reason, the yeast juice from a single pressing was used in Exps. 9 (*a–d*) and 10 (*a–d*), and suitable control fermentations (autofermentation alone and in the presence of added phosphate) were carried out under conditions similar to those for the galactose fermentations. Exp. 11 (*a–d*) shows a similar series of fermentations with a sample of the air-dried (25°) adapted yeast.

These yeast preparations fermented glucose rapidly giving a well-defined phosphate rate. They gave much lower rates of fermentation and phosphorylation with galactose but these rates were considerably higher than those attributable to the autofermentation, alone or in the presence of added phosphate

(Figs. 1, 2). Thus, phosphoric esters accumulate during the fermentation of galactose in much larger amounts than can be derived from the reserve polysaccharides in the yeast preparation used.

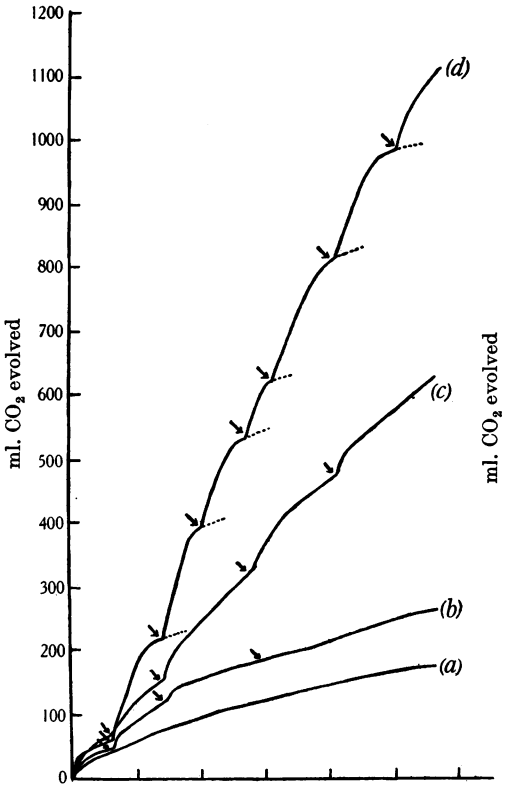

Fig. 1.

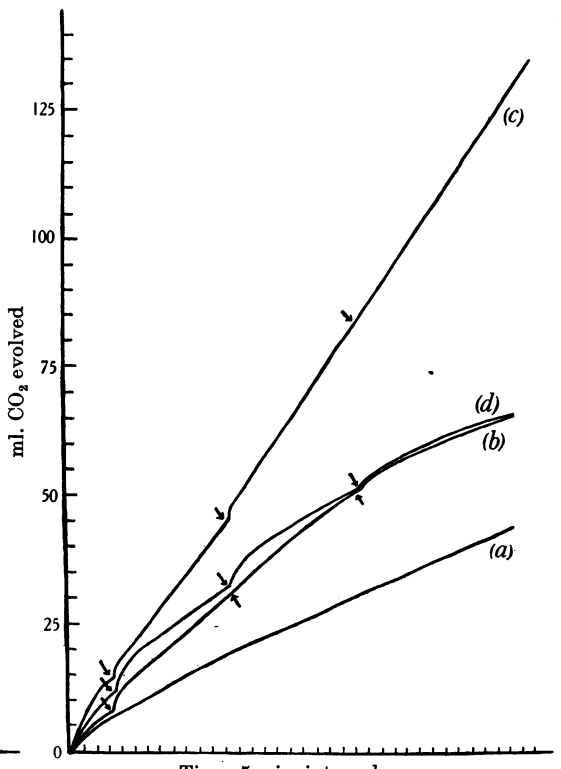

Fig. 2.

Fig. 1. Showing the rates of fermentation of glucose and of galactose by yeast-juice (40 ml.) from an adapted yeast (Exp. 9, *a–d*). (*a*) Autofermentation; (*b*) autofermentation + added phosphate; (*c*) galactose (100 millimols.) fermentation; (*d*) glucose (133 millimols.) fermentation. (The dotted lines indicate the basal rate of fermentation in the absence of added phosphate.)

Fig. 2. Showing the rates of fermentation of galactose and of galactose-6-phosphate by yeast-juice (8 ml.) from an adapted yeast. (Exp. 10, *a–d*.) (*a*) Autofermentation; (*b*) autofermentation + added phosphate; (*c*) galactose (8 millimols.) fermentation; (*d*) galactose-6-phosphate (8 millimols.) fermentation.

The properties of synthetic galactosemonophosphoric ester.

From the nature of the hexosemonophosphates formed during the fermentation of glucose, fructose or mannose it might be inferred that, if a galactose-phosphate is formed, the phosphate group will enter position 6 in the galactose molecule. A galactosemonophosphoric ester has been prepared by Levene and Raymond [1931] by the phosphorylation of diacetonegalactose and subsequent removal of the acetone groups. Its method of preparation and specific rotation were considered by Levene and Raymond to suggest that the synthetic mono-

phosphate obtained was galactose-6-phosphate. This ester has been synthesised in the present research and a further study of its properties made. In Table VI the analytical values of this ester are compared with those for the known natural hexosemonophosphoric esters. Its specific rotation is in good agreement with that previously reported by the above investigators. The Hagedorn-Jensen (H. J.) value for the galactosemonophosphate is much lower than that of glucose-6-phosphate in agreement with the fact that galactose itself possesses only 73 % of the reducing value of glucose towards this reagent. The sugar obtained by hydrolysis of the ester with purified bone phosphatase was identified as galactose.

Hydrolysis by N *HCl at 100°*. The rate of hydrolysis of the free ester in acid solution is shown in Table II. It is hydrolysed more rapidly than glucose-, or mannose-, but less rapidly than fructose-6-phosphate [Robison, 1932].

Table II. *Hydrolysis of synthetic galactosemonophosphoric ester (0·01* M) *in* N *HCl at 100°.*

Time hours	Hydrolysis %	$k \times 10^3$	
1	6·7	0·43	
3	14·6	0·32	
5	23·3	0·38	
8	35·7	0·42	
11	44·8	0·37	
23	73·1	0·43	
58	94·2	0·32	Average 0·38

Phenylosazone. Synthetic galactosemonophosphoric ester when heated with phenylhydrazine in acetic acid solution, yielded a phospho-osazone which crystallised in small rosettes. (P, 5·52 %; $C_{24}H_{31}O_7N_6P$ requires 5·69 %.) It was recrystallised twice from boiling alcohol to which an equal volume of boiling chloroform was added, the solution being then left at 0° for 24 hours; M.P. 135–137°. The formation of a phospho-osazone precluded the possibility of the phosphate being attached to carbon atom 1 or 2 of the hexose molecule.

Formation of an insoluble methylphenylhydrazone. The galactosemonophosphoric acid formed an insoluble methylphenylhydrazone when added to a solution of methylphenylhydrazine in glacial acetic acid at 0°. Crystallisation began in less than an hour and was completed in three hours. The hydrazone, recrystallised three times from 80 % aqueous alcohol, yielded colourless needle-shaped crystals; M.P. 134–136° (corr.; temperature rise 6–8° per minute). (Found, P, 6·34, 6·43 %; $C_{20}H_{31}O_8N_4P$ requires 6·38 %.) By decomposition of the hydrazone with benzaldehyde at room temperature the galactosemonophosphate was regenerated and isolated as the neutral barium salt.

Galactosemonophosphate very closely resembles glucosemonophosphate in the solubility of its neutral barium, lead and basic lead salts. The brucine salt crystallises readily from its aqueous alcohol solution in elongated hexagonal plates.

The separation of a galactosemonophosphate from the other esters (glucose-, fructose-, and mannose-6-monophosphate and trehalosemonophosphate) which might occur in the soluble B fractions of a galactose fermentation presents a problem of considerable difficulty. The separation of the Robison ester into glucose- and fructose-monophosphates was only attained after a prolonged fractional crystallisation of the brucine salts [Robison and King, 1931].

The formation of the insoluble phosphomethylphenylhydrazone is therefore a valuable method for the detection of small amounts of galactosephosphoric esters.

The fermentation of galactose-6-phosphate. In Exps. 10, *a–d*, the fourth fermentation (10, *d*) is that of the potassium salt of the synthetic galactosemonophosphate, equimolar with the galactose in the galactose fermentation of the same series (8 millimols). The results obtained indicated that the galactose-6-phosphate was not readily fermentable in conditions in which an active fermentation of galactose was observed. (Table I.)

The rate of fermentation of the ester (in the presence of added phosphate) was not greater than that of the autofermentation under similar conditions (Fig. 2). The galactosemonophosphate was not converted into a diphosphate but was recovered unchanged in the monophosphate fraction (Tables III and VI). No evidence was obtained of the presence of a phosphohexokinase capable of converting the galactose-6-phosphate (Table VI) into an equilibrium mixture of aldose $\rightleftharpoons$ ketose esters, which would have a considerably decreased iodine value (28 %) and a much higher Selivanoff value (6 %). This change readily takes place with the monophosphates of the normally fermentable sugars, glucose, fructose and mannose.

THE NATURE OF THE PHOSPHORIC ESTERS ACCUMULATING DURING THE FERMENTATION OF GALACTOSE.

The hexosediphosphate.

It has been shown that the same hexosediphosphate is formed, whether the sugar undergoing fermentation by yeast preparations is glucose, fructose or mannose [Harden and Young, 1908; Young, 1909]. To this compound the constitution 1:6-diphosphofructofuranose has been ascribed [Morgan and Robison, 1928; Robison and King, 1931; Levene and Raymond, 1928; 1931; Morgan, 1929].

The general analytical values for the neutral barium salts of the diphosphates formed in the galactose fermentations (Exps. 1; 2–8; 9, *a–d*; 10, *a–d*), are given in Table III.

Table III. *Analyses of the neutral barium salts of the diphosphoric esters formed during the fermentation of galactose.*

Exp.	Amount* g.	P %	Reducing power as glucose H.J.†	Reducing power as glucose Iodine	Fructose (Selivanoff) %	$[\alpha]^{20°}_{5461}$ ‡
1	4·0	10·0	11	1·5	8	+1·4
2–8	30·0	10·0	11	2·1	7	+4·2
9 *a–d*						
a Autofermentation	0·6	10·0	11	2·3	7	+4·0
b Autofermentation +inorganic PO_4	0·8	9·9	10	2·4	6	+4·6
c Galactose ferm.	4·3	9·8	10	2·6	7	+4·1
d Glucose ferm.	5·9	10·0	10	2·2	7	+4·3
10 *a–d*						
a Autofermentation	0·2	9·2	9	3·3	6	+5·5
b Autofermentation +inorganic PO_4	0·4	9·4	8	2·0	6	+4·8
c Galactose ferm.	0·7	9·9	11	2·4	6	+3·9
d Galactose-6-phosphate fermentation	0·4	9·4	11	4·7	8	+5·2
1:6-Diphosphofructofuranose§	—	10·0	12	1·5	9	+2·0

* As acid barium salt.
† Determined with the addition of 0·5 ml. 0·5*N* NaOH.
‡ In acid solution. § Macleod and Robison [1933].

In Exps. 1 and 2–8 the diphosphates were especially purified by seven reprecipitations of the acid barium salts (Macfarlane and Robison, unpublished results). The analytical values for these purified samples are in excellent agreement with the analysis of 1:6-diphosphofructofuranose.

Exps. 9 and 10 indicate that the amounts of diphosphate accumulating during the fermentation of the galactose are much in excess of that attributable to the autofermentation of the polysaccharides in the yeast preparations used. This has also been found true for the fermentation with dried yeast (Exps. 11, *a–d*, Table I) where the galactose fermentation (11, *c*) yielded 2·0 g. of the acid salt as compared with 0·5 g. and 0·7 g. for the autofermentation alone (11, *a*) and in the presence of added phosphate (11, *b*) respectively.

Osazone formation. The phospho-osazone (P, 5·50 %; $C_{24}H_{31}O_7N_6P$ requires, P, 5·68 %) melted at 153–153·5°; a mixture of the osazone with that prepared from a sample of 1:6-diphosphofructofuranose melted at the same temperature. The mutarotation of the osazone in alcohol-pyridine agrees closely with that found for the osazone of glucosemonophosphate (Table IV).

Table IV. *Mutarotation in alcohol-pyridine of the phospho-osazone prepared from the diphosphate of the galactose fermentations.*

Time mins.	Rotation From diphosphate of galactose fermentation $[\alpha]^{20°}_{5461}$	Rotation* From glucose-monophosphate $[\alpha]^{20°}_{5461}$
15	−59·5°	−60°
60	−44·5	—
85	−39·8	−38
17 hours	−37·4	—
24 hours	−35·1	−35

* Robison and King [1931].

Rate of hydrolysis. The rate of hydrolysis of the diphosphoric acid was also in good agreement with that of fructose-1:6-diphosphate (Table 5). Fructose-6-phosphate (Neuberg ester) was isolated from the products of fractional hydrolysis by acid of the fructosediphosphate from fermentations 2–8. (Analysis: P, 7·72 %; H.J. 33 %; iodine, 3·7 %; fructose (Selivanoff) 26 %; $[\alpha]^{20°}_{5461}$ +0·8.)

Table V. *Rate of hydrolysis of the diphosphoric ester (0·02 M) in N HCl at 100°.*

Time mins.	% Hydrolysis (1) Diphosphate from galactose fermentation	% Hydrolysis (2) Fructose-diphosphate*	$k \times 10^3$ (1)	$k \times 10^3$ (2)*
0	0	0	—	—
5	26·8	23·3	27·3	23·0
10	38·3	36·8	15·1	16·8
30	58·1	—	8·4	8·2
60	71·0	68·8	5·3	4·6
90	79·7	79·1	5·2	5·8
120	83·2	83·5	2·8	3·4
180	89·2	90·2	3·2	3·8
240	94·6	—	5·0	—
300	—	96·6	—	3·8
480	—	99·4	—	4·2

* Macleod and Robison [1933].

These results show conclusively that the diphosphate formed when galactose is the sugar fermented is the same as that formed during the fermentation of glucose, fructose and mannose, namely 1:6-diphosphofructofuranose. The ester constitutes the major portion of the phosphorylated products and is found in amounts considerably in excess of those which can be attributed to the yeast polysaccharides.

The monophosphoric esters.

The amounts of the monophosphate fractions ("soluble B") isolated from the galactose fermentations and the analyses of the neutral barium salts are shown in Table VI.

Table VI. *Analyses of the neutral barium salts of the soluble B fractions obtained in the fermentations.*

Exp.		Amount g.	Trehalose monophosphate isolated Ba salt g.	P %	Reducing power as glucose (%) H.J.	Reducing power as glucose (%) Iodine	Fructose (Selivanoff) %	$[\alpha]_{5461}^{20°}$	N %
1		1·75	0·67	5·0	10	11	2	+60·5°	
2		1·32	0	3·3	12	25	3	+ 2·8	
3		2·19	0·10	4·8	12	16	3	+31·6	
4		0·53	0·05	4·6	13	22	4	+29·3	
5		1·53	0	3·5	13	31	1	+ 3·8	
6		2·90	0·20	4·5	7	16	2	+68·0	
7		2·10	0·98	4·2	13	20	5	+55·8	
8		2·69	0·10	4·1	14	20	3	+27·0	
9 *a–d*									
a	Autofermentation	0·20	0·02	1·9	12	33	(0·1)	− 3·8	5·2
b	Autofermentation + inorganic PO_4	0·11	0·01	2·2	15	24	1	−16·9	5·2
c	Galactose ferm.	0·54	0·11	4·1	13	16	2	+37·5	2·4
d	Glucose ferm.	2·55	1·11	5·3	17	25	3	+49·3	
d'	Glucose ferm. after trehalose monophosphate removal	—	—	7·0	19	21	3	+26·3	
10 *a–d*									
a	Autofermentation	0·14	—	2·4	9	20	(0·4)	+16·9	
b	Autofermentation + inorganic PO_4	0·14	—	2·8	8	18	(0·3)	−12·5	
c	Galactose ferm.	0·34	—	4·9	12	19	(0·4)	+47·6	
d	Galactose-6-phosphate ferm.	2·21*	—	—	—	—	—	—	
	Before fermentation	—	—	7·8	28	41	1	+30·0	
	After fermentation	—	—	7·7	25	41	2	+27·5	
11 *a–d*									
a	Autofermentation	0·12	—	0·9	12	23	(0·3)	−13·0	
b	Autofermentation + inorganic PO_4	0·11	—	0·9	9	22	(0·2)	−18·0	
c	Galactose ferm. 25°	0·42	—	4·5	12	29	1	+52·5	
	" " 37°	0·20	—	4·4	14	34	2	+ 3·0	
d	Glucose ferm.	0·83	—	5·4	17	15	5	+40·3	
	Robison ester[1]	—	—	7·85	30	25	6	+14·4	
	Glucose-6-phosphate	—	—	7·86	36	46	(0·5)	+20·6	
	Mannose-6-phosphate[2]	—	—	7·82	36	27	2	+ 3·6	
	Fructose-6-phosphate	—	—	7·73	35	1·6	22	+ 2·3	
	Trehalosemonophosphate[3]	—	—	5·61	0	0	0	+132·0	
	Fructose-1-phosphate[4]	—	—	7·85	23	1·5	25	−35·0	
	Galactose-6-phosphate (present synthesis)	—	—	7·86	28	41	(0·7)	+30·0	

[1] Robison [1922].
[2] Macleod and Robison [1933].
[3] Robison and Morgan [1930].
[4] Tankó and Robison [1935].

* The galactosemonophosphate recovered after two basic lead precipitations; the amount of the galactose ester added as potassium salt was equivalent to 3·1 g. neutral barium salt.

In Exps. 9, 10 and 11, additional data are presented to indicate the amounts and nature of the soluble B fractions produced during the autofermentation alone and in the presence of added phosphate. The composition of this fraction when glucose is the sugar fermented is recorded in Exps. 9 (*d*) and 11 (*d*).

The data given for Exps. 9–11 indicate the real source of the difficulty in obtaining a hexosemonophosphate (P, 7·84 %) from the "soluble B" fraction of the galactose fermentations. In all cases this fraction represents a relatively small portion of the total esterified phosphorus and is seriously contaminated by concomitant impurities introduced by the yeast.

The latter material has a high nitrogen content, a high iodine value, is low in P and usually possesses a negative rotation.

Isolation of trehalosemonophosphate. Where the low P content of the monophosphate fraction occurred together with a high dextrorotation, trehalosemonophosphate was identified. It was separated by crystallisation from the aqueous alcoholic (10–20 %) solution of the monophosphate fraction. Under these conditions the trehalosemonophosphate crystallises as the sparingly soluble barium salt [Robison and Morgan, 1930]. The amounts are shown in Table VI.

Several recrystallisations of the crude salt yielded in each case pure crystalline trehalosemonophosphate as judged by the analyses of the neutral barium salt. [P, 5·57 %; $[\alpha]^{20°}_{5461}+132°$, for the anhydrous salt; no reducing power (H.J. and iodine) or Selivanoff value.] However, the whole of the ester was not removed from the monophosphate fractions by the above separations.

In Exps. 9 (*a–d*), the amounts of trehalosemonophosphate isolated from the glucose and galactose fermentations (*c*, *d*) were far in excess of those which could be attributed to the autofermentations (*a*, *b*) of the yeast preparation used. The laevorotatory "soluble B" fractions obtained from the autofermentation experiments did, however, contain detectable amounts of this ester.

Preparations from the unadapted yeast also possessed the mechanism for the synthesis of this ester from the normally fermentable sugars, glucose, fructose and mannose. The formation of this diglucosemonophosphate during the fermentation of galactose by preparations of the adapted yeast is of particular interest in view of the interconversion of hexoses involved.

Further examination of the residual soluble B fractions. After the removal of the trehalosemonophosphate, three methods were employed to effect a separation of the remaining hexosemonophosphoric esters in the soluble B fractions.

In the first method the monophosphates were precipitated from their solutions in aqueous alcohol (10 %) as the neutral lead salts [Robison, 1922]. The analyses of the regenerated neutral barium salts for some of the experiments are given in Table VII. A certain amount of purification was achieved, for

Table VII. *Analytical values of the neutral barium salts of the hexosemonophosphates isolated from the soluble B fractions*[1].

Exp.	Method of isolation	Amount mg.	P %	Reducing power as glucose (%) H.J.	Reducing power as glucose (%) Iodine	Fructose (Selivanoff) %	$[\alpha]^{20°}_{5461}$
4	Ppt. as neutral lead salt	50	6·0	16	32	3	+25·0
5	,, ,,	30	5·3	13	21	3	+ 3·0
8	,, ,,	415	5·2	16	23	4	+13·3
12	,, ,,	40	5·4	14	18	5	+19·9
7	Ppt. obtained by fractionation of the acid barium salt (40–70 % alcohol)	190	6·0	13	16	2	+70·0
		b	5·6	(0·3)	(0·8)	—	+131
		b'	6·5	16	20	—	+62·0

[1] Methylphenylhydrazine test negative in all cases.

the P content approximates more closely to that required for a hexosemonophosphate.

It was also found possible to effect a purification of the soluble B fractions by fractionation in alcohol of their acid barium salts. The monophosphate fractions were obtained at p_H 3·6 by increasing the alcohol content from 40–70 %. The analysis of one of these intermediate fractions is given for Exp. 7 and also its further separation into trehalosemonophosphate and a hexosemonophosphate fraction (Table VII, *b*, *b'*). In each case only small amounts of the hexosemonophosphates were obtained by either of the above methods.

The methylphenylhydrazine test. These small amounts of hexosemonophosphates were treated with a solution of methylphenylhydrazine in acetic acid, but there was no separation of an insoluble methylphenylhydrazone. The crude soluble B fractions were also subjected to this test but negative results were obtained in every case offering additional evidence of the absence of phosphoric esters of galactose from the phosphorylated products isolated from the fermentations.

The residual monophosphoric ester of the glucose fermentation (10*d'*) after removal of the trehalosemonophosphate possesses the general properties of the Robison ester though still contaminated with a little trehalosemonophosphate, as shown by the higher specific rotation. (See Table VI.)

The above results support the view that the monophosphoric esters which accumulate during the fermentation of galactose by preparations of adapted yeast are not the phosphoric esters of galactose, but those common to the fermentation of glucose, fructose and mannose. Trehalosemonophosphate has been definitely obtained, together with smaller amounts of material which resembles a hexosemonophosphate, yields a negative methylphenylhydrazine test and corresponds in its general analysis with the Robison ester found in the glucose fermentation.

Fermentation of galactose by dried yeast at 37°. Phenomenal yields of mannose-6-phosphate are obtained during the fermentation of mannose by dried yeast, if the temperature is 37°. This occurs only with dried yeast and does not result with glucose or fructose [Jephcott and Robison, 1933]. For this reason the fermentation of galactose by dried yeast at 37° was investigated in an attempt to increase the yield of monophosphate. This proved unsuccessful, the normal ratio of diphosphate to monophosphate being obtained. Similar results were obtained with fresh yeast + toluene at 37°. The monophosphate fraction isolated possessed a lower rotation suggesting that less trehalosemonophosphate was formed at the higher temperature of fermentation.

The polysaccharides synthesised during the metabolism of galactose by the living yeast cell.

The sugar polymerides built up when the adapted yeast was grown upon galactose as the sole source of carbohydrate offered additional evidence of the changes involved during the metabolism of this hexose.

Method of preparation. The yeast (69 g. moist yeast) obtained after three days' growth in a medium of yeast extract *plus* phosphate containing 4 % galactose, was washed free from reducing substances. It was then autoclaved for 30 minutes at 120° in 400 ml. of *N*/3 KOH. The insoluble residue ("insoluble polysaccharide") was filtered off and dried *in vacuo* over H_2SO_4, after several washings with 96 %, followed by absolute alcohol (5·31 g.; reducing power (H.J.) 0·2 %).

The "soluble polysaccharide" was obtained by precipitating the above

filtrate with three volumes of alcohol. It was further purified by two reprecipitations. (3·11 g.; $[\alpha]_{5461}^{20°}+132°$; H.J. 0·2 %.)

Hydrolysis of the polysaccharide fractions. 1 g. portions of the two fractions were hydrolysed for 5 hours in *N* H_2SO_4 at 100°. The hydrolysates were brought with baryta to p_H 6 and the filtrates and washings concentrated to a small volume, under reduced pressure, at a temperature of 40–50°. Final concentration to syrups was carried out at room temperature *in vacuo* over H_2SO_4.

Nature of the sugars in the above hydrolysates. The procedure of Harding and Grant [1931–32] was used to estimate the "fermentable sugar" (glucose, fructose and mannose) and galactose content of the hydrolysates from the soluble and insoluble polysaccharide fractions. The mannose was estimated as insoluble phenylhydrazone; the fructose by the Selivanoff value in excess of that developed for an amount of glucose equivalent to the reducing power of the syrups.

Mannosephenylhydrazone was obtained only from the hydrolysate of the "soluble polysaccharide" fraction, M.P. 200–201°; a sample mixed with pure mannosephenylhydrazone melted at the same temperature.

Galactose, if present, could be isolated as the insoluble methylphenylhydrazone. The hydrolysate from the "soluble polysaccharide" yielded an insoluble methylphenylhydrazone, not, however, that of galactose but of mannose which also forms an insoluble hydrazone in the concentrations present in the hydrolysate; M.P. 179–180°; a sample mixed with pure mannosemethylphenylhydrazone melted at the same temperature; while a sample mixed with galactosemethylphenylhydrazone (M.P. 188–190°) melted at a much lower temperature, 170–172°.

The osazone formed from the hydrolysates of both the soluble and insoluble polysaccharide fractions was almost entirely glucosazone. The combined results are summarised in Table VIII.

Table VIII. *Nature of the hexoses in the hydrolysates from the adapted yeast polysaccharides.*

	% of the total hydrolysate as				
Hydrolysate from 1 g. polysaccharide	Glucose	Fructose	Mannose	Galactose	Residual reduction (as glucose)
Insoluble polysaccharide	95·5	3	0	0	1·4
Soluble polysaccharide	77	3·3	16	1·8 (?)	1·9

The polysaccharides synthesised by the adapted yeast grown on galactose as the only carbohydrate, and isolated in the above manner, gave upon hydrolysis only the normally fermentable sugars, mainly glucose and mannose, with traces of fructose. Galactose was not found in definitely recognisable quantities by either the yeast analysis or the methylphenylhydrazine test. In this connection the detection of very small amounts of galactose, only demonstrable by indirect methods is of doubtful value as evidence of a galactose-containing polysaccharide in view of the possible contamination of the normally occurring polysaccharides by strongly adsorbed galactolipins (see recent findings of Heidelberger and Menzel [1935] upon lipo-carbohydrate complexes).

DISCUSSION.

The specific mechanism developed in certain yeasts during the process of adaptation on galactose and required for the fermentation of this sugar is largely, though not wholly, destroyed when the adapted yeast is treated with toluene, or dried, or in the preparation of a cell-free juice. The slow rate of fermentation of galactose by such yeast preparations, compared with that of glucose, contrasts strikingly with the equal rates of fermentation of these two sugars by the living yeast.

During the fermentation of galactose, and of glucose, by these preparations of adapted yeast a concomitant phosphorylation takes place. The phosphorylated products which accumulate during the fermentation of galactose are not the esters of this sugar but of glucose and fructose. A hexosediphosphoric ester constitutes the major portion of the esterified phosphate and has been shown by a detailed study of its properties to be identical with the ester produced from glucose, fructose or mannose, namely 1:6-diphosphofructofuranose. From the monophosphate fraction it was possible to isolate the diglucose ester, trehalosemonophosphate, together with small amounts of a monophosphate closely resembling in its properties the Robison ester.

In an effort to detect any small amounts of galactosephosphate which might be present, the methylphenylhydrazine test was applied to the monophosphate fractions obtained in the fermentation of galactose, since it was shown that the synthetic galactose-6-phosphate forms an insoluble methylphenylhydrazone. The failure to obtain any evidence by this test of the presence of galactose-6-phosphate coupled with the non-fermentability of this ester by an active preparation of the adapted yeast, offers strong evidence that this galactosemonophosphate is not an intermediate product of the fermentation process. If any other galactose ester which gives an insoluble methylphenylhydrazone is formed it must undergo subsequent transformation at such a rate as to preclude its accumulation.

The living yeast cell continues to build up the same polysaccharides when galactose is the sole carbohydrate metabolised as when the carbohydrate is glucose. The polysaccharides produced are polymerides chiefly of glucose, and to a lesser extent of mannose and fructose. Moreover, 1:6-diphosphofructofuranose is the chief ester formed during the fermentation of the polysaccharides occurring in preparations of the adapted yeast.

The nature of the mechanism for this important physiological transformation by which the adapted yeast converts galactose into derivatives of glucose, fructose and mannose remains unknown. It may be that a direct galactose → glucose conversion takes place and that the latter sugar is then metabolised in its usual manner. The nature of the hexosephosphoric esters found during the fermentation of galactose, and of the polysaccharides synthesised during the metabolism of the sugar by the living yeast cell, are in agreement with such a view. The appearance of derivatives of fructose and mannose can easily be accounted for by the action of the enzyme phosphohexokinase which is present in the yeast, and which can convert glucose-6-phosphate into an equilibrium mixture of the aldose-ketose esters of the three sugars, glucose, fructose and mannose [Martland and Robison, 1929; Lohmann, 1933; Macleod and Robison, 1933]. As previously mentioned a monophosphate fraction closely resembling this equilibrium mixture (the Robison ester) was obtained during the fermentation of galactose. Subsequent phosphorylation of the Robison ester occurring during its fermentation

by yeast preparations may result in the formation of the 1:6-diphosphofructofuranose [Meyerhof and Lohmann, 1927; Euler and Myrbäck, 1928; Harden and Robison, 1932].

Robinson [1927] has introduced an interesting theory to account for the changes occurring during the conversion of one sugar to another. He suggested that a Walden inversion might take place during the dephosphorylation of a 4-phosphohexose, producing glucose from galactose or *vice versa*. According to such a view the interconversion of the hexoses occurring naturally is conditioned by the enzymic hydrolysis of the phosphoric esters of the hexoses concerned. Mathers and Robertson [1933] and Robertson and Oldham [1934] have found that such inversions take place during the alkaline hydrolysis of certain synthetic sugar esters having two adjacent OH groups in the sugar molecule both esterified. It is also worthy of note that in cases where a Walden inversion was proved to occur, such inversion was accompanied by anhydro-formation. These investigators suggest that anhydro-formation may be a necessary precursor of this type of inversion, which then follows as a consequence of the opening of the anhydro-ring.

If the above findings are applied to galactose, the conversion into glucose could take place upon dephosphorylation of a 3:4-galactosediphosphate. The anhydro-formation during the removal of the phosphate groups could be accompanied by a Walden inversion on one or other of the substituted carbons (3 and 4) or on both of them. In this connection a 3:4-enol of the hexosediphosphate would be of importance in the metabolism of this ester. Thus from *d*-galactose, besides *d*-glucose, variable amounts of anhydro-sugars and of *d*-sorbose, *d*-gulose and *d*-allose could result. *d*-Glucose is the only one of these sugars readily fermented by yeast.

Definite evidence has been obtained by Oldham and Robertson [1935] that the opening of a 3:4-anhydro-ring in a glucose molecule can result in the transformation of this sugar into *d*-galactose and *d*-gulose. They emphasise the relationship of these newly formed hexoses with lactose and ascorbic acid respectively.

Tankó and Robison [1935] have considered the possibility of the occurrence of natural hexoses phosphorylated in positions other than 1 and 6. These investigators find evidence which seems to point to the formation of such esters during the fractional hydrolysis of 1:6-diphosphofructofuranose by acids.

Nilsson [1930] has postulated an entirely different mechanism to account for the formation of the same diphosphate from galactose as from the normally fermentable sugars. He suggested that the galactose molecule was first decomposed into 3-carbon compounds, a procedure which would destroy the spatial specificity of the fourth carbon atom, and that these 3-carbon compounds, upon phosphorylation, could be built up into the hexosediphosphate. Meyerhof and Lohman [1934] have obtained evidence of the existence of a zymohexase in yeast and muscle preparations capable of converting triosemonophosphoric ester into fructosediphosphate. Their results suggest that dihydroxyacetonephosphoric acid is the main triose ester to be formed *in vivo*, and it was possible to demonstrate its production from and conversion into hexosediphosphoric acid.

Cattaneo [1933] has obtained additional evidence pointing to the common path of fermentation of galactose and glucose by isolating phosphoglyceric acid from the phosphorylated products formed during the fermentation of galactose by preparations of adapted yeast in the presence of added phosphate, acetaldehyde and sodium fluoride.

Whatever the nature of the mechanism for the transformation of galactose into derivatives of glucose, fructose and mannose, it is remarkably specific for this sugar, for a yeast capable of fermenting galactose, does not possess the power readily to ferment such closely related aldose and ketose isomerides as talose and tagatose [Reichstein and Bossard, 1934].

The reverse change glucose → galactose takes place in the active mammary gland, and it also appears to be quite specific, for in conditions in which added glucose was almost completely synthesised *in vitro* to the galactose-containing disaccharide, lactose, little evidence of synthesis from added mannose, galactose or fructose could be demonstrated. There is also a hexosephosphatase present in the active gland capable of hydrolysing the naturally occurring hexosephosphoric esters and the synthetic galactose-6-phosphate. Moreover, there is evidence of a slight synthesis of organic phosphates, by the active gland preparations, from added glucose and inorganic phosphate, provided hexokinase is also added (Grant, unpublished results).

The decision as to whether the phosphorylation occurring during the metabolism of galactose is also the primary process concerned in effecting the interconversion of hexoses observed, or whether a specific mechanism is developed for the direct interconversion of galactose $\rightleftharpoons$ glucose prior to the phosphorylation of the latter hexose, must await the accumulation of further experimental evidence.

Summary.

1. The specific mechanism developed in certain yeasts during the process of adaptation on galactose and required for the fermentation of this sugar, is largely destroyed when the adapted yeast is treated with toluene or dried, or in the preparation of a cell-free juice.

2. During the fermentation of galactose, and of glucose, by these preparations of adapted yeast a concomitant phosphorylation takes place. The phosphorylated compounds which accumulate during the fermentation of the galactose are not the esters of this sugar but those of glucose and fructose.

3. The major portion of the esterified phosphorus is present as 1:6-diphosphofructofuranose, while the monophosphate fraction consists of the diglucose ester, trehalosemonophosphate, and possibly smaller amounts of the typical mixed ester of fermentation, the Robison ester. No evidence was obtained of the production of a galactosephosphoric ester.

4. Synthetic galactose-6-phosphate is not readily fermented by an adapted yeast preparation capable of fermenting galactose.

5. The polysaccharides synthesised by the adapted yeast grown upon galactose as the sole carbohydrate are mainly polymerides of the normally fermentable sugars, glucose, fructose and mannose.

6. The bearing of these results upon the nature of the mechanism developed in the adapted yeast for the metabolism of galactose is discussed.

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