THE METABOLISM OF FRUCTOSE IN LIVER. ISOLATION OF FRUCTOSE-1-PHOSPHATE AND INORGANIC PYROPHOSPHATE

by

GERTY T. CORI, SEVERO OCHOA,
MILTON W. SLEIN, AND CARL F. CORI
Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri (U.S.A.)

GEMMILL AND HOLMES[1] showed that the total fermentable carbohydrate content of slices of rat liver increased during 3 hours of incubation in oxygenated bicarbonate-Ringer's solution. This finding was confirmed by CORI AND SHINE[2,3] and led to the use of liver slices as a means of testing the convertibility of various substances to glucose. The following order in the rate of conversion to glucose was found, fructose being taken as 100. Dihydroxyacetone 71, DL-glyceraldehyde 58, α-glycerophosphate 56, β-glycerophosphate 31, glycerol 30, galactose 20, mannose 9. BACH AND HOLMES[4] added pyruvate, lactate, and a number of amino acids to this list.

These observations showed that the liver slice technique gave results in accord with those obtained on intact animals, but left open the question of the mechanism of these interconversions. GODA[5] who investigated the conversion of fructose to glucose in liver slices was unable to find such a conversion in minced liver or in liver extract. Not until this was accomplished[6] could one hope to find the intermediary steps involved.

It will be shown in this paper that the conversion of fructose to glucose in liver homogenates involves the intermediary formation of fructose-1-phosphate. An incidental observation in the course of this work is the formation of inorganic pyrophosphate in respiring liver homogenates.

EXPERIMENTAL

*Experiments with liver slices**

In the experiment in Fig. 1 liver slices of a fasted rat were shaken in oxygenated bicarbonate-CO_2 buffered Ringer's solution of p_H 7.4, containing 0.5% fructose. At stated intervals 1 ml samples of the medium were removed, deproteinized, and analyzed for total fermentable sugar (*i.e.*, the sum of glucose and fructose) by the SHAFFER-SOMOGYI[7] method and for fructose by CAMPBELL'S[8] method. Fructose disappeared at the rate of 6.6 mg per g of liver per hour, the rate remaining linear until 80% of the added fructose had disappeared. The total fermentable sugar content, after a small initial rise, remained constant. The fact that most of the fructose which disappeared had been converted to

* Carried out by W. M. SHINE in 1935.

References p. 317.

glucose was confirmed by polarimetric analysis at the end of the experiment. In other experiments it was found that a small amount of fructose (14 to 18%) was converted to lactic acid. Analyses for inorganic and organic phosphate gave no indication for the accumulation of a phosphate ester.

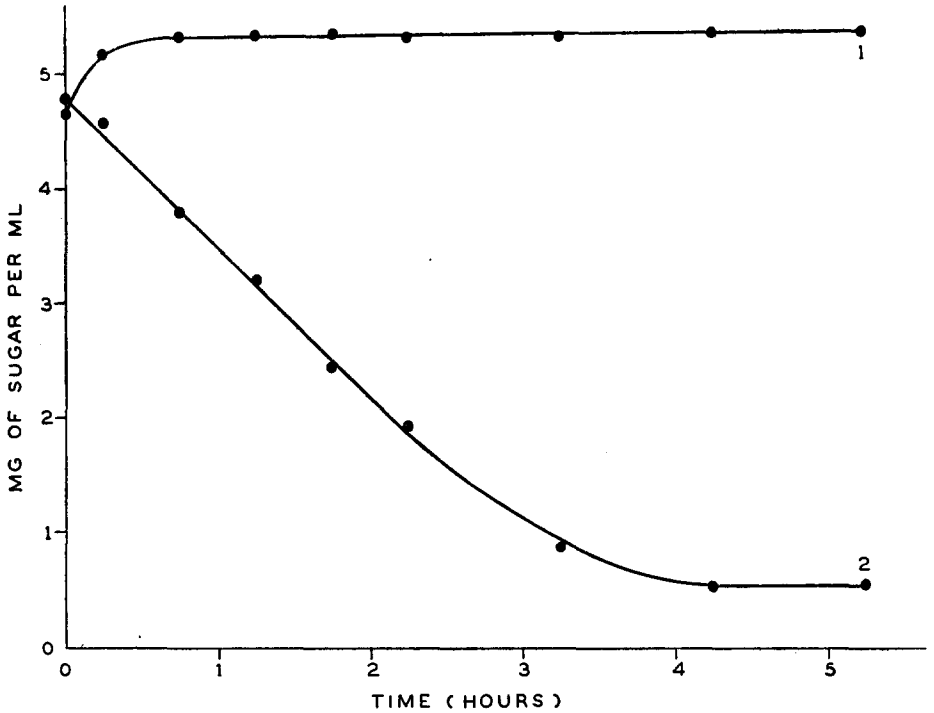

Fig. 1. Conversion of fructose to glucose in rat liver slices. Curve 1, fermentable sugar; curve 2, fructose. Values are expressed in mg per ml of medium. 4.06 g of slices were shaken in 20 ml of oxygenated bicarbonate-CO_2 buffered Ringer's solution at 37°.

Fig. 2 shows that when fructose-6-phosphate was incubated with liver slices under anaerobic conditions, fructose, as determined by CAMPBELL's method, disappeared much more rapidly than organic phosphate. This is due to the activity of LOHMANN's isomerase[9] which catalyzes the reaction, fructose-6-phosphate $\rightleftharpoons$ glucose-6-phosphate, and which acts more rapidly than the specific phosphatase which splits glucose-6-phosphate. Through the activity of these two enzymes, most of the fructose-6-phosphate is converted to glucose and inorganic phosphate, and only traces of free fructose are formed. The rate of glucose formation, calculated from the decrease in organic P, amounted to 10.5 mg per g of liver per hour. This rate is greater than that found in the experiment of Fig. 1 and hence an accumulation of phosphate esters in liver slices during the conversion of fructose to glucose was not to be expected.

*Experiments with liver homogenates**

The livers of rats fasted previously for 24 hours were perfused with ice-cold Ringer's solution in order to remove blood (which is the main source of α-amylase). Broken cell

* Carried out in 1940 by S. OCHOA, G. T. CORI, AND C. F. CORI.

References p. 317.

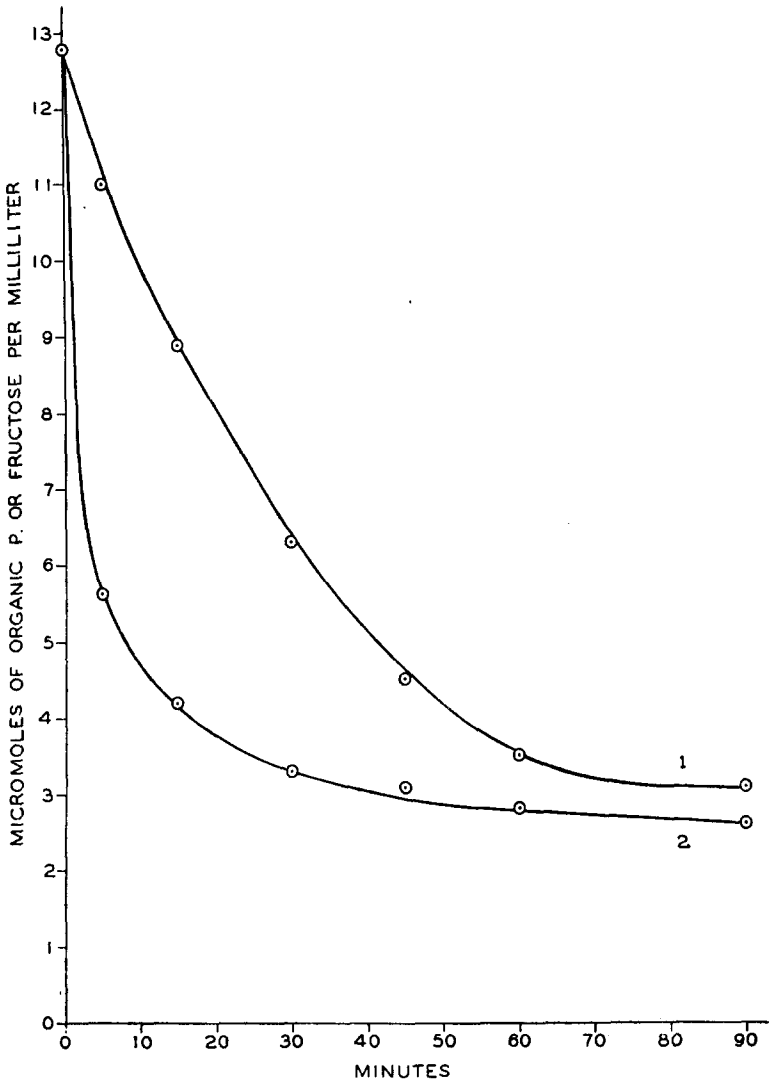

Fig. 2. Rate of disappearance of fructose-6-phosphate in rat liver slices. Curve 1, organic P; curve 2, fructose. Values are expressed in micromoles per ml of reaction mixture. 3.09 g of liver slices were incubated anaerobically in 20 ml of bicarbonate-CO_2 buffered Ringer's solution

dispersions were prepared by grinding the tissue in a stainless steel homogenizer with four volumes of ice-cold 0.9% KCl, followed by filtration through muslin. The dispersions were dialyzed for 3 to 4 hours at 1 to 4° in narrow cellophane tubes in a rocking device against a large volume of 0.4% KCl.

One and a half ml samples of the dialyzed dispersions (equivalent to 300 mg of fresh tissue) were made up with additions to 2.2 ml and shaken in Warburg vessels at 38° with oxygen in the gas phase. Fructose was determined by the method of ROE[10] and total reducing sugar by the method of SHAFFER AND SOMOGYI[7] in aliquots of the reaction mixture after deproteinization by the method of WEST *et al.*[11]; the difference

References p. 317.

between total reducing sugar and fructose corresponds to the amount of glucose present. Since inorganic and organic phosphates are precipitated in the WEST method, determination of the phosphate fractions was carried out in aliquots of a trichloroacetic acid filtrate.

The experiments in Table I show the following points: 1. In the absence of an oxidizable substrate (Exp. 2, samples 1 and 2) or of adenosinetriphosphate (Exp. 2, samples 3 and 4) there is little disappearance of fructose whether or not fluoride is added.

TABLE I

AEROBIC CONVERSION OF FRUCTOSE TO GLUCOSE IN DIALYZED RAT LIVER DISPERSIONS

All samples contained 0.2 mg of Mg^{++}, and 0.025 *M* phosphate buffer p_H 7.3 in 2.2 ml of reaction mixture. 10.0 mg of l(+) glutamate, 8.5 mg of fructose, and 0.06 mg of adenosine triphosphate (as labile P) were added, except when noted. Sodium fluoride, when added, was present in a final concentration of 0.04 *M*.

Experiment No.	Sample No.	NaF	Incubation period	Oxygen uptake	P esterified	Fructose disappeared	Glucose formed	Fructose converted to glucose
			min	μl	mg	mg	mg	percent
1	1*	+	60	286	0.22			
	2**	—	60	962	0.05			
	3**	+	60	950	0.49			
	4	—	60	932	0.14	3.42	2.67	78
	5	+	60	938	1.10	4.17	1.37	33
2	1***	—	60	126	0.00	0.00	0.00	0
	2***	+	60	237	0.15	0.25	0.00	0
	3§	—	60	99	0.00	0.26	0.00	0
	4§	+	60	660	0.61	1.59	0.19	12
	5	—	60	1320	0.35	4.95	2.49	50
	6	+	60	648	1.18	3.86	0.00	0
3	1	—	10	172	0.06	1.13	0.24	21
	2	—	20	311	0.09	1.46	0.87	60
	3	—	30	462	0.02	1.46	0.87	60
	4	—	120	582	0.00	1.18	1.18	100
	5	+	120	810	0.90	2.60	0.00	0
4	1	—	10	228	0.15	2.80	1.00	35
	2	—	20	445	0.28	2.32	1.32	57
	3	—	30	615	0.25	3.80	1.65	43
	4	+	10	151	0.53	2.57	0.43	17
	5	+	20	310	0.66	2.46	0.08	3
	6	+	30	438	0.77	3.06	0.06	2

* No glutamate or fructose added ** No fructose added *** No glutamate added
§ No ATP added

2. In the complete system but in the absence of fluoride, fructose is rapidly converted to glucose with little or no accumulation of phosphate esters (Exp. 1, sample 4; Exp. 2, sample 5; Exp. 3 and 4). The rate of conversion is higher at the beginning of incubation; thus in experiment 4 (sample 1), 1.0 mg of glucose was formed in 10 minutes or 20 mg per g of liver per hour, while after 30 minutes of incubation the rate had dropped to 11 mg of glucose per g of liver per hour. These rates of conversion are higher than those observed in liver slices. 3. In the presence of fluoride, fructose disappears at about the same rate as in the absence of fluoride, but its conversion to glucose is decreased or altogether prevented; instead there occurs an accumulation of a phosphate ester (Exp. 1,

References p. 317.

sample 5; Exp. 2, sample 6; Exp. 3, sample 5; Exp. 4, samples 4, 5, and 6). The ratio of inorganic P esterified to fructose utilized (after subtracting the fructose converted to glucose) is about 2 atoms of P to one molecule of fructose. This suggested the formation of fructosediphosphate, but since the ester which accumulated was more easily hydrolyzed by acid than fructosediphosphate, it was decided to purify the material for further characterization.

*Isolation of fructose-1-phosphate and inorganic pyrophosphate**

To 90 ml of dialyzed rat liver dispersion were added 12 mg of Mg^{++} (as $MgCl_2$), 0.02 *M* phosphate buffer p_H 7.3, adenosinetriphosphate with 3 mg of labile P, 500 mg of fructose, 0.044 *M* NaF and 300 mg of sodium succinate as oxidizable substrate, giving a final volume of 136 ml. The mixture was shaken in a conical flask of 1 liter capacity in an atmosphere of oxygen for 1 hour at 38° and then deproteinized with trichloroacetic acid. It was found that 29.3 mg of inorganic phosphate and 84 mg of fructose had disappeared; of the latter 14 mg had been converted to glucose. This gives a ratio of 2 atoms of P to 0.8 molecule of fructose for the esterified product. In 10 minutes in 1.0 *N* H_2SO_4 at 100°, 75% of the product was hydrolyzed, while fructosediphosphate under the same conditions was hydrolyzed only 20%.

A separation into 2 phosphate fractions showing different rates of hydrolysis in acid was achieved by means of calcium. The fraction of the calcium salts which was insoluble at p_H 6 contained 11 mg of a phosphate compound which was hydrolyzed completely in 10 minutes in 1.0 *N* H_2SO_4 at 100°. The soluble calcium salts (13 mg P) were fractionated with alcohol and yielded a product which was hydrolyzed 44.7% in 10 minutes in 1.0 *N* H_2SO_4 at 100°. Further characterization of this partially purified product showed that it was a hexosemonophosphate which reduced alkaline copper solution, gave a strong ketose test before and after acid hydrolysis and exhibited a high levo-rotation ($[\alpha]_D = -43.2°$, as the free acid). Furthermore, inorganic phosphate was liberated quantitatively when the osazone was formed. This indicated that the phosphate group was attached to carbon 1 of a ketose. Finally, the value for the hydrolysis constant in 1.0 *N* HCl at 100° agreed very well with that given by TANKÓ AND ROBISON[12] for fructose-1-phosphoric acid, when the initial concentration of the ester was calculated from polarimetric readings, assuming $[\alpha]_D = -52.5°$. An authentic sample of this ester was synthesized by the method of RAYMOND AND LEVENE[13] and was found to show the same biological behavior as the natural product isolated from several preparations (see below).

This left the insoluble calcium salts to be accounted for. They were converted to the sodium salts by double decomposition with sodium oxalate and crystallized from dilute alcohol. After several recrystallizations, the phosphate content of the material before and after drying at high temperature corresponded to that of sodium pyrophosphate. Inorganic pyrophosphate was also isolated in a large-scale experiment similar to that in Table I, Exp. 1, sample 3, where it is shown that inorganic phosphate disappears in the presence of fluoride even when no phosphate acceptor is added. The formation of inorganic pyrophosphate in tissue homogenates incubated under aerobic conditions has since been confirmed[14] and the mechanism of its formation has been explained[15].

* A preliminary account was given in an article by C. F. CORI in *Biological Symposia*, Vol. V, The Jaques Cattell Press, Lancaster, Pennsylvania, 1941. Fructose-1-phosphate was also isolated from a liver autolysate by J. PANY (*Z. physiol. Chem.*, 272 (1942) 273).

References p. 317.

The observations presented in this section may be summarized by saying that an apparent formation of fructosediphosphate from fructose in liver homogenates was simulated by the formation of a mixture of inorganic pyrophosphate and fructose-1-phosphate, a finding which emphasizes the danger of superficial characterization of phosphorylated products.

*Fate of hexose phosphates in liver dispersions**

TANKÓ AND ROBISON[12] obtained about equal amounts of fructose-1-phosphate and fructose-6-phosphate by the action of bone phosphatase on fructose-1,6-diphosphate. In order to investigate whether this might be the mechanism of formation of fructose-1-phosphate in liver dispersions, fructose-1,6-diphosphate and fluoride were added, both under anaerobic and aerobic conditions. The results shown in Tables II and III indicate that fructose-1,6-diphosphate is converted almost completely to glucose and inorganic phosphate without the formation of any free fructose. In the anaerobic experiment (Table II, Exp. 1) there was no easily hydrolyzable P left after incubation with fructose-1,6-diphosphate. Had any fructose-1-phosphate been formed, it would have been hydrolyzed over 90% by heating in 1.0 *N* H_2SO_4 for 25 minutes. The easily hydrolyzable P which appears in the aerobic experiment (Table III), consisted of inorganic pyrophosphate, as shown by the fact that it formed an insoluble calcium salt. The amount of acid-labile P in the soluble calcium salts (the fraction which would contain fructose-1-phosphate if it were present) was negligible.

TABLE II

BREAKDOWN OF HEXOSE DIPHOSPHATE IN DIALYZED RAT LIVER DISPERSIONS (ANAEROBIC)

Samples contained Mg^{++} (as $MgCl_2$), 0.04 *M* sodium fluoride and phosphate buffer p_H 7.3. Nitrogen in the gas phase. Values expressed per 2 ml of reaction mixture.

Experiment No.	Hexose diphosphate P added	Incubation time	Inorganic P	Additional P hydrolyzed in 1.0 *N* H_2SO_4 at 100°		Total acid soluble P	Free hexose	Free fructose
				10 minutes	25 minutes			
	mg	min	mg	mg	mg	mg	mg	mg
1	0.45*	0	1.77	0.11	0.27	2.36	0.01	0.00
	0.00	60	1.80	0.07	0.07	1.94	0.03	
	0.45*	60	2.21	0.01	0.03	2.43	1.32	0.00
2	0.00	0	0.70				0.11	
	0.00	90	0.74				0.22	
	0.55**	0	0.76				0.11	
	0.55**	90	1.26				1.43	
	0.55**	150	1.33				1.63	0.13

* Equivalent to 1.3 mg of hexose
** Equivalent to 1.6 mg of hexose

Added hexose-6-phosphate (the equilibrium mixture of fructose-6- and glucose-6-phosphate) was also split to glucose and inorganic phosphate in dialyzed rat liver dispersions; here again no or only traces of free fructose were formed.

These results can be explained by the following series of reactions: Fructose-1,6-diphosphate → fructose-6-phosphate + phosphate → glucose-6-phosphate → glucose

* Experiments carried out in 1940 by S. OCHOA, G. T. CORI AND C. F. CORI.

References p. 317.

TABLE III

BREAKDOWN OF HEXOSE DIPHOSPHATE IN DIALYZED RAT LIVER DISPERSIONS (AEROBIC)

15 ml of dispersion were diluted to 20.5 ml with additions including 0.025 *M* phosphate buffer p_H 7.3, 6 mg of Mg^{++} (as $MgCl_2$), 0.0004 *M* adenosine triphosphate, 0.0007 *M* cozymase, 0.043 *M* sodium pyruvate 0.005 *M* sodium fumarate, and 0.02 *M* sodium fluoride. Values expressed per 2 ml of reaction mixture Oxygen in the gas phase.

Sample No.	Hexose diphosphate P added	Incubation time	Oxygen uptake	Inorganic P	Additional P hydrolyzed in 1.0 *N* HCl at 100°		Total acid soluble P	Free hexose	Free fructose
					7 minutes	30 minutes			
	mg	min	μl	mg	mg	mg	mg	mg	mg
1	0.00	0		1.47	0.05		1.62		
2	0.00	90	960	1.10	0.51				
3	0.52	0		1.54	0.22	0.34	2.21	0.16	0.00
4	0.52	90	842	1.56	0.56	0.62		1.38	0.00

Fractionation of Ca salts at p_H 6

Sample No.	P in soluble Ca salts			P in insoluble Ca salts		
	Inorganic	Additional P hydrolyzed in 10 minutes*	Residual organic	Inorganic	Additional P hydrolyzed in 10 minutes*	Residual organic
	mg	mg	mg	mg	mg	mg
2				0.44	0.46	0.03
4	1.01	0.03	0.06	0.45	0.43	0.08

* In 1.0 *N* H_2SO_4 at 100°

+ phosphate. Specific phosphatases for the phosphate in position 1 of fructose-1,6-diphosphate[16] and for glucose-6-phosphate[17] have been shown to occur in the liver. The unspecific (acid or alkaline) phosphatases of the liver which would dephosphorylate fructose-1,6-diphosphate in position 6 to yield fructose-1-phosphate were apparently not active at p_H 7.3 in the presence of fluoride.

The inhibition of the conversion of fructose to glucose by fluoride and the simultaneous accumulation of fructose-1-phosphate suggested that this ester was an intermediary in this conversion. In order to test this, fructose-1-phosphate (enzymatically or synthetically[13] prepared) was added to dialyzed liver dispersions and incubated anaerobically in the presence and absence of fluoride. It was found that up to 80% of the ester which disappeared was converted to glucose and inorganic phosphate in the absence of fluoride and that fluoride, although not completely suppressing it, had an inhibitory effect on the reaction.

*Pathway of conversion of fructose-1-phosphate to glucose**

The extent of conversion of fructose-1-phosphate to glucose in a centrifuged KCl homogenate of the liver of a fasted rat is shown in the following example.

* Experiments carried out in 1948 by M. W. SLEIN.

References p. 317.

2.4 ml of reaction mixture, p_H 7.6, contained homogenate equivalent to 800 mg of liver, 0.008 *M* $MgCl_2$ and 6 μM of fructose-1-phosphate. A control sample without fructose-1-phosphate was incubated simultaneously, (both 30 minutes at 30°). Chemical analysis was according to the scheme outlined below. Of 2.9 μM of fructose-1-phosphate (determined as easily hydrolyzable fructose) which disappeared, 2.2 μM or 76% appeared as free glucose, while formation of free fructose was insignificant (about 0.15 μM) According to P analysis, 3.5 μM of inorganic P were formed and 3.2 μM of easily hydrolyzable P (15 minutes in 1 *N* HCl at 100°) disappeared during incubation, in fair agreement with the disappearance of fructose-1-phosphate calculated from analysis of easily hydrolyzable fructose.

An intermediary formation of glucose-6-phosphate was found in experiments with partially purified liver extracts. The livers of rats or rabbits were perfused with cold 1% KCl and homogenized in a Waring blendor with 2 volumes of 1% KCl. The supernatant fluid obtained after centrifugation for 10 minutes at 7000 r.p.m. was fractionated with saturated ammonium sulfate solution adjusted to p_H 7.8. Highest activity was found in the fraction between 0.45 and 0.55 saturation with ammonium sulfate. The dissolved fractions were dialyzed in the cold against 0.02 *M* sodium bicarbonate.

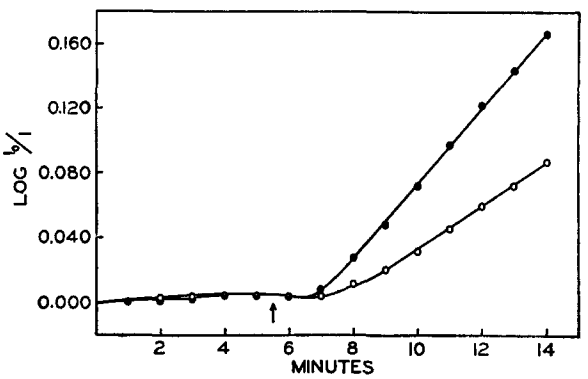

Fig. 3. Spectrophotometric determination of phosphofructomutase activity at p_H 8 in veronal buffer. Total volume, 3 ml. 1 μM of fructose-1-phosphate was added at 5.5 minutes. Upper curve shows rate of reduction of TPN in the presence of Zwischenferment, LOHMANN's isomerase, and 2.8 mg of a protein fraction from rat liver; lower curve shows rate with 1.4 mg of protein

The conversion of fructose-1-phosphate to glucose-6-phosphate was measured in a Beckman spectrophotometer by observing the reduction of triphosphopyridine nucleotide (TPN) at 340 mμ in the presence of Zwischenferment[18] and purified LOHMANN's isomerase*. Fig. 3 gives an example of many such measurements. There is a lag period after the addition of fructose-1-phosphate, followed by the attainment of a constant rate which is proportional to protein concentration. Mg^{++} has an accelerating and NaF an inhibitory effect on the reaction**.

A more detailed analysis of the products formed from fructose-1-phosphate with the 0.45–0.55 ammonium sulfate fraction is shown in Table IV. The scheme of analysis was as follows. One aliquot of the reaction mixture was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ to precipitate phosphorylated sugars; the filtrate was analyzed for free fructose by the method of ROE[10] and for total reduction (fructose + glucose) by the method of NELSON[19]. Another aliquot was precipitated with trichloroacetic acid and the filtrate divided into 2 parts, one was analyzed for inorganic phosphate, while the other was hydrolyzed for 15 minutes in 1 *N* HCl at 100°, neutralized, precipitated with $Ba(OH)_2$–$Zn\ SO_4$ and the filtrate analyzed for fructose. Fructose-1-phosphate is hydrolyzed over

* Purified according to an unpublished method by A. A. GREEN.

** Crystalline phosphoglucomutase[20] does not act on fructose-1-phosphate.

References p. 317.

90% under these conditions, while glucose-6- and fructose-6-phosphate are not appreciably hydrolyzed and are removed by the Ba–Zn precipitation. Hence one can calculate from the analysis of this sample how much fructose-1-phosphate has disappeared (after applying a correction for the free fructose formed).

TABLE IV

BALANCE FOR DISAPPEARANCE OF FRUCTOSE-1-PHOSPHATE

The reaction mixture contained 0.025 *M* veronal buffer p_H 8.1 and 0.01 *M* $MgCl_2$, 32 μM of fructose-1-phosphate (F-1-P) and 28 mg of rat liver protein (fraction between 0.45 and 0.55 saturation with ammonium sulfate). Total volume 10.5 ml, incubated at 30°. Values are given in micromoles.

Time in minutes	F-1-P disappeared	Hexose-6-P formed	F-1-P dephosphorylated			Percent F-1-P accounted for*
			P	Glucose	Fructose	
30	7.2	4.5	2.7	0.9	0.5	82
60	9.6	5.2	4.4	1.5	1.1	81
120	13.1	6.7	6.4	1.9	1.7	79
180	15.3	7.6	7.7	1.8	2.4	77

* Sum of hexose-6-phosphate, glucose, and fructose

Table IV shows that a large part of the fructose-1-phosphate which disappears is converted to acid-stable esters (a mixture of fructose-6- and glucose-6-phosphate according to spectrophotometric analysis with the Zwischenferment system). Some dephosphorylation still takes place as shown by the formation of inorganic phosphate and of free glucose and fructose. This is due to incomplete separation from glucose-6-phosphatase and fructose-1,6-diphosphatase. The latter enzyme has been found to act also on fructose-1-phosphate. That free fructose and glucose were formed by independent enzymatic processes was shown by a large variation in the ratio, free fructose/free glucose, in different ammonium sulfate fractions. Although the mutase system of the liver has not so far been purified sufficiently to prevent these side reactions or to permit a demonstration of the reversibility of the reaction, one may explain the results by the following tentative scheme*.

$$\begin{array}{ccc} \text{Fructose-1-P} & \rightarrow \text{Fructose-6-P} & \rightleftharpoons \text{Glucose-6-P} \\ \downarrow & & \downarrow \\ \text{Fructose} + \text{P} & & \text{Glucose} + \text{P} \end{array}$$

Rabbit muscle and sheep brain extracts do not contain an enzyme for the conversion of fructose-1-phosphate to hexose-6-phosphate, as shown by tests carried out in the spectrophotometer with Zwischenferment and TPN. In the case of muscle extracts the reaction was also negative in the presence of small amounts of fructose-1,6-diphosphate (which might have been required as co-enzyme in a manner analogous to glucose-1,6-diphosphate in the phosphoglucomutase reaction). Muscle, but not brain, contains an enzyme which converts fructose-1-phosphate to fructose-1,6-diphosphate in the presence of ATP[21].

* Another possibility is: fructose-1-P → glucose-1-P → glucose-6-P. Fractionations with the aim of eliminating the second reaction were not successful. In spectrophotometric tests, the rate of formation of glucose-6-P was more rapid with glucose-1-P than with fructose-1-P as substrate.

References p. 317.

Fructokinase

It has been shown that crystalline yeast hexokinase and purified brain hexokinase phosphorylate glucose, fructose, mannose and glucosamine[22] in position 6, and that these sugars in mixtures act as competitors for the same center of the enzyme, the degree of mutual inhibition being determined by the relative affinities of these sugars for the enzyme. In the case of liver and muscle, it has been possible to obtain enzyme preparations which act on fructose but not on glucose and *vice versa*[21]. The utilization of fructose by muscle or liver fructokinase was not inhibited by glucose, in contrast to the strong inhibition which glucose exerts on the utilization of fructose by yeast or brain hexokinase. Another property which distinguishes the fructokinase of muscle is that it is saturated with fructose only at unusually high concentrations[21].

Another unusual behavior of this enzyme in liver homogenates consists in its much greater activity under aerobic than anaerobic conditions. The aerobic system was similar to that used in the experiments in Table I, except that the homogenates were not dialyzed and that the reaction mixture was shaken in 50 ml flasks with air as the gas phase. The anaerobic system differed in the omission of glutamate and DPN and in the addition ot a large amount of ATP; it was incubated in test tubes without shaking and no special precautions were taken, except in a few cases, to make conditions strictly anaerobic. Deproteinization was carried out with barium hydroxide and zinc sulfate and aliquots were analyzed for fructose by ROE's method[10] before and after incubation. The addition of fluoride served the double purpose of inhibiting ATP-ase and of preventing the conversion of the products of fructose phosphorylation to glucose.

TABLE V

AEROBIC VERSUS ANAEROBIC DISAPPEARANCE OF FRUCTOSE IN RAT LIVER HOMOGENATE

The reaction mixture contained in 2.5 ml, fructose 10 μM, $MgCl_2$ 20 μM, phosphate buffer 50 μM, NaF 150 μM, ATP (10 μM anaerobic, 2 μM aerobic), glutamate 50 μM (aerobic), DPN 0.5 μM (aerobic) and 1 ml of homogenate, equivalent to 200 mg of fresh tissue. Incubation was for 20 to 30 minutes at 30° at p_H 7.5.

Homogenate No.	μg fructose disappeared		Ratio a/b	Remarks
	aerobic (a)	anaerobic (b)		
1	1145	340	3.4	
	130	135	1.0	Glucose instead of fructose added
2	1230	560	2.2	1.6 μM ATP split by ATP'ase
3	1105	395	2.8	
4	1065	410	2.6	(b) kept under nitrogen
	615			No glutamate added to (a)
5	995	320	3.0	
6	575	375	1.5	No ATP added to (a)
		780		1 μg yeast hexokinase added
7	330 (225)*	295	1.1	No ATP added to (a)
	615 (385)*		2.1	1 μM ATP added to (a)
	705 (585)*		2.4	2 μM ATP added to (a)
	720 (890)*		2.4	10 μM ATP added to (a)
8	295	70	4.2	Rabbit liver
9	980	950	1.0	Rabbit kidney cortex
10	915	935	1.0	Rabbit brain cortex

* Values in parentheses correspond to the rate of glucose disappearance, when glucose and 2.5 μg of yeast hexokinase were added to homogenate.

References p. 317.

Some representative experiments are shown in Table V. Fructose disappeared several times more rapidly under aerobic than anaerobic conditions in rat and rabbit liver homogenates, the latter containing less fructokinase than the former. Neither kidney nor brain homogenates showed this phenomenon (Exp. 9 and 10). In contrast to fructose, there was no difference in the rate of glucose utilization in liver homogenates under aerobic and anaerobic conditions (Exp. 1).

The possibility that ATP became limiting under anaerobic conditions was explored, but was not found to be the explanation for the difference in the rate of fructose utilization in liver homogenates. In Exp. 2, the amount of inorganic P formed from ATP was determined; it corresponded to a splitting of 16% of the 10 μM of ATP added anaerobically. When a small amount of yeast hexokinase was added to the anaerobic system, the rate of fructose utilization was increased, showing again that ATP was not limiting (Exp. 6).

In the aerobic system 2 μM of ATP were optimal for fructose utilization, and the addition of 10 μM did not cause a falling off in activity, showing that the ATP sample used was free of inhibitory impurities (Exp. 7). Glucose and yeast hexokinase were also added to the aerobic system, as shown in Exp. 7. The aim was to compare the relative affinity of ATP for liver fructokinase and yeast hexokinase under comparable conditions. The results obtained in this and in another experiment show a somewhat greater affinity of ATP for the liver enzyme.

In experiments designed to test proportionality between amount of liver added and amount of fructose used, another difference between the aerobic and anaerobic system was noted. Table VI shows that there was good proportionality in the aerobic system when the concentration of liver was increased from 200 to 400 mg per 2.5 ml of reaction mixture. In the anaerobic system, the ratio of ATP to homogenate was kept constant in order to insure that ATP would not become limiting. It may be seen that an increase in the concentration of liver above 200 mg per 2.5 ml had only a small effect on the rate of fructose utilization in the anaerobic system. Without additional work, it is impossible to say what is the significance of these observations. The ester formed in a large scale anaerobic experiment was isolated; it had the properties of fructose-1-phosphate. The product of fructose phosphorylation is therefore the same anaerobically and aerobically.

Other properties of liver fructokinase have recently been described[23, 24].

TABLE VI

EFFECT OF VARYING AMOUNTS OF RAT LIVER HOMOGENATE ON AEROBIC AND ANAEROBIC DISAPPEARANCE OF FRUCTOSE

Experimental conditions were the same as in Table V.

Homogenate No.	ATP added	Amount of liver	Fructose disappeared	Remarks
	μM	mg	μg	
1	2	200	680	Aerobic
	1	400	1095	Aerobic
	2	400	1265	Aerobic
2	5	100	175	Anaerobic
	10	200	450	Anaerobic
	15	300	550	Anaerobic
	20	400	620	Anaerobic

References p. 317.

DISCUSSION

A summary of the enzymatic reactions concerned with the metabolism of fructose, as described in this and in a preceding paper[21] is given in Table VII. In liver, enzyme reactions 2, 3, and 4 lead to the conversion of fructose to hexose-6-phosphate. It is significant that the enzyme which catalyzes reaction 5 is present in relatively low concentration in liver; this favors the conversion of hexose-6-phosphate to glycogen or to glucose. As is well known, fructose is a good glycogen former in the liver of normal animals and gives rise to extra glucose in the diabetic animal.

TABLE VII

ENZYMES CONCERNED WITH FRUCTOSE METABOLISM

Enzyme and Source	Substrates	Product Formed	Remarks
1. *Hexokinase* Yeast Brain	Glucose Fructose Mannose Glucosamine + ATP	The respective 6-phosphates	Substrate competition in mixtures of hexoses
2. *Fructokinase* Liver Muscle	Fructose + ATP	Fructose-1-phosphate	Not inhibited by glucose
3. *Phosphofructomutase* Liver	Fructose-1-phosphate	Glucose-6-phosphate via unknown intermediate	Inhibited by fluoride. Not found in muscle or brain.
4. *Isomerase* Most tissues	Fructose-6-phosphate	Glucose-6-phosphate	Separate isomerase for mannose-6-phosphate[25]
5. 6-*phosphofructokinase* Most tissues	Fructose-6-phosphate + ATP	Fructose-1,6-diphosphate	Present in low concentration in liver
6. 1-*phosphofructokinase* Muscle	Fructose-1-phosphate + ATP	Fructose-1,6-diphosphate	Not present in brain
7. *Fructose-1,6-diphosphatase* Liver Muscle	Fructose-1,6-diphosphate	Fructose-6-phosphate + phosphate	Acts also on fructose-1-phosphate

The existence of a separate enzyme for the phosphorylation of fructose in the liver may have the following significance. Phosphorylation of fructose by yeast or brain hexokinase is completely inhibited by an equal concentration of glucose and is still inhibited 85% when the glucose concentration is ¼ that of fructose*. Liver tissue contains free glucose in a concentration approximately equal to that in the blood and this would inhibit fructose phosphorylation by a yeast-type of hexokinase. However, the fructokinase found in liver and muscle is not inhibited by glucose. Intestine may contain a similar type of fructokinase, since KJERULF-JENSEN[26] isolated fructose-1-phosphate from the mucosa of the small intestine during fructose absorption. Kidney

* The reason for this strong inhibition is a greater affinity of glucose for the enzyme and the low concentration of fructofuranose which is the form of fructose in solution with which the enzyme reacts. It is possible that the ineffectiveness of fructose as an antidote during hypoglycemia may be explained in this manner.

References p. 317.

homogenates phosphorylate fructose rapidly and convert some fructose to glucose, but the primary product formed from fructose could not be ascertained because fructose-1,6-diphosphate accumulated in the presence of fluoride, owing to the great rapidity of reaction 5. The same situation was encountered when purified fractions of muscle fructokinase were examined; reaction 5 was 100 times and reaction 6 ten times more rapid than reaction 2[21]. The fact that muscle contains a separate enzyme for the disposal of fructose-1-phosphate speaks somewhat in favor of this ester's being the primary phosphorylation product of fructose. To complete the present status of our knowledge, it should be pointed out that both muscle* and liver were shown to contain a glucokinase, but this enzyme has not been purified sufficiently to examine its specificity.

SUMMARY

Fructose is converted to glucose in a dialyzed liver homogenate fortified with an oxidizable substrate, Mg^{++} and a catalytic amount of ATP. In the presence of fluoride, the conversion of fructose to glucose is inhibited, and an acid-labile phosphate ester accumulates. Isolation and characterization by its strong negative specific rotation, hydrolysis constant in acid, and liberation of inorganic phosphate on forming the osazone, identified this ester as fructose-1-phosphate. Another acid-labile phosphate ester which accumulated even in the absence of a phosphate acceptor was isolated as the crystalline sodium salt and identified as inorganic pyrophosphate.

A protein fraction precipitated from liver extract between 0.45 and 0.55 saturation with ammonium sulfate catalyzed the conversion of fructose-1-phosphate to hexose-6-phosphate. This mutase reaction was accelerated by Mg^{++} ions and inhibited by fluoride. In liver homogenate, in the absence of fluoride, the mutase reaction is followed by dephosphorylation of hexose-6-phosphate to free glucose and inorganic phosphate. These three enzymatic reactions explain the mechanism of conversion of fructose to glucose in the liver.

The formation of fructose-1-phosphate by liver fructokinase is not inhibited by glucose, in contrast to the strong inhibition glucose exerts on the phosphorylation of fructose by yeast and brain hexokinase. Fructose utilization in the liver is thus independent of glucose utilization, the latter being catalyzed by a separate enzyme. The liver fructokinase is much more active in an aerobic system with catalytic amounts of ATP than in an anaerobic system with an excess of ATP.

A summary of the enzymatic reactions concerned with the metabolism of fructose in various tissues is presented.

RÉSUMÉ

Le fructose est transformé en glucose dans un homogénat de foie dialysé additionné d'un substrat oxydable, de Mg^{++} et d'une quantité catalytique d'ATP. En présence de fluorure la transformation de fructose en glucose est inhibée et un ester phosphorique labile en milieu acide s'accumule. L'isolement et la caractérisation par son pouvoir rotatoire spécifique fortement négatif, la constante d'hydrolyse en milieu acide et la mise en liberté de phosphate inorganique lors de la formation de l'osazone, identifient cet ester comme fructose-1-phosphate. Un autre ester phosphorique labile en milieu acide qui s'accumule même en absence d'un accepteur de phosphate a été isolé comme sel de sodium cristallin et identifié comme pyrophosphate inorganique.

Une fraction de protéine précipitée à partir d'un extrait de foie entre 0.45 et 0.55 de saturation par le sulfate d'ammonium catalysait la transformation de fructose-1-phosphate en hexose-6-phosphate. Cette réaction mutasique était accélérée par les ions Mg^{++} et inhibée par le fluorure. Dans un homogénat de foie en l'absence de fluorure la réaction mutasique est suivie de la déphosphorylation de l'hexose-6-phosphate en glucose libre et phosphate inorganique. Ces trois réactions expliquent le mécanisme de la transformation du fructose en glucose dans le foie.

La formation de fructose-1-phosphate par la fructokinase de foie n'est pas inhibée par le glucose en contradiction avec la puissante inhibition exercée par le glucose sur la phosphorylation du fructose par l'hexokinase de levure et de cerveau. L'utilisation du fructose dans le foie est donc indépendante de l'utilisation du glucose, cette dernière étant catalysée par un autre enzyme. La fructokinase de foie est beaucoup plus active dans un système aerobie contenant des quantités catalytiques d'ATP que dans un système anaerobie contenant un excès d'ATP.

Nous présentons un résumé des réactions enzymatiques liées au métabolisme du fructose dans divers tissus.

* An independent assimilation of glucose and fructose has been demonstrated in the artificially perfused hind-leg preparation of the cat. At equal concentrations in the blood, the rate of uptake of the two sugars was about equal[27].

References p. 317.

ZUSAMMENFASSUNG

Fructose wird in einem dialysierten Leberhomogenat, das mit einem oxydierbaren Substrat, Mg^{++} und katalytischen Mengen von ATP verstärkt ist, in Glucose verwandelt. In Gegenwart von Fluorid wird die Umwandlung von Fructose in Glucose gehemmt und ein säurelabiler Phosphatester sammelt sich an. Dieser wurde durch Isolierung und Charakterisierung durch seine stark negative spezifische Drehung, seine Hydrolysekonstante in saurer Lösung und das Freiwerden von anorganischem Phosphat bei der Osazonbildung als Fructose-1-phosphat identifiziert. Ein anderer säurelabiler Phosphatester, welcher sich selbst in Abwesenheit eines Phosphatacceptors ansammelt, wurde als kristallinisches Natriumsalz isoliert und als anorganisches Pyrophosphat identifiziert.

Eine Eiweissfraktion, welche aus Leberextrakt zwischen 0.45 und 0.55 Sättigung mit Ammoniumsulphat gefällt war, katalysierte die Umwandlung von Fructose-1-phosphat in Hexose-6-phosphat. Diese Mutasereaktion wurde durch Mg^{++}-Ionen beschleunigt und durch Fluorid gehemmt. In Leberhomogenat, in Abwesenheit von Fluorid, ist die Mutasereaktion von der Dephosphorylierung des Hexose-6-phosphates zu freier Glucose und anorganischem Phosphat gefolgt. Diese drei Enzymreaktionen erklären den Mechanismus der Umwandlung von Fructose in Glucose in der Leber.

Die Bildung von Fructose-1-phosphat durch Leber-Fructokinase wird nicht durch Glucose gehemmt, zum Unterschied von der starken Hemmung, welche Glucose auf die Phosphorylierung der Fructose durch Hefe- und Hirnhexokinase ausübt. Die Verwertung von Fructose in der Leber ist also von der Verwertung der Glucose unabhängig, da letztere durch ein anderes Enzym katalysiert wird. Die Leber-Fructokinase ist in einem aeroben System mit katalytischen Mengen ATP viel aktiver als in einem anaeroben System mit ATP-Überschuss.

Eine Zusammenfassung der Enzymreaktionen, welche mit dem Metabolismus der Fructose in verschiedenen Geweben zusammenhängen, wird gegeben.

REFERENCES

1 C. L. GEMMILL AND E. G. HOLMES, *Biochem. J.*, 29 (1935) 338.
2 C. F. CORI AND W. M. SHINE, *Science*, 82 (1935) 134.
3 C. F. CORI AND W. M. SHINE, *J. Biol. Chem.*, 114 (1936) XXI.
4 S. J. BACH AND E. G. HOLMES, *Biochem. J.*, 31 (1937) 89.
5 T. GODA, *Biochem. Z.*, 294 (1937) 259; 297 (1938) 134.
6 C. F. CORI, *A Symposium on Respiratory Enzymes*, The University of Wisc. Press, Madison 1942.
7 P. A. SHAFFER AND M. SOMOGYI, *J. Biol. Chem.*, 100 (1933) 695.
8 W. R. CAMPBELL, *J. Biol. Chem.*, 67 (1926) 59.
9 K. LOHMANN, *Biochem. Z.*, 262 (1933) 137.
10 J. H. ROE, *J. Biol. Chem.*, 107 (1934) 15.
11 E. W. WEST, F. H. SCHARLES, AND V. L. PETERSON, *J. Biol. Chem.*, 82 (1929) 137.
12 B. TANKO AND R. ROBISON, *Biochem. J.*, 29 (1935) 961.
13 A. L. RAYMOND AND P. A. LEVENE, *J. Biol. Chem.*, 83 (1929) 619.
14 R. J. CROSS, J. V. TAGGART, J. V. COVO, AND D. E. GREEN, *J. Biol. Chem.*, 177 (1949) 655.
15 A. KORNBERG, *J. Biol. Chem.*, 182 (1950) 779.
16 G. GOMORI, *J. Biol. Chem.*, 148 (1943) 139.
17 M. A. SWANSON, *Federation Proc.*, 8 (1949) 258; *J. Biol. Chem.*, 184 (1950) 647.
C. DE DUVE, J. BERTHET, H. G. HERS, AND L. DUPRET, *Bull. Soc. chim. biol.*, 31 (1949) 1242.
18 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 254 (1932) 438.
E. NEGELAIN AND W. GERISCHER, *Biochem. Z.*, 284 (1936) 289.
19 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
20 V. A. NAJJAR, *J. Biol. Chem.*, 175 (1948) 281.
21 M. W. SLEIN, G. T. CORI, AND C. F. CORI, *Federation Proc.*, 6 (1947) 245; *J. Biol. Chem.*, 186 (1950) 763.
22 R. P. HARPUR AND J. H. QUASTEL, *Nature*, 164 (1949) 693.
D. H. BROWN, *Biochim. Biophys. Acta*, 7 (1951) in press.
23 C. S. VESTLING, A. K. MILROIE, U. IRISH, AND N. H. GRANT, *J. Biol. Chem.*, 185 (1950) 789.
24 I. LEUTHARDT AND E. TESTA, *Helv. Chim. Acta*, 33 (1950) 1919.
25 M. W. SLEIN, *J. Biol. Chem.*, 186 (1950) 753.
26 K. KJERULF-JENSEN, *Acta Physiol. Scand.*, 4 (1942) 225.
27 A. GEMMELTOFT, P. KRUHOFFER, AND E. LUNDSGAARD, *Acta Physiol. Scand.*, 8 (1944) 162.

Received January 30th, 1951