Decrease and Inhibition of Liver Glycogen Phosphorylase After Fructose

An Experimental Model for the Study of Hereditary Fructose Intolerance

Jean Holowach Thurston, B.A., M.D., Elizabeth M. Jones, B.S., and Richard E. Hauhart, B.S., St. Louis

SUMMARY

Inhibition of liver phosphorylase may play an important role in the fructose-induced hypoglycemia of hereditary fructose intolerance. This report is an in vivo study of the effects of fructose on selected metabolic intermediates and on phosphorylase activity in the livers of young mice. Phosphorylase activity in liver homogenate was measured in the direction of glycogen breakdown. The Km for Pi was 1.12 mM and the Ki for fructose-1-phosphate was 1.19 mM. This finding is particularly relevant since twenty minutes after fructose injection (30 mmoles per kilogram intraperitoneally) liver Pi was reduced 50 per cent, $p < 0.001$ and fructose-1-phosphate was increased fiftyfold, $p < 0.001$. Liver glucose was unchanged.

In controls, activity of phosphorylase was dependent on the state of the animals: 2.43 ± 0.31 μmoles/gm. min.$^{-1}$ in anesthetized mice, 9.55 ± 0.55 in excited animals. Under these same conditions phosphorylase activity in fructose-injected littermates was reduced 40 to 80 per cent (mean 48 per cent, $p = 0.002$). At the concentrations of Pi and fructose-1-phosphate found in liver after fructose, phosphorylase activity in vitro was inhibited 88 per cent ($p < 0.001$). In vivo the activity of liver phosphorylase is apparently reduced by two mechanisms, a conversion to the inactive form and an inhibition of the remaining active enzyme by reduced Pi and elevated fructose-1-phosphate levels. We have postulated that de novo synthesis of glucose from fructose in normal animals could mask any hypoglycemia which might result from reduced liver phosphorylase activity.

The in vivo effect of glucose injection on liver phosphorylase was also studied. At high levels of enzyme activity, glucose reduced phosphorylase activity by 43 per cent, $p = 0.012$; at low levels of enzyme activity, glucose had no effect. DIABETES *23*:597-604, July, 1974.

It is well established that the hypoglycemia which results from fructose injection in patients with hereditary fructose intolerance is due to a decrease in the output of glucose from the liver.[1-5] Froesch, Wolf, Baitsch, Prader and Labhart[4] were the first to suggest that reduced liver glycogen phosphorylase activity may play an important role in this phenomenon. These authors postulated that Pi levels might be seriously reduced in liver as well as in plasma, thereby limiting glycogen phosphorylase activity. In addition, Cornblath, Rosenthal, Reisner, Wybregt and Crane[5] considered the possibility of reduced liver glycogen phosphorylase activity due to the effects of fructose-1-phosphate (which accumulates as a result of the primary enzymatic defect in hereditary fructose intolerance) on activation or inactivation of the enzyme. Reports of reduced Pi levels in rat liver after fructose injection,[6-8] in vitro inhibition of liver phosphorylase by fructose-1-phosphate[9] and of decreased liver phosphorylase in patients with hereditary fructose intolerance[10] support both these hypotheses.

Fructose-1-phosphate increases to extraordinary levels in the liver after fructose injection in normal animals.[6,7,11-13] Since it is believed that the accumu-

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From the Edward Mallinckrodt Department of Pediatrics; Division of Neurology, St. Louis Children's Hospital; Washington University School of Medicine, St. Louis, Missouri 63110.

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lation of fructose-1-phosphate is directly or indirectly responsible for the clinical and pathophysiologic features of hereditary fructose intolerance, this finding suggested the possibility of a living experimental model for the study of this disorder. The prime objective was to determine in vivo effects of fructose loading on phosphorylase enzyme activity in liver homogenate. To this end the effect of fructose injection on levels of metabolic intermediates in liver and an investigation of a few basic kinetic properties of the enzyme preparation were highly pertinent.

METHODS

Preparation of Animals

Ten litters of nursing white mice, aged seventeen to twenty-three days were used in this study. Animals weighed 6 to 12 gm. We had hoped to be able to use newborn mice, but fructose-1-phosphate does not accumulate in the livers of young mice to the extent that it does in older animals with an equivalent fructose load (unpublished observations). It is probable that fructokinase activity is limited in young mice, as it is in young rats.[14] In each in vivo experiment the dose of fructose (or glucose) used was 30 mmoles per kilogram injected intraperitoneally in a volume of 20 to 30 ml. per kilogram of water; control littermates received NaCl of equal osmolality (15 mmoles per kilogram in a similar volume of water). In all but one experiment (thirty minutes) animals were killed twenty minutes after injection by plunging the whole animal into liquid N_2 or by decapitation. In the latter case, samples of liver were dissected quickly at room temperature and dropped into liquid N_2 for weighing at $-20°$ C. or weighed at room temperature.

In some experiments, animals were anesthetized with Na phenobarbital, 150 mg. per kilogram subcutaneously, thirty to forty minutes prior to injection of fructose (or glucose).

Preparation of Tissue and Plasma Extracts and Liver Homogenates

Frozen animals and liver samples were stored at $-75°$ C. until the time of dissection. Dissection of the liver with removal of as much blood and membranes as possible was carried out in a cryostat at -35° C. For metabolite measurements, frozen tissue was powdered and weighed at -20° C. and PCA extracts prepared according to the procedure used by Lowry, Passonneau, Hasselberger and Schulz[15] for brain. For determination of phosphorylase activity, liver samples were homogenized in Tenbroeck tissue grinders at 4° C. in 100 volumes of 12 mM potassium phosphate buffer pH 7.0 which contained 1 mM EDTA, 2 mM dithiothreitol, 50 mM NaF and 0.01 per cent bovine plasma albumin. Till the time of assay, PCA extracts and liver homogenates were stored at -75° C. Liver homogenate was frozen in small aliquots and a "first thaw" sample was used in each assay. The enzyme preparation is very stable with no loss of activity when re-assayed two years after storage.

MATERIALS

Rabbit liver glycogen was obtained from Mann Research, Inc. AMP-free lots were selected by testing for AMP contamination with purified rabbit muscle phosphorylase B, a gift from Dr. Barbara Illingworth Brown. After acid hydrolysis and neutralization, glycogen was assayed as glucose with hexokinase and glucose-6-phosphate dehydrogenase.[16] Most of the enzymes used for determination of metabolite levels were obtained from Boehringer and Sons or Sigma. Ox heart lactate dehydrogenase was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. Fructose-1-phosphate aldolase from rabbit liver was a gift from Sigma.

Methods of Metabolite Assay

ATP, glucose-6-phosphate, lactate and glycogen were measured fluorometrically by the specific enzymatic methods described by Lowry et al.[15] Glucose and fructose were measured after the procedure of Matschinsky, Ellerman, Landgraf, Krzanowski, Kotler-Brajtburg and Fertel.[16] In this method glucose and fructose are first measured together with the aid of hexokinase, glucose-6-phosphate dehydrogenase and glucosephosphate isomerase. In another sample glucose is removed with glucose oxidase and catalase in a 0.05 M citrate buffer, pH 5.5 at 37° C. for two hours. After treatment with heat (100° C., two minutes) fructose is measured in this sample as described above. The difference in the values of these two determinations is equal to the glucose concentration. Fructose-1-phosphate was assayed with fructose-1-phosphate aldolase and glycerolphosphate dehydrogenase after the method of Burch et al.,[7] and protein was determined by the method of Lowry, Rosebrough, Farr and Randall.[17]

Measurement of Phosphorylase Activity

With minor modifications, measurement of phosphorylase activity in liver homogenates was similar to that used by Lowry, Schulz and Passonneau.[18] Enzyme activity was measured in the direction of glycogen breakdown. The reaction was carried out at room temperature in 50 mM imidazole-HCl buffer pH 7.0 containing 10 mM $MgCl_2$, 0.5 mM EDTA, 0.01 per cent bovine plasma albumin and 0.05 mM

NADP. Glycogen and Pi concentrations were varied as required. AMP was not used. A great excess of auxiliary enzymes (8 μg. of glucose-6-phosphate dehydrogenase and 20 μg. of phosphoglucomutase per milliliter of reagent) was used so that the rate-limiting step in the production of NADPH was the phosphorolysis of glycogen by phosphorylase activity. $(NH_4)_2SO_4$ was removed from these auxiliary enzymes by centrifuging the commercial preparations and replacing the supernatant fluid with 20 mM tris buffer pH 7.0 containing 0.02 per cent bovine plasma albumin.

Phosphorylase activity was measured by the appearance of NADPH in a Farrand fluorometer. The sensitivity of the instrument was adjusted so that the full scale on the galvanometer was equivalent to about 2.5 x 10^{-6} M NADPH. The reaction was started by the addition of homogenate. The volume of homogenate used was 2.5 to 10.5 μl. per milliliter of reagent, equivalent to 0.025 to 0.1 mg. liver per milliliter of reaction mixture. In view of the extreme dilution of liver (1/40,000 to 1/10,000) the possibility of inhibition or activation of enzyme activity by tissue metabolites was most unlikely.

With saturating concentrations of glycogen and Pi (10 mM each), there was a steady rate of NADP reduction for at least ten minutes. Enzyme activity was proportional to the amount of tissue added up to and including a dilution of 1/5,000.

RESULTS

Kinetic Studies of Phosphorylase from Liver Homogenate

The kinetics of liver phosphorylase from crude homogenates in mice of this age have not previously been reported. It was therefore of interest to determine the interactions of phosphorylase in such a preparation with its substrates and with the inhibitor fructose-1-phosphate.[9] The data show that both the maximal velocities and the apparent Michaelis constants for each of the two substrates are affected by the concentrations of the other substrate (figures 1 and 2). The kinetic constants for glycogen are lowered by increases in Pi, and those for Pi are lowered by increasing levels of glycogen. In this respect the activity of glycogen phosphorylase in mouse liver homogenate resembled the activity of the purified enzyme from rabbit liver.[9] However, the apparent Km values for both substrates were considerably less than those reported for the purified enzyme. In view of the great dilution of liver in the final enzyme assay system, it is unlikely that this difference could be attributed to the

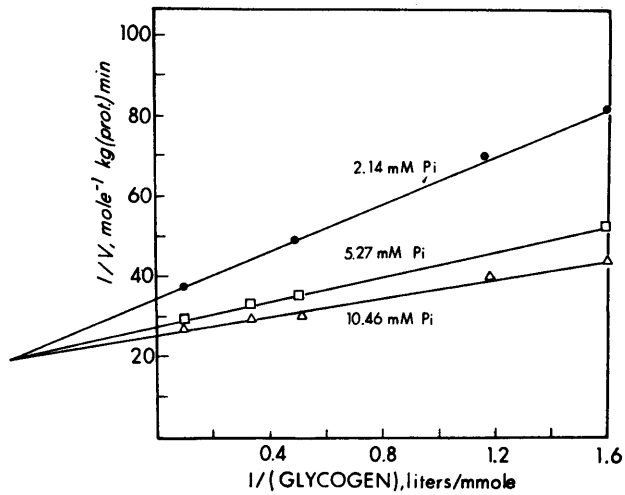

FIG. 1. Velocity of glycogen phosphorylase activity in liver homogenate as a function of glycogen concentration at three levels of Pi. Concentrations of Pi and corresponding apparent Michaelis constants for glycogen are as follows: 2.14 mM Pi, 0.875 mM glycogen; 5.27 mM Pi, 0.561 mM glycogen; 10.46 mM Pi, 0.461 mM glycogen. The intersection gives a value for -1/(glycogen) = -1/Kg, where Kg represents the Michaelis constant for glycogen when the enzyme is free of Pi.

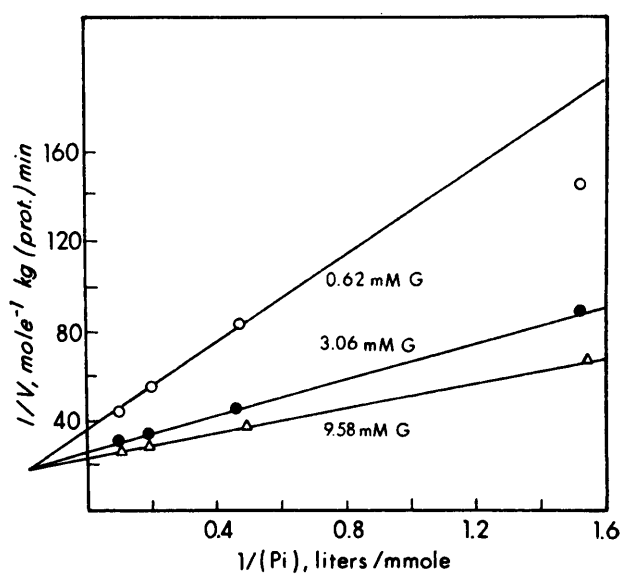

FIG. 2. Velocity of glycogen phosphorylase activity in liver homogenate as a function of Pi concentration at three levels of glycogen (G). Concentrations of glycogen and corresponding apparent Michaelis constants for Pi are as follows: 0.625 mM glycogen, 2.72 mM Pi; 3.06 mM glycogen, 1.56 mM Pi; 9.58 mM glycogen, 1.12 mM Pi. The intersection gives a value for -1/(Pi) = -1/Kp, where Kp represents the Michaelis constant for Pi when the enzyme is free of glycogen.

influence of other metabolic constituents in the crude tissue sample. It is possible that liver phosphorylase could be altered in some fashion during the multiple steps of purification or the discrepancy could lie in species (mouse vs. rat) or age (young vs. adult animals) differences.

The in vitro inhibition of glycogen phosphorylase from normal liver homogenate by fructose-1-phosphate is shown in figure 3. With fructose-1-phosphate concentrations of 0.95 mM or 2.55 mM, phosphorylase activity was linear at all concentrations of Pi tested (range 1.29 to 10.3 mM). With fructose-1-phosphate at 5.12 mM, linearity was observed only at high concentrations of Pi. It is apparent that at the concentration of glycogen used in these studies (10 mM) the inhibition was competitive with respect to Pi. The mean Ki for fructose-1-phosphate calculated from the three curves in figure 3 was 1.19 mM ± 0.12 (S.E.M.). It should be noted that at saturating concentrations of glycogen (10 mM) this Ki was equal to the Km for Pi.

Again in keeping with the lower Km values for glycogen and Pi in these crude enzyme preparations the Ki was considerably lower than that reported by Maddaiah and Madsen[9] for purified rabbit liver phosphorylase. These authors found fructose-1-phosphate to be competitive with Pi with a Ki of 4 mM.

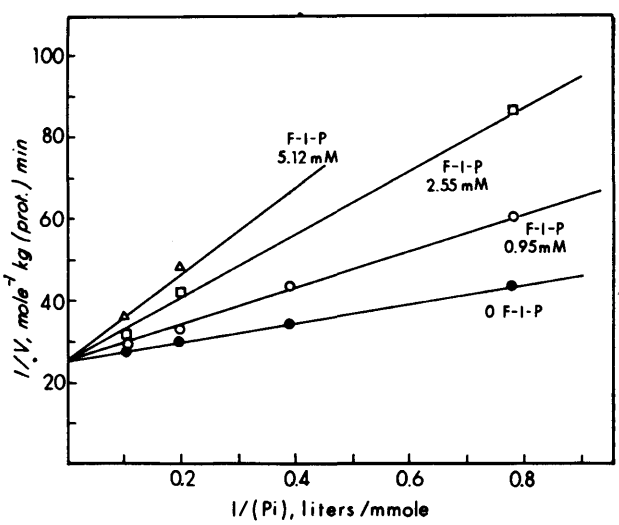

FIG. 3. Velocity of glycogen phosphorylase activity in liver homogenate as a function of Pi concentration at 10 mM glycogen and three levels of fructose-1-phosphate (F-1-P). Concentrations of fructose-1-phosphate and corresponding apparent Michaelis constants for Pi are as follows: 0 fructose-1-phosphate, 0.95 mM Pi; 0.95 mM fructose-1-phosphate, 1.81 mM Pi; 2.55 mM fructose-1-phosphate, 3.14 mM Pi; 5.12 mM fructose-1-phosphate, 4.34 mM Pi.

Effect of Fructose on Selected Metabolic Intermediates in the Liver

Twenty minutes after fructose injection (30 mmoles per kilogram), fructose and fructose-1-phosphate levels were enormously elevated (table 1). As others have shown, these elevations were associated with profound reductions in the concentrations of liver ATP[6-8,13] and Pi[6-8] values were down to 50 per cent of the control levels.

TABLE 1

Effect of Fructose on Selected Metabolites in Liver

Metabolite	Control (5)	Fructose (7)	P value
	mmoles/kg.		
ATP	2.53 ± 0.17	1.36 ± 0.05	< 0.001
Glucose	6.02 ± 0.25	6.84 ± 0.75	N.S.
Fructose	0	21.31 ± 1.93	< 0.001
Fructose-1-phosphate	0.221 ± 0.037	10.56 ± 0.92	< 0.001
Lactate	2.09 ± 0.34	3.84 ± 0.67	N.S.
Glycogen	82.8 ± 23.1	117.4 ± 20.5	N.S.
Pi	4.57 ± 0.16	2.07 ± 0.14	< 0.001

Liver samples were obtained twenty minutes after injection. The dose of fructose was 30 mmoles per kilogram intraperitoneally, controls received 15 mmoles per kilogram NaCl. Values are given as the mean ± S.E.M.; number of animals in parentheses.

It is of considerable interest that liver glucose levels were not changed twenty minutes after fructose injection. With a slightly larger dose of fructose (40 mmoles per kilogram intraperitoneally), liver glucose levels in adult rats were almost doubled at fifteen minutes and by thirty minutes three- to fourfold increases were seen.[7] In view of this finding additional studies were made. In four of the eight litters examined (including the two used for table 1) liver glucose concentration was not changed after fructose injection; values were increased in the others (figure 4). To explain this difference, other parameters were examined. Of the several variables—anesthesia, time of sacrifice after injection,' manner of sacrifice, and method of tissue preparation, the most consistent factor was the interval before sacrifice. Liver glucose values were not increased twenty minutes after fructose injection; only after thirty minutes were significant increases seen.

In Vitro Liver Phosphorylase Activity with in Vivo Concentrations of Fructose-1-phosphate and Pi

To mimic the in vivo situation phosphorylase activity in normal liver homogenate was measured in vitro in the presence of the concentrations of fructose-1-phosphate and Pi seen after fructose injec-

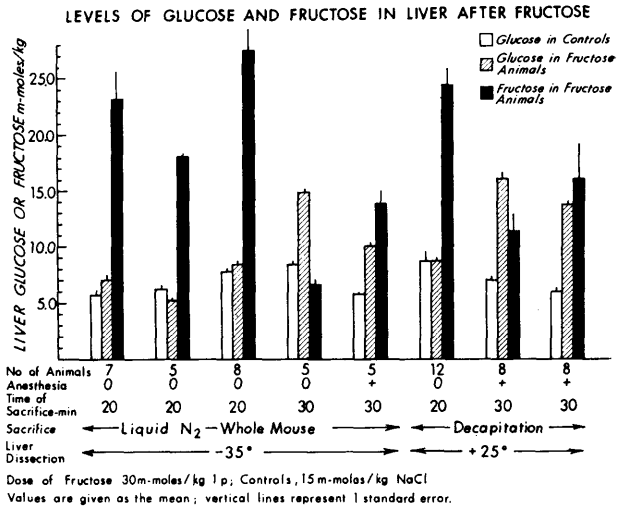

FIGURE 4

tion (table 1) and 10 mM glycogen. Compared with the control value, the activity of the enzyme was reduced 88 per cent by these additions (from 5.9 to 0.74 μmoles/gm. min.$^{-1}$, $p < 0.001$).

In Vivo Effect of Fructose and Glucose on Liver Phosphorylase Activity

The in vivo level of active phosphorylase in normal animals was strongly influenced by the state of excitement of the animals; by just gently handling the animals for several seconds prior to sacrifice, activity was increased from 7.16 ± 0.13 to 9.55 ± 0.55 μmoles/gm. min.$^{-1}$ (N = 4), probably the result of epinephrine release and phosphorylase activation.[19-23] As might be expected, the lowest values, 2.43 ± 0.31 μmoles/gm. min.$^{-1}$ were seen in the deeply anesthetized mice (table 2). The manner of sacrifice[23] and the method of tissue preparation[22] also affect the level of enzyme activity. However, under all experimental conditions of the study, whether the animals were awake or deeply anesthetized, whether they were killed by decapitation or by quick freezing in liquid N_2, whether the liver was dissected at room temperature or at -35° C. and whether the samples were weighed at room temperature or at -20° C., there was a highly significant decrease in phosphorylase activity in the livers of fructose-injected mice (table 2).

Since increased concentrations of glucose in the liver are known to reduce the level of active phosphorylase,[24,25] glucose levels were measured in additional PCA-treated samples of liver from three of the four litters used for table 2 (Experimental Groups A, B and D). In fructose-injected Group A and B mice, liver glucose values did not differ from controls but in Group D values were more than twice the control (14.70 ± 0.70 vs. 6.04 ± 0.24 mmoles per kilogram; $p < 0.001$). From the preceding experiments (see figure 4) the explanation for this difference is the time interval between fructose injection and sacrifice; Group A and B animals were killed twenty minutes after fructose injection, Group D at thirty minutes. Since the lowest levels of phosphorylase activity were seen in the fructose-treated mice with the highest liver glucose values, the possibility that the latter played a role in this finding had to be considered. In an attempt to clarify this issue, the effect of glucose injection on liver phosphorylase activity in vivo was investigated (table 3). When the level of active liver phosphorylase was high, glucose produced a striking reduction of enzyme activity. This effect in the intact animal confirms the findings in the isolated perfused rat liver after glucose infusion.[24,25] However, when anesthesia greatly reduced liver phosphorylase activity, the same dose of glucose had no effect on further lowering the reduced level of enzyme activity. Furthermore, since the concentration of glucose in the livers of the anesthetized animals after glucose injection, 22.70 ± 1.54 mmoles per kilogram, was even higher than that seen in the livers of anesthetized animals after fructose injection, 14.70 ± 0.70 mmoles per kilogram, it seems unlikely that glucose played a significant role in the reduction of phosphorylase activity (82 per cent) in the anesthetized fructose-injected mice.

DISCUSSION

The administration of a fructose load to normal nursing mice of weanling age produces profound changes in levels of metabolic intermediates in liver. Particularly relevant to the enzyme, liver glycogen phosphorylase, are the changes in Pi and fructose-1-phosphate concentrations. The critically reduced levels of Pi in the liver after fructose injection are in keeping with the consistent fall of Pi levels in plasma of patients with hereditary fructose intolerance during a fructose tolerance test.[2-5,10,26] As others have reasoned, decreased Pi levels in liver are probably due to sequestration of Pi and the large accumulation of fructose-1-phosphate (almost a fiftyfold increase). (This binding of phosphate could also explain the 50 per cent decrease in liver ATP content.)

The findings in this study confirm the postulates of early investigators that reduced Pi levels[4] and increased fructose-1-phosphate levels[5] might affect liver

TABLE 2

In Vivo Effect of Fructose on Phosphorylase Activity in Liver Homogenate

	Procedure	Control	Fructose	Decrease	P Value
		μmoles/gm. min.$^{-1}$			
A	Decapitation 20 min. after injection;* liver removed and weighed at R.T.†	7.16 ± 0.13 (3)‡	4.34 ± 0.89 (4)	39.4%	0.03
B	Decapitation 20 min. after injection; liver removed at R.T., dropped into liquid N_2, and weighed at -20° C.	4.34 ± 0.44 (5)	1.90 ± 0.23 (7)	56.2%	0.002
C	Whole mouse dropped into liquid N_2 20 min. after injection; liver dissected at -35° C. and weighed at -20° C.	5.42 ± 0.20 (4)	3.19 ± 0.57 (5)	41.2%	0.007
D	Anesthetized animals (see methods). Decapitation 30 min. after injection; preparation of tissue as in B	2.43 ± 0.31 (5)	0.43 ± 0.11 (6)	82.3%	< 0.001

*The dose of fructose was 30 mmoles per kilogram, intraperitoneally; controls received 15 mmoles per kilogram NaCl.
†Room temperature.
‡Values are given as the mean ± S.E.M.; number of animals in parentheses.

glycogen phosphorylase activity in hereditary fructose intolerance patients. At the concentrations of Pi and fructose-1-phosphate found in the liver after fructose injection, phosphorylase activity in normal liver homogenate was reduced 88 per cent. Since the concentration of hepatic Pi in fructose-injected mice was reduced to just twice the apparent Km value for Pi and the fructose-1-phosphate concentration was ten times the determined Ki value, this result is not surprising. The concentrations of fructose-1-phosphate in the livers of patients with hereditary fructose intolerance after a test dose (0.25 gm. per kilogram intravenously; 0.5 to 1.75 mg. per kilogram per os) is not known. Although the dose of fructose used in this study is by comparison quite large (30 mmoles per kilogram or 5.4 gm. per kilogram intraperitoneally), it should be noted that in vitro as little as 0.95 mM fructose-1-phosphate, equivalent to a tissue concentration of 0.267 gm. per kilogram, caused a doubling of the apparent Km for Pi (figure 3).

TABLE 3

In Vivo Effect of Glucose on Phosphorylase Activity in Liver Homogenate

Procedure	Control	Glucose	Decrease	P Value
	μmoles/gm. min.$^{-1}$			
Decapitation 20 min. after injection*; liver removed and weighed at R.T.†	8.59 ± 0.36 (3)‡	4.85 ± 0.60 (4)	43.5%	0.012
As above except with anesthesia (see Methods)	2.00 ± 0.04 (4)	2.56 ± 0.39§ (4)	—	N.S.

*The dose of glucose was 30 mmoles per kilogram intraperitoneally; controls received 15 mmoles per kilogram NaCl.
†Room temperature.
‡Values are given as the mean ± S.E.M.; number of animals in parentheses.
§Liver glucose concentration 22.70 ± 1.54 mmoles per kilogram vs 4.89 ± 0.13 in controls, $p < 0.001$.

Apart from the ill effects of decreased Pi and increased fructose-1-phosphate levels on normal active liver phosphorylase in vitro, as Cornblath et al.[5] conjectured, there appears to be another mechanism for reduced enzyme activity in vivo. Under all experimental conditions tested there was a consistent, highly significant decrease in the level of the active form of the enzyme in fructose-injected mice. The cause of this finding is not immediately apparent. NaF was used in the homogenizing solution to inhibit phosphorylase phosphatase[27] and the enzyme was measured with optimal concentrations of glycogen and Pi. The extreme dilution of liver in the assay reagent would virtually eliminate the possibility of inhibition due to the abnormal accumulation of metabolites seen in vivo; in particular, fructose-1-phosphate was diluted to noninhibitory levels. The presence of inhibitors in the enzyme preparation was further excluded by combined assays of liver homogenates from control and fructose-injected animals—results were additive. As in the case for muscle phophorylase A, it is generally believed that the formation of the active form of the liver enzyme involves a number of interdependent enzyme steps. Any of the metabolic changes in liver observed in this and other reported

studies after fructose injection (increased fructose and its phosphorylated intermediates, increased glucose and glucose-6-phosphate, increased IMP, decreased ATP, UTP, UDPG and Pi, to name a few) could influence this activation process. Effects of fructose injection on phosphorylase phosphatase[27,28] or other inactivating enzymes must also be considered. Recently Van den Berghe, Hue and Hers reported observations similar to ours using older animals (20 gm. vs. 6 to 12 gm. mice used in our experiments). As an explanation for the inactivation of liver phosphorylase after fructose these authors postulated a decrease of free cyclic AMP levels despite the fact that total cyclic AMP levels were unchanged. The possibility of inactivation of liver phosphorylase due to rapid conversion of fructose to glucose in these older animals must also be considered.[7,13]

In conclusion, it appears that in vivo, liver glycogen phosphorylase activity in fructose-injected mice would be seriously reduced by two mechanisms; first by a reduction in the level of active enzyme and secondly by diminished activity of the remaining active enzyme by reduced Pi and elevated fructose-1-phosphate levels.

However, an important issue remains. If diminished glycogenolysis due to a decrease and inhibition of liver phosphorylase does indeed play a role in the fructose-induced hypoglycemia of patients with hereditary fructose intolerance, then why do we not see reduced liver glucose levels after fructose injection? Blood glucose levels were not measured in this study, but it is generally accepted that plasma glucose values are accurately reflected in the liver glucose concentration. Although there is some evidence that fructose injection inhibits fructose-1-phosphate aldolase in normal animals (by the accumulation of IMP[8]) and fructose-1-phosphate inhibits fructose diphosphate aldolase in vitro,[2] at this time our only explanation is that in normal mice there is enough activity of these and other gluconeogenic enzymes to permit sufficient conversion of fructose to glucose to mask any hypoglycemia which might result from a decrease in glycogen breakdown in liver by reduced glycogen phosphorylase activity. The fact that liver glucose levels were not elevated more quickly after fructose injection may perhaps support this speculation. It is hoped that this experimental model of hereditary fructose intolerance will be useful to others in future investigations of the unsolved problems of this disorder.

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