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The Cause of Hepatic Accumulation of Fructose 1-Phosphate on Fructose Loading

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1. The changes in the metabolite content in freeze-clamped livers of fed rats occurring on perfusion with 10mM-D-fructose have been examined. 2. The most striking effects of fructose were an accumulation of fructose 1-phosphate, as already known, up to 8.7 μmol/g of liver within 10min, a loss of total adenine nucleotides (up to 35% after 40min) with a decrease in the ATP content to 23% within 10min, a sevenfold rise in the concentration of IMP to 1.1 μmol/g and an eightfold rise of α-glycerophosphate to 1.1 μmol/g. 3. There was a transient decrease in P_i from 4.2 to 1.7 μmol/g. Within 40min the P_i content recovered to the normal value, probably because of an uptake of P_i from the perfusion medium. 4. The degradation of the adenine nucleotides beyond the stage of AMP can be accounted for by the decrease of ATP and P_i. As ATP inhibits 5-nucleotidase, and as P_i inhibits AMP deaminase any AMP arising in the tissue is liable to undergo dephosphorylation or deamination under the conditions occurring after fructose loading. 5. The content of lactate increased to 4.3 μmol/g at 80min; pyruvate also increased and the [lactate]/[pyruvate] ratio remained within physiological limits. 6. The concentration of free fructose within the liver remained much below that in the perfusion medium, indicating that the rate of penetration of fructose into the tissue was lower than the rate of utilization. 7. The fission of fructose 1-phosphate by liver aldolase is inhibited by several phosphorylated intermediates, especially by IMP. This inhibition is competitive with a K_i of 0.1mM. 8. The maximal rates of the enzymes synthesizing and splitting fructose 1-phosphate are about equal. The accumulation of fructose 1-phosphate on fructose loading is due to the inhibition of the fission of fructose 1-phosphate by the IMP arising from the degradation of the adenine nucleotides.

Kjerulf-Jensen (1942) found that fructose 1-phosphate accumulates in the mammalian liver when the body is loaded with D-fructose (see also Heinz, 1968; Burch, Max, Chyu & Lowry, 1969). Fructose 1-phosphate is formed by the ketohexokinase (EC 2.7.1.3) reaction:

Fructose + ATP → fructose 1-phosphate + ADP (1)

and the removal of fructose 1-phosphate is brought about by the ketose 1-phosphate aldolase (EC 4.1.2.7) reaction:

Fructose 1-phosphate → dihydroxyacetone phosphate + D-glyceraldehyde (2)

Assays of the two enzymes indicate that under the conditions that exist in rat liver the rates of reaction of the two enzymes (when maximally active) are about the same and in view of this the accumulation of fructose 1-phosphate is unexpected. The experiments reported in this paper show that the aldolase is in fact partially inhibited after fructose loading because of an increase in the concentrations of IMP, an effective inhibitor of liver aldolase. The accumulation of IMP is connected with the hepatic depletion of the adenine nucleotides after fructose loading which was discovered by Mäenpää, Raivio & Kekomäki (1968) and Raivio, Kekomäki & Mäenpää (1969) (see also Burch *et al.* 1969). This depletion of the adenine nucleotides in turn is connected with the high rate of reaction (1) which causes a rapid decrease in the concentration of ATP and of P_i. Since ATP is an inhibitor of 5-nucleotidase and P_i an inhibitor of AMP deaminase, the decrease in the concentration of ATP and P_i results in an increased rate of the reactions that cause an irreversible degradation of AMP. Under the conditions used in the present work the concentration of total adenine nucleotides decreased to one-quarter

within 10min of adding fructose to the perfused rat liver whereas that of IMP rose sevenfold. There are thus far-reaching disturbances of liver metabolism on fructose loading.

MATERIALS AND METHODS

Animals and diets. Female Wistar rats each weighing 170–215g obtained from Carworth (Europe) Ltd., Alconbury, Hunts., U.K., were used and were fed on a standard small-animal diet (Spillers Mills Ltd., Gainsborough, Lincs., U.K.).

Reagents. D-Glyceraldehyde was supplied by Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. L-α-Glycerophosphate (dicylohexylammonium salt) was prepared by Mr N. Gascoyne of the Department of Biochemistry, University of Oxford. When analysed by the method of Hohorst (1963*b*) it had a purity of 96% of the calculated L-form. Other substrates, nucleotides, coenzymes, and crystalline enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K.

Liver perfusion. The method of liver perfusion used was that described by Hems, Ross, Berry & Krebs (1966). The perfusion medium was the physiological saline (Krebs & Henseleit, 1932), with 2.6% bovine serum albumin fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.), and washed aged human erythrocytes. The bovine serum albumin was dialysed as a 10% solution (at 4°C) against three changes of physiological saline gassed with CO_2+O_2 (5:95).

The perfusion medium described by Hems *et al.* (1966) contains initially about 1mM-L-lactate [0.87±0.05S.E.M. (14) μmol/ml] derived from the erythrocytes. To decrease the initial lactate concentration the erythrocytes were washed five times with ten times their volume of physiological saline. This lowered the initial lactate concentration in the perfusion medium to 0.23±0.02S.E.M. (16) μmol/ml. The medium was gassed with CO_2+O_2 (5:95) during perfusion.

Sampling of liver. For the analysis of liver, samples were rapidly frozen *in vivo* or during perfusion by using the deep cooled clamps of Wollenberger, Ristau & Schoffa (1960). The resulting disc of liver tissue was ground to a fine powder in a cooled mortar with frequent additions of liquid N_2. The liver powder was transferred to a tared centrifuge tube cooled in liquid N_2 and 4ml of ice-cold 6% (w/v) $HClO_4$ was then added to each gram of liver powder with constant stirring. The resulting slurry was allowed to thaw and then homogenized in the centrifuge tube at low speed with a glass pestle. The homogenate was kept ice-cold for 30min, centrifuged and the resulting supernatant was brought to pH6–7 with 20% (w/v) KOH to precipitate the excess of $HClO_4$ as $KClO_4$. The assays were carried out on the clear supernatant.

Preparation of liver aldolase. Livers of large (300–450g) rats were bled by perfusion *in situ* with cold isoosmotic KCl and then homogenized with 4vol. of KCl. After centrifugation at 30000*g* for 20min the supernatant was fractionated with $(NH_4)_2SO_4$ described by Leuthardt & Wolf (1955). The final precipitate was taken up in a small volume of water (0.3ml/g of original liver) and dialysed against 200vol. of water at 0°C, changed every hour for 4h. The cloudy preparation was centrifuged and 0.1ml of 0.1M-EDTA was added to every 4ml of clear supernatant. Incubation for 1h at 25°C completely inactivated sorbitol dehydrogenase (EC 1.1.1.14) (Hers, 1956), which would otherwise react with fructose. The final preparation, containing 35–45mg of protein/ml was stored at −18°C and lost only 30% activity in 1 year. In addition to aldolase activity it also contained glycerol 1-phosphate dehydrogenase (EC 1.1.1.8) activity and triose phosphate isomerase (EC 5.3.1.1.) activity.

Other aldolase preparations. Chilled fresh rat and rabbit tissues were homogenized with 14vol. of 1mM-EDTA and centrifuged for 20min at 30000*g*. The supernatant obtained was used in assays without further purification. A crystalline preparation of rabbit muscle aldolase was supplied by the Boehringer Corp. (London) Ltd.

Aldolase assays. These were based on the measurement of dihydroxyacetone phosphate formed from the fission of fructose 1-phosphate or fructose 1,6-diphosphate (see Leuthardt, Testa & Wolf, 1953). Reaction mixtures were: 50mM-tris–HCl buffer, pH7.4, 0.12mM-NADH, substrates, inhibitors, and enzymes in a total vol. of 3ml. Similar mixtures without substrate were run simultaneously as blanks, to measure any NADH removal by other reactions in the tissue preparations. The dialysed liver aldolase preparation already contained adequate amounts of glycerol 1-phosphate dehydrogenase and triose phosphate isomerase, but with other rat and rabbit tissue preparations and crystalline rabbit muscle aldolase, 5μl of a glycerol 1-phosphate dehydrogenase–triose phosphate isomerase mixture (Boehringer) was added to the cuvettes. Reactions were started by the addition of the aldolase preparation and changes in extinction at 340nm were measured at 25°C. Readings were taken at 3min intervals. The progress curves were approximately linear. The percentage inhibition was calculated from corrected extinction changes of about 0.25 to obtain reliable values.

Analytical methods. ATP was determined by the method described by Lamprecht & Trautschold (1963), ADP and AMP were determined in the combined assay of Adam (1963). P_i was determined by the method described by Berenblum & Chain (1938) as modified by Martin & Doty (1949). Fructose 1-phosphate was determined by the method of Eggleston (1970). Fructose 1,6-diphosphate was measured together with total triose phosphates in the combined assay of Bücher & Hohorst (1963); pyruvate, phosphoenolpyruvate, 2- and 3-phosphoglycerate were determined in sequence (Czok & Eckert, 1963). The references to other analytical methods are as follows: α-glycerophosphate (Hohorst, 1963*b*); L-(+)-lactate (Hohorst, 1963*c*); glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963*a*); glucose 1-phosphate (Bergmeyer & Klotzsch, 1963); glucose and fructose (Klotzsch & Bergmeyer, 1963); the sum of D-glyceraldehyde and glycerol (Pinter, Hayashi & Watson, 1967). For the fluorimetric determination of very low concentrations of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by the method of Veech, Raijman, Dalziel & Krebs (1969) a portion of the neutralized supernatant was shaken for 1min with Florisil (100–200U.S. mesh) to remove flavins and then recentrifuged before use. In livers perfused with fructose where the concentration of dihydroxyacetone phosphate was increased it was determined by the spectrophotometric method of Bücher & Hohorst (1963).

IMP was determined by a combination of paper chromatographic separation (Krebs & Hems, 1953) and a spectrophotometric assay. A portion of deproteinized liver extract (0.1 or 0.2ml) was dried on to a 1cm area on Whatman no. 1 chromatograph paper under a current of hot air. Duplicates, with and without added IMP standards (10μl, 2mM solutions) on the same spot, were developed by descending chromatography with the isobutyric acid–ammonia solvent mixture described by Krebs & Hems (1953) for 45–48h at room temperature. After drying in a current of air the papers were examined under u.v. light from a Chromatolite lamp (Hanovia Ltd., Slough, Bucks., U.K.) and absorbent areas were ringed by pencil. Average distances run from the starting line were: IMP 23cm, ATP 27cm, ADP 32cm, AMP and inosine 37cm. IMP areas, and a blank area of similar size before the starting line, were cut out and dropped into 4ml of 10mM-potassium phosphate buffer, pH7.0. After gentle mixing at intervals for 1h 3ml was removed and the extinction at 248nm in 1cm wide silica cells in a Zeiss spectrophotometer was determined. At this wavelength the $\epsilon_{max.} \times 10^{-3}$ for IMP is 12.3 (Deutsch, 1952). Recovery of standards by the whole procedure was 93–104%.

RESULTS

Concentrations of intermediary metabolites in the freeze-clamped perfused rat liver after fructose loading. The changes in the concentrations of liver metabolites occurring at different time-intervals after fructose loading (addition of 10mM fructose to the perfusion medium) are shown in Table 1. Apart from the accumulation of fructose 1-phosphate the changes in the adenine nucleotides, as described by Mäenpää *et al.* (1968), were, from the quantitative point of view, the most noteworthy effects.

The concentration of ATP decreased to 23% within 10min and then slowly increased, reaching 48% of the original value at 80min. The sum of the adenine nucleotides decreased to 42% in 10min and was 50% at 80min. Some of the lost adenine nucleotides appeared as IMP, the concentration of which increased sevenfold within 10min and then decreased, but the lost adenine nucleotides were not fully accounted for by the substances analysed. This deficit increased with time. The findings of Mäenpää *et al.* (1968) (made under slightly different conditions) suggest that the adenine nucleotides were partly broken down to uric acid and allantoin.

Among the phosphorylated products fructose 1-phosphate increased 40-fold within 10min to 8.7μmol/g and α-glycerophosphate rose eightfold to 1.06μmol/g. Dihydroxyacetone phosphate and glyceraldehyde 3-phosphate increased about fourfold. The changes in the concentrations of other phosphorylated compounds, though substantial on a percentage basis in the case of fructose 1,6-diphosphate, glucose 6-phosphate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate, were small in absolute terms.

There was a large decrease in the concentration of P_i at 10min. The value of P_i had returned to normal at 40min and was increased above normal at 80min. The total phosphorus content of the liver, calculated from the mean concentration of phosphorylated intermediates, adenine nucleotides, IMP and P_i, was increased at 10min and at 40min on perfusion with D-fructose. Livers freeze-clamped *in vivo* contained 13.8μmol of total phosphorus/g whereas those perfused with fructose contained 16.3μmol/g at 10min and 40min. The total phosphorus content returned to normal (14.3μmol/g) at 80min. The transient increase in total phosphorus content was due to an increase in the concentration of phosphorylated intermediates. The values for total phosphorus content are approximate because a few intermediates such as UTP and UDP-glucose that decrease in concentration during treatment with D-fructose (Burch *et al.* 1969) were not determined in the freeze-clamped livers. However the contribution of these intermediates is small. At 10min and 40min the increase in total phosphorus content represents an uptake of 19.68μmol of phosphorus from the perfusion medium by 8g of liver. This hepatic phosphorus uptake may account for the transient lowering of serum P_i in human subjects during intravenous D-fructose infusion (Miller, Drucker, Owens, Craig & Woodward, 1952).

The [fructose 6-phosphate]/[glucose 6-phosphate] ratio, [glucose 6-phosphate]/[glucose 1-phosphate] ratio and [2-phosphoglycerate]/[3-phosphoglycerate] ratio were all of the order expected for equilibrium, 0.25–0.57, 27.5 and 0.14–0.20. At 25°C the equilibrium constants are 0.43, 18 and 0.17 respectively (Burton, 1957) and they must be similar at 38°C for which temperature no values are available. The mass action ratio of the adenylate kinase system, $[ATP][AMP]/[ADP]^2$, which varies somewhat with the concentration of Mg^{2+}, was outside the expected range after 10 and 40min. Adenylate kinase is taken to be located in the outer mitochondrial membrane and to act slowly upon the cytoplasmic adenine nucleotides (Chappell & Crofts, 1965; Brdiczka, Pette, Brunner & Miller, 1968). As ketohexokinase, AMP deaminase and 5-nucleotidase are cytoplasmic enzymes the depletion of the adenine nucleotides after fructose loading is expected to concern the cytoplasm in the first instance. If the adenine nucleotides that remain after fructose loading were mainly mitochondrial some deviations of the mass action ratio from equilibrium would be understandable.

The concentrations of lactate and pyruvate increased steadily and in parallel. Lactate reached an average concentration of 4.3μmol/g after 80min which is much higher than the concentrations obtained in the absence of fructose when, under

Table 1. *Contents of intermediary metabolites in the perfused rat liver*

Livers of fed rats were perfused with a medium containing D-fructose (initial concentration 10mM) and were freeze-clamped at various times after the start of the perfusion. The initial values refer to livers of fed rats freeze-clamped *in vivo* after cervical dislocation. The results are means±S.E.M.

	Livers not perfused	Livers perfused with D-fructose		
Time of perfusions (min)...	0	10	40	80
Number of observations...	4	4	4	3
Content of metabolites (μmol/g wet wt.)				
Fructose 1-phosphate	0.23 (2)	8.72±0.83	7.68 ±0.98	3.27 ±1.31
ATP	2.22 ±0.07	0.51±0.16	0.77 ±0.11	0.99 ±0.01
ADP	0.78 ±0.08	0.66±0.07	0.20 ±0.03	0.50 ±0.10
AMP	0.26 ±0.05	0.20±0.03	0.17 ±0.02	0.14 ±0.002
Total adenine nucleotides	3.27 ±0.12	1.37±0.08	1.14 ±0.13	1.64 ±0.13
P_i	4.25 ±0.25	1.67±0.29	3.97 ±0.26	5.62 ±0.28
IMP	0.165 (2)	1.14±0.08	0.37 ±0.13 (3)	0.26 ±0.02
α-Glycerophosphate	0.13 ±0.10	1.06±0.23	0.60 ±0.05	0.45 ±0.07
Free fructose	<0.01	3.86±0.51	0.82 ±0.20	0.22 ±0.11
Glucose	6.99 (2)	2.29±0.55	5.01 ±0.45	5.91 ±0.16
Lactate	0.79 ±0.09	1.34±0.15	3.57 ±0.34	4.32 ±0.68
Pyruvate	0.08 ±0.01	0.15±0.03	0.35 ±0.02	0.36 ±0.05
Fructose 1,6-diphosphate	0.02 ±0.002	0.10±0.02	0.03 ±0.01	0.02 ±0.002
Glucose 6-phosphate	0.25 ±0.03	0.14±0.04	0.38 ±0.04	0.20 ±0.06
Fructose 6-phosphate	0.06 ±0.01	0.06±0.01	0.11 ±0.01	0.07 ±0.02
Glucose 1-phosphate	<0.01	<0.01	0.01 ±0.001	<0.01
Phosphoenolpyruvate	0.16 ±0.02	0.05±0.02	0.19 ±0.03	0.22 ±0.01
2-Phosphoglycerate	0.04 ±0.01	0.03±0.01	0.07 ±0.01	0.08 ±0.003
3-Phosphoglycerate	0.26 ±0.06	0.16±0.03	0.47 ±0.06	0.57 ±0.06
Glyceraldehyde 3-phosphate	0.006±0.0002	0.02±0.004	0.006±0.0003	0.007 (2)
Dihydroxyacetonephosphate	0.04 ±0.003	0.16±0.04	0.06 ±0.002	0.06 (2)
[Lactate]/[pyruvate]	9.60 ±0.96	10.1 ±1.23	10.4 ±1.24	12.0 ±0.73
[Fructose 6-phosphate]/[glucose 6-phosphate]	0.25 ±0.02	0.57±0.12	0.30 ±0.01	0.35 ±0.06
[Glucose 6-phosphate]/[glucose 1-phosphate]	—	—	27.5 ±3.8	—
[2-Phosphoglycerate]/[3-phosphoglycerate]	0.20 ±0.06	0.19±0.02	0.14 ±0.01	0.15 ±0.01
[α-Glycerophosphate]/[dihydroxyacetone phosphate]	3.55 ±0.40	9.38±3.32	9.61 ±1.08	8.52 ±1.95
$[ATP][AMP]/[ADP]^2$	0.97 ±0.17	0.31±0.17	3.80 ±0.70	0.60 ±0.17

Table 2. *Balance of fructose metabolism in the perfused rat liver*

The amounts of products formed represent the sum of metabolites in medium and liver. The quantities of most other intermediates in medium and liver (listed in Table 1) were negligibly small and have therefore been omitted from the calculation of the balance.

Expt. no	Liver wt. (g)	Duration of perfusion (min)	Fructose removed (μmol)	Products formed					
				Fructose 1-phosphate (μmol)	Lactate (μmol)	Pyruvate (μmol)	Glucose (μmol)	Sum of products (expressed in C_6 units) (μmol)
1	9.36	40	1048	62	594	30	701	1075
2	7.51	40	756	79	456	45	559	888
3	8.00	40	1065	59	707	40	749	1182
4	10.80	40	1049	66	696	87	702	1160
5	10.14	80	1328	27	1419	52	898	1660
6	9.30	80	1175	54	748	78	930	1397
7	9.95	80	1074	14	545	67	907	1227

the same conditions, 2mM was rarely exceeded. The [lactate]/[pyruvate] ratio remained fairly constant throughout the perfusion period at a value near 10.

Balance between fructose disappearance and metabolites formed. The sum of the products formed from fructose, as calculated in Table 2, was somewhat greater (up to 10% at 40min and up to 23% at 80min) than the amounts of fructose removed. This surplus is explained by the inclusion of some glucose formed from glycogen. The calculations indicate that the greater part of the fructose removed was converted into glucose. It is unlikely that there were significant quantities of metabolites other than those listed in Table 1, and the complete oxidation of fructose could at most have contributed 10% to the removal of fructose because the rate of oxygen consumption was 2.6μmol/min per g of liver. This could have oxidized 0.43μmol of fructose/min whereas the rate of removal was 2.63μmol/min per g of liver.

The values for the mean initial rates of fructose removal and presence of glucose, lactate and pyruvate in the perfusion medium (Table 3) were obtained for the balance experiments of Table 2 from a plot of metabolites in the total medium versus time. Linearity usually extended for about 60min. The values for the rates confirm that virtually all the fructose removed during the earlier period of perfusion is accounted for by the formation of glucose, lactate and pyruvate.

A comparison of the fructose concentration in the freeze-clamped perfused liver with that in the perfusion medium at the time of clamping shows that a gradient existed for this metabolite between the medium and the liver (Table 4). The concentrations in the liver were several-fold lower. The values for fructose content of the freeze-clamped liver have been corrected for the fructose content and volume of the entrapped medium by using the value 22.2% obtained by Krebs, Freedland, Hems

Table 3. *Rates of metabolite changes in the medium of the perfused rat liver*

The initial rates of metabolite changes during perfusion with D-fructose (initial concentration 10mM) were calculated from a plot of each metabolite in the total perfusion medium versus time. The results are means ± S.E.M. for seven observations. The experiments were the same as those in Table 2.

Metabolite changes	Rate (μmol/min per g wet wt.)
Fructose removed	2.63 ± 0.25
Glucose formed	1.67 ± 0.22
Lactate formed	1.74 ± 0.16
Pyruvate formed	0.15 ± 0.01

Table 4. *Fructose content of liver and perfusion medium*

The values for fructose content of the liver have been corrected for the fructose content of the extracellular space using the figure of 22.2% (Krebs *et al.* 1969). The bulk of the extracellular space was the perfusion medium within the hepatic vascular system.

		Fructose content			
			Perfused liver		
Time (min)	Expt. no.	Perfusion medium (μmol/ml)	Uncorrected (μmol/g wet wt.)	Corrected for fructose content and volume of the extracellular space (μmol/g wet wt.)	Fructose in medium / Fructose in tissue (corrected) ratio
10	1	8.53	5.23	4.29	2.0
	2	8.05	4.04	2.89	2.8
	3	7.33	3.05	1.83	4.0
	4	8.74	3.13	1.53	5.7
40	1	2.60	0.55	<0.01	
	2	3.05	0.82	0.18	16.9
	3	4.52	1.39	0.50	9.0
	4	2.73	0.53	<0.01	
80	1	1.60	0.36	<0.01	
	2	0.23	<0.01	<0.01	
	3	1.13	0.29	<0.01	

& Stubbs (1969) for the extracellular space, which consists mainly of the liver vascular system and its contents.

The initial rate of fructose removal was 2.63 μmol/min per g wet wt. of liver and the existence of the concentration gradient implies that the rate of penetration of fructose is less than the rate of its metabolism.

Cause of accumulation of fructose 1-phosphate. The results outlined above can serve as a basis for the calculation of the rates of fructose 1-phosphate formed by the tissue compared with that accumulated. Within 10 min 8.7 μmol of fructose 1-phosphate had accumulated per g of tissue. Since the capacity of hexokinase is very low in relation to that of ketohexokinase (Heinz, Lamprecht & Kirsch, 1968) most of the other products formed from fructose (at a collective rate of 2.63 μmol/min per g, Table 3) must have passed through the stage of fructose 1-phosphate, about 30% of the fructose 1-phosphate formed during the first 10 min had accumulated, and although the rate of fructose removal remained constant for another 35–50 min the concentration of fructose 1-phosphate did not continue to increase. This fact, together with the observation that the potential capacity of the aldolase is high enough to split more than 2.6 μmol of fructose 1-phosphate/min per g (Table 8), suggests that the accumulation of fructose 1-phosphate is caused by an inhibition of the aldolase that is overcome when the concentration of fructose 1-phosphate rises. The following experiments bear out this hypothesis.

A systematic search for inhibitors of the aldolase reaction among liver constituents (Table 5) shows that several phosphorylated metabolites inhibit liver aldolase and that the fission of fructose 1-phosphate is much more sensitive to inhibitions than the fission of fructose 1,6-diphosphate. However, the concentration of the majority of potential inhibitors is not sufficiently high in the liver to be effective. Possible exceptions are IMP, AMP, GMP, UMP, CMP and α-glycerophosphate. The tri- and di-phosphates of adenosine, guanosine, uridine and cytidine are powerful inhibitors in the absence of Mg^{2+} but since these substances are present in the tissue mainly as Mg^{2+} chelates they cannot be major inhibitors *in vivo*. The concentrations of GMP, UMP and CMP are probably too low for them to be major inhibitors so that IMP and α-glycerophosphate must be taken to be the main agents that affect the activity of aldolase, because the sum of the concentrations of GTP, GDP, GMP, UTP, UDP, UMP, CTP, CDP, CMP is less than 1.5 μmol/g of liver (Bücher, Krejci, Rüssman, Schnitger & Weseman, 1964; Hurlbert, Schmitz, Brumm & Potter, 1954; Schmitz, Hurlbert & Potter, 1954). Adenosine, inosine, adenine, hypoxanthine, fructose and glucose were not inhibitory.

The extent of the inhibition of fructose 1-phosphate fission by a particular inhibitor depends on the presence of other inhibitory phosphorylated compounds in the incubation medium. In general the inhibitions recorded in Table 5 were not additive. Measurements of the rate of fission in solutions designed to imitate the essential metabolite

Table 5. *Inhibitions by cell constituents of the rat liver aldolase reactions in tris–hydrochloric acid buffer at* pH7.4

The preparation of the liver extract and details of the assay are given in the Materials and Methods section. The effect of Mg^{2+} was tested when it was found that Mg^{2+} can abolish the inhibition of activity on fructose 1-phosphate by ATP. The substances marked with an asterisk contained, according to the makers, between 1 and 6% of the mono- and di-phosphates.

Substances tested (1mM)	Percentage inhibition of aldolase reactions with: Fructose 1-phosphate (0.067mM)	Fructose 1-phosphate (0.067mM) $MgCl_2$ (7.5mM)	Fructose 1,6-diphosphate (0.033mM)
ATP	63	0	0
ADP	42	17	0
AMP	54	44	0
ITP*	68	36	0
IDP	56	71	0
IMP	90	74	0
GTP*	82	27	0
GDP	75	52	0
GMP	80	57	0
UTP*	58	13	0
UDP	59	38	0
UMP	67	50	0
CTP	78	0	0
CDP	51	14	0
CMP	57	37	0
L-α-Glycerophosphate	35	37	20
2-Phosphoglycerate	44	37	6
3-Phosphoglycerate	41	38	0
Phosphoenolpyruvate	31	36	36
Glucose 1-phosphate	78	30	0
Glucose 6-phosphate	32	27	15
Fructose 6-phosphate	22	30	18
P_i	29	35	0
D-Glyceraldehyde	49	49	0

concentrations of the liver (as recorded in Table 1) show that the inhibition in a liver environment equivalent to perfusion with 10mM-fructose for 10min is 62% and that equivalent to 40min is 22% provided that the fructose 1-phosphate concentration is at the 'normal' value of 0.2μmol/g. Increasing the fructose 1-phosphate concentration to 5μmol/g decreased these inhibitions to 44% and 16% respectively at the two times. The main inhibitor under these conditions is IMP. Although P_i alone is inhibitory this inhibition is not additive to that by IMP. In further experiments not recorded in the Tables combinations of the four purine and pyrimidine mono-, di- and tri-phosphates at physiological concentrations had no significant effects on the inhibition of fructose 1-phosphate fission by IMP and α-glycerophosphate.

Detailed information on the degree of inhibition of fructose 1-phosphate fission by IMP at different substrate and inhibitor concentrations is given in Table 6. A plot of $1/v$ against $1/s$ (Lineweaver & Burk, 1934) from the data in Table 6 indicates a competitive nature of the inhibition and a plot of $1/v$ against $[I]$ according to Dixon (1953) from this and other experiments gave an average K_I of 0.1mM-IMP. The K_m for the substrate from results in Table 6 (graphical methods of Schwimmer, 1950; Dixon, 1953) was approximately 0.18mM-fructose 1-phosphate, but other experiments gave values ranging from 0.14 to 0.66 (average 0.35mM-fructose 1-phosphate). This is lower than the K_m values found for rabbit liver aldolase: 0.8mM (Rutter, Woodfin & Blostein, 1963) and 2mM (Spolter, Adelman & Weinhouse, 1965).

Effect of IMP on aldolases of various tissues. The aldolase activity of the supernatant of homogenized rat and rabbit tisues was tested under the conditions given in Tables 5 and 6 except that higher concentrations of substrate and inhibitor were used. As shown in Table 7 IMP inhibited the fission of

Table 6. *Effect of substrate and inhibitor concentration on the inhibition by IMP of rat liver aldolase fission of fructose 1-phosphate*

The preparation of the liver extract and method of the assay are given in the Materials and Methods section. Corrections have been made for a blank without fructose 1-phosphate.

Concentration of fructose 1-phosphate (mM)	Rate of fission of fructose 1-phosphate ($10^3 \times \Delta E$ in 6 min at 25°C)		
	Control (no IMP)	0.5 mM-IMP	1.0 mM-IMP
0.25	118	30	17
0.5	145	53	27
1.0	163	82	52
2.5	180	115	89
5.0	186	142	121
10.0	180	153	138

Table 7. *Effect of IMP on the rate of fission of fructose 1-phosphate by the aldolases of various tissues*

Details of the tissue preparation and the assay method are given in the Materials and Methods section. Each cuvette contained 0.2 ml of supernatant of a 6.6% (w/v) homogenate. The temperature was 25°C. The concentration of fructose 1-phosphate was 3.3 mM.

Enzyme material	Percentage inhibition by IMP	
	1 mM-IMP	3.3 mM-IMP
Rat liver	43	90
Rat kidney cortex	51	78
Rat brain	22	67
Rat leg muscle	17	45
Rabbit liver	41	71
Rabbit kidney cortex	46	78
Rabbit brain	29	50
Rabbit leg muscle	10	23
Crystalline rabbit muscle aldolase (Boehringer)	0	0

fructose 1-phosphate in the four tissues tested but the degree of inhibition varied, being lower particularly in leg muscle. The effects of IMP were similar in the rat and rabbit tissue extracts and whereas the activity of rabbit leg muscle supernatant was inhibited by IMP there was no effect of IMP when a crystalline rabbit muscle aldolase was tested. This indicates that rabbit muscle contains more than one enzyme that can split fructose 1-phosphate. Under the same conditions the fission of fructose 1,6-diphosphate by the four tissues was not affected by IMP (not recorded in Table 7).

DISCUSSION

Adenine nucleotide depletion after fructose loading. The experiments confirm the finding that loading a rat liver with fructose either by intravenous injection into the living animal or by adding fructose to the medium perfusing the isolated liver causes a rapid loss of hepatic adenine nucleotides (Mäenpää *et al.* 1968; Raivio *et al.* 1969). The loss of adenine nucleotides is accompanied by a large accumulation of fructose 1-phosphate in the liver. The purine component of the adenine nucleotides, as the Finnish authors found, appears partly in the form of allantoin and uric acid in the blood plasma and in the perfusion medium, and partly, as the present experiments show, in the form of IMP in the liver. At 10 min after the addition of fructose 36% of the original adenine nucleotides were recovered as IMP which had reached a concentration of 1.14 μmol/g (Table 1). Despite the loss of adenine nucleotides and the liberation of P_i from the nucleotides there is a transient decrease in P_i content in the tissues evidently because the formation of fructose 1-phosphate and other phosphorylated intermediates outweighed the dephosphorylations.

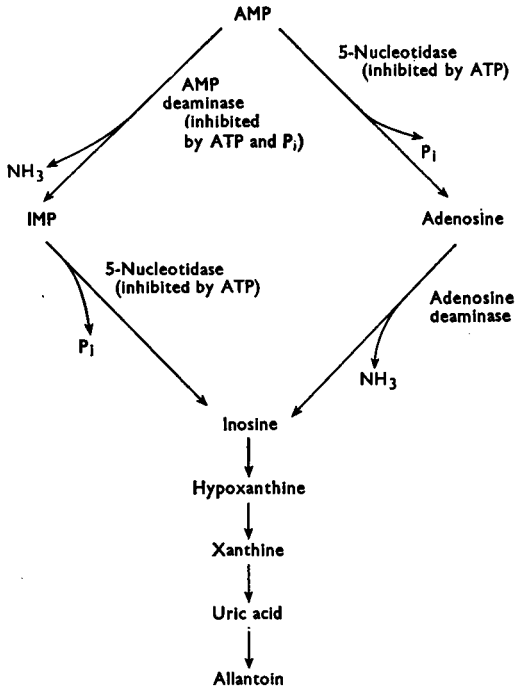
The hepatic changes on fructose loading can be satisfactorily explained on the basis of the properties of the enzymes of fructose metabolism and of adenine nucleotide degradation. The primary step, as suggested by Mäenpää *et al.* (1968), is the rapid reaction of ATP with fructose catalysed by ketohexokinase. This causes a decrease in the concentration of ATP and P_i both of which are essential in stabilizing AMP and therefore the total adenine nucleotide content of the tissue. They inhibit the enzymes that cause virtually irreversible degradation of AMP. ATP inhibits 5-nucleotidase (Baer, Drummond & Duncan, 1966) and P_i inhibits AMP deaminase (Nikiforuk & Colowick, 1956) and at higher concentrations also 5-nucleotidase (Segal & Brenner, 1960). When the inhibition of AMP deaminase becomes less effective the hepatic IMP concentration increases and both AMP and IMP undergo dephosphorylation with the formation of adenosine and inosine and eventually hypoxanthine, uric acid and allantoin, as shown in Scheme I.

Concluding remarks. Whereas the accumulation of fructose 1-phosphate after fructose loading has been known for sometime (Kjerulf-Jensen, 1942) the reasons for the accumulation were hitherto not properly understood. It could not be explained simply on the basis of the relative activities of ketohexokinase and ketose 1-phosphate aldolase as summarized in Table 8 because under the conditions existing in the normal liver the *in vivo* activities of the two enzymes should be about the same. If anything the activity of the ketohexokinase may be expected to be lower than that of ketose 1-phos-

Table 8. *Properties of enzymes of fructose metabolism in rat liver*

The results refer to normal well-fed rats, but the strains and diets used by different authors were not identical. The assays of ketohexokinase in this laboratory were carried out as described by Adelman, Ballard & Weinhouse (1967) and those of ketose 1-phosphate aldolase as described in the Materials and Methods section.

	Ketohexokinase	Ketose 1-phosphate aldolase	References
Maximal rates (μmol/min per g of tissue) at 25°C and pH 7.4–7.5, mean ± S.D. and number of observations	3.12		Adelman *et al.* (1967)
	3.14 ± 0.81 (7)	3.40 ± 0.36 (3)	Present work
	2.20 ± 0.39 (8)	1.63 ± 0.11 (5)	Heinz *et al* (1968)
K_m (mM) (mean, S.D. and no. of observations)	0.2–0.5		Adelman *et al.* (1967)
	0.4		Parks, Ben-Gershom & Lardy (1957)
		0.35 ± 0.18 (8)	Present work



Scheme 1. Pathway of AMP degradation. For the inhibition of AMP deaminase see Ronca-Testoni, Raggi & Ronca (1970).

phate aldolase as soon after loading the concentration of fructose is lower than that of fructose 1-phosphate. The finding that IMP accumulates and that this product is a powerful inhibitor of ketose 1-phosphate aldolase fully accounts for the accumulation of fructose 1-phosphate.

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