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Role of mitochondria in hepatic fructose metabolism

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During metabolism of fructose at concentrations exceeding 5 mM, isolated liver cells accumulate fructose 1-phosphate and lose ATP. At added bicarbonate concentrations below 10 mM in the incubation medium, the addition of atractyloside (or carboxyatractyloside) causes a significant net accumulation of 2-phosphoglycerate, resulting in an increase in the ratio 2-phosphoglycerate : 3-phosphoglycerate from below 1 to greater than 5. Digitonin fractionation revealed that virtually all this 2-phosphoglycerate is associated with the mitochondrial fraction, where it achieves a concentration estimated to be about 40 mM. The amount of 2-phosphoglycerate that accumulates is directly related to the initial concentration of fructose. With DL-glyceraldehyde in place of fructose, an even greater accumulation of 2-phosphoglycerate occurs, and this is also dependent upon both the presence of atractyloside and low bicarbonate. Formation of 2-phosphoglycerate is also observed when isolated mitochondria from rat liver are incubated together with glyceraldehyde and an energy source. The obligatory role of atractyloside for the accumulation of 2-phosphoglycerate within intact cells indicates the involvement of the mitochondrial adenylate translocator in this process, possibly as a carrier directly responsible for 2-phosphoglycerate egress from the mitochondrial matrix. If this is so, competition between 2-phosphoglycerate and ATP for egress from the matrix would be predicted to further exaggerate the fructose-induced depletion of cytosolic ATP.

Introduction

Fructose is rapidly metabolised by mammalian liver [1], the principal end-products being glucose and lactate. Various intermediates also increase significantly, notably fructose 1-phosphate and pyruvate [2–4], particularly when supra-physiological levels of fructose are employed. The rapid removal of fructose is associated with a significant fall in total adenine nucleotides and inorganic phosphate, a decline in the ATP : ADP ratio and a decrease in the ratio of lactate:pyruvate, which may approach half of the normal fasting steady-state value [2–4]. This fall in the concentrations of adenine nucleotides and phosphate has been ascribed to a very rapid rate of fructose phosphorylation which is believed to both 'trap' inorganic phosphate as fructose 1-phosphate and to deplete cytosolic ATP and GTP. Because AMP deaminase is inhibited by GTP and inorganic phosphate [5], the decrease in GTP and inorganic phosphate is thought to lead to a de-inhibition of this enzyme that results in increased formation of IMP and enhanced purine degradation [1,5,6]. It has been suggested that the lowered lactate : pyruvate ratio, reflecting a net accumulation of pyruvate, may be a secondary consequence of the decreased ATP : ADP ratio [7].

Following formation of fructose 1-phosphate, ketose 1-phosphate aldolase catalyses its cleavage to yield dihydroxyacetone phosphate and glyceraldehyde. Whereas the main fate of dihydroxyacetone phosphate is immediate entry into the glycolytic and gluconeogenic pathways, three alternative pathways exist for the removal of glyceraldehyde [2,8]: phosphorylation to form glyceraldehyde 3-phosphate, reduction to glycerol followed by phosphorylation then oxidation to dihydroxyacetone phosphate, or oxidation to glyceric acid followed by phosphorylation to 2-phosphoglycerate. Measurements of activities of the relevant enzymes [9,10] and isotope dilution experiments [11] have indicated that, at physiological concentrations of fructose,

Abbreviations: 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; ATR, atractyloside.

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the predominant route of disposal of the glyceraldehyde moiety is most likely that involving phosphorylation to glyceraldehyde-3-phosphate, which is assumed to be further metabolised via the normal glycolytic and gluconeogenic sequences.

During studies on the function of the mitochondrial adenine nucleotide translocator in replenishment of the cytosolic ATP depleted during fructose metabolism, some unexpected accumulation of intermediary metabolites was noted which led to the conclusion that metabolism of fructose at high concentrations might involve a mitochondrial pathway. This is consistent with the observation that glyceraldehyde oxidation and glycerate phosphorylation can occur within the mitochondrion [12]. The experiments reported here throw new light on this facet of fructose metabolism, and contribute a further and novel explanation for the marked depletion of cytosolic ATP.

Materials and Methods

Materials

Collagenase and enzymes for metabolite determination were from Boehringer-Mannheim (F.R.G.). Digitonin, atractyloside and carboxyatractyloside were obtained from Sigma (St. Louis, MO). Silicone oil (types 200 and 550) were from Dow Corning Corporation (Midland, MI). Other chemicals were of the highest quality commercially available.

Methods

Isolated hepatocytes from normal, male Hooded Wistar rats (250–300 g body weight), starved for 24 h to deplete liver glycogen, were prepared according to a modification [13] of the method of Berry and Friend [14]. More than 90% of the cells excluded 0.2% trypan blue. Hepatocytes (90–120 mg wet wt) were incubated at 37 °C in 2 ml of isotonic 'phosphate-buffered medium' (pH 7.4) of composition: 136 mM Na^+, 5.4 mM K^+, 0.8 mM Mg^{2+}, 2.55 mM Ca^{2+}, 0.8 mM SO_4^{2-}, 11.4 mM phosphate and Cl^-, the concentration of which was normally 130 mM, but which was reduced proportionately to maintain isotonicity when $NaHCO_3$ (0 to 25 mM) was added to the medium. At the end of the incubation period, the vessel contents were deproteinized with an equal volume of 1 M perchloric acid and neutralized. Metabolites were measured by standard enzymatic techniques as described in Ref. 15 by means of a Cobas FARA automated analyser (Roche, Basle), the data being transferred to a PDP11/73 computer (D.E.C., U.S.A.) for subsequent processing. Because of their lability, 2PG and 3PG were always analysed within 6 h of the completion of an experiment.

Mitochondria were isolated by the method described by Hughes and Barritt [16], and incubated, with supplements, in the respiration medium described by Greenawalt [17]. Medium plus mitochondria was gassed with O_2 before incubation. At the completion of incubation, but prior to acid-quenching, particulate and supernatant fractions were separated within 10 s by centrifugation at $12000 \times g$ in an Eppendorf air-fuge. Deproteinisation with perchloric acid, neutralisation and metabolite analysis were the same as for experiments with intact cells.

For the determination of D-fructose 1-phosphate, liver aldolase was prepared as described by Eggleston in Ref. 15, but with the following modifications. Liver homogenisation was by means of a loose-fitting Teflon-in-glass homogeniser, the crude homogenate being centrifuged at $180000 \times g$ for 20 min. On completion of the polyol dehydrogenase-inactivation by incubation with EDTA, Tris-HCl buffer (pH 7.4) was added to a final concentration of 0.1 M and the preparation incubated at 37 °C for 30 min, after which it was centrifuged, filtered and stored frozen in conveniently small portions. In some experiments, separation of cellular cytoplasmic and mitochondrial compartments was achieved by centrifuging samples of digitonin-treated cells layered over silicone oil, according to Ref. 19. The activities of glutamate and lactate dehydrogenases were measured spectrophotometrically [15] at 37 °C in a Cobas FARA analyser.

Protein was determined by the method of Pesce and Strande [18], using a commercial serum-protein standard.

All tabulated values are means ± S.E. Statistical comparisons were made using the two-tailed *t*-test.

Results

In initial experiments hepatocytes, isolated from fasted rats, were incubated for periods of up to 90 min in the presence of 12 mM fructose, either with or without inclusion of atractyloside in the incubation medium, which was buffered with 10 mM phosphate (pH 7.4) and contained no added bicarbonate. Levels of phosphorylated intermediates, and major products accumulating, are shown in Table I, the intermediates present in substantial concentration being fructose 1-phosphate, 3-phosphoglycerate (3PG) and 2-phosphoglycerate (2PG). A notable feature of these experiments was the effect of atractyloside, an inhibitor of the mitochondrial adenine nucleotide translocase [20,21]. Atractyloside inhibited both the rate of removal of fructose and the rate of formation of glucose, and induced a very marked accumulation of 2PG and a corresponding increase of the ratio 2PG:3PG (Table I). This observation was not anticipated because of the expectation that phosphoglycerate mutase would maintain a ratio close to 0.2, the equilibrium position for the reaction [4]. Other inhibitors of mitochondrial en-

TABLE I

Influence of fructose, atractyloside and bicarbonate on selected parameters of intermediary metabolism

Freshly prepared hepatocytes were incubated for 30 min in phosphate-buffered medium as described in Materials and Methods, with the following additions where indicated in the table: 12 mM fructose, 25 μM atractyloside, 25 mM bicarbonate. Measurements, presented as mean values ± S.E. are the mean of 4–7 measurements. Significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) are between treatments and the 'fructose' treatment.

Additions	Rate of change (μmol/min per g wet wt)				Metabolite concentration (μmol/g wet wt)				Mean ratio: $\frac{2PG}{3PG}$
	fructose	glucose	lactate	pyruvate	fructose 1-phosphate	ATP	3PG	2PG	
Nil	–	0.06 *** ±0.01	0.01 *** ±0.006	0.003 *** ±0.002	0.32 ±0.06	1.72 *** ±0.12	0.21 * ±0.04	0.11 *** ±0.03	0.5
ATR	–	0.05 *** ±0.01	0.05 *** ±0.02	0.008 *** ±0.005	0.26 ±0.05	0.93 *** ±0.05	0.16 * ±0.03	0.08 *** ±0.02	0.5
Fructose	−3.23 ±0.12	1.50 ±0.09	1.19 ±0.09	0.06 ±0.01	9.8 ±0.4	0.44 ±0.04	0.49 ±0.09	0.68 ±0.05	1.4
Fructose, ATR	−1.97 *** ±0.11	0.37 *** ±0.02	1.82 *** ±0.05	0.21 *** ±0.01	7.3 * ±0.3	0.36 ±0.01	0.41 ±0.03	3.18 *** ±0.12	7.8
Fructose, HCO_3^-	−4.31 ** ±0.21	2.02 * ±0.11	1.52 ±0.17	0.41 *** ±0.04	10.1 ±0.5	0.76 ** ±0.05	0.28 ±0.02	0.16 *** ±0.02	0.5
Fructose, ATR, HCO_3^-	−4.31 ** ±0.20	1.45 ±0.08	4.06 *** ±0.13	0.59 *** ±0.05	4.9 *** ±0.2	1.03 *** ±0.05	0.38 ±0.05	0.40 ** ±0.05	1.0

ergy transduction (dinitrophenol, cyanide, rotenone and oligomycin) also decreased the uptake of fructose and the formation of glucose to the same extent as atractyloside but, in contrast to atractyloside, did not cause an elevation of the 2PG level (data not presented).

The time-dependent accumulation of 2PG in cells incubated in phosphate-buffered medium (with and without added bicarbonate) is illustrated in Fig. 1. In the presence of atractyloside but absence of bicarbonate, 2PG increased almost linearly until about 30 min, approaching a maximal value only after a further 30 to 60 min incubation. In the absence of atractyloside, the accumulation of 2PG was very much slower, whereas no 2PG accumulated in bicarbonate-buffered medium irrespective of the presence of 25 μM atractyloside. Preincubation of cells with atractyloside before introduction of fructose did not alter the response (data not presented).

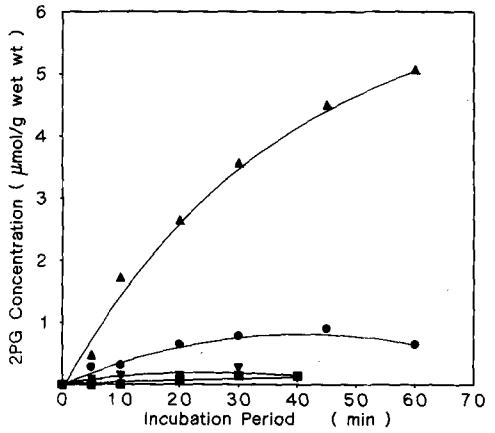

Fig. 1. Effect of atractyloside and bicarbonate on the accumulation of 2PG in the presence of fructose. Hepatocytes were incubated, as described in Materials and Methods, for the times indicated, in phosphate-buffered medium containing 12 mM fructose. Further additions to the medium were: ●, Nil; ■, 25 mM bicarbonate; ▲, 25 μM ATR; ▼, 25 mM bicarbonate, 25 μM ATR.

Influence of fructose concentration

It was found that the atractyloside-dependent accumulation of 2PG in phosphate-buffered incubation medium was dependent upon the fructose concentration (Fig. 2). At initial concentrations of fructose less than 4 mM the level of 2PG (and the ratio 2PG:3PG) were not influenced by the presence of 25 μM atractyloside. However, at fructose concentrations of 4 mM or more the atractyloside-dependent accumulation of 2PG became marked.

After an initial rapid removal of fructose, which was complete by 10 min, the rate of subsequent fructose uptake in the presence of atractyloside was not greatly dependent upon the initial fructose concentration provided that this was greater than 4 mM. However, the decrease of steady-state concentration of ATP and accumulation of 2PG were much larger at higher fructose concentrations (Fig. 2).

Influence of atractyloside concentration

The dependence of the extent of 2PG accumulation on the concentration of atractyloside was examined (Fig. 3). Both fructose removal and glucose formation were depressed, by 1.5- and 4-fold, respectively, by 25–100 μM atractyloside in the absence of added bicarbonate. The level of 2PG was elevated more than

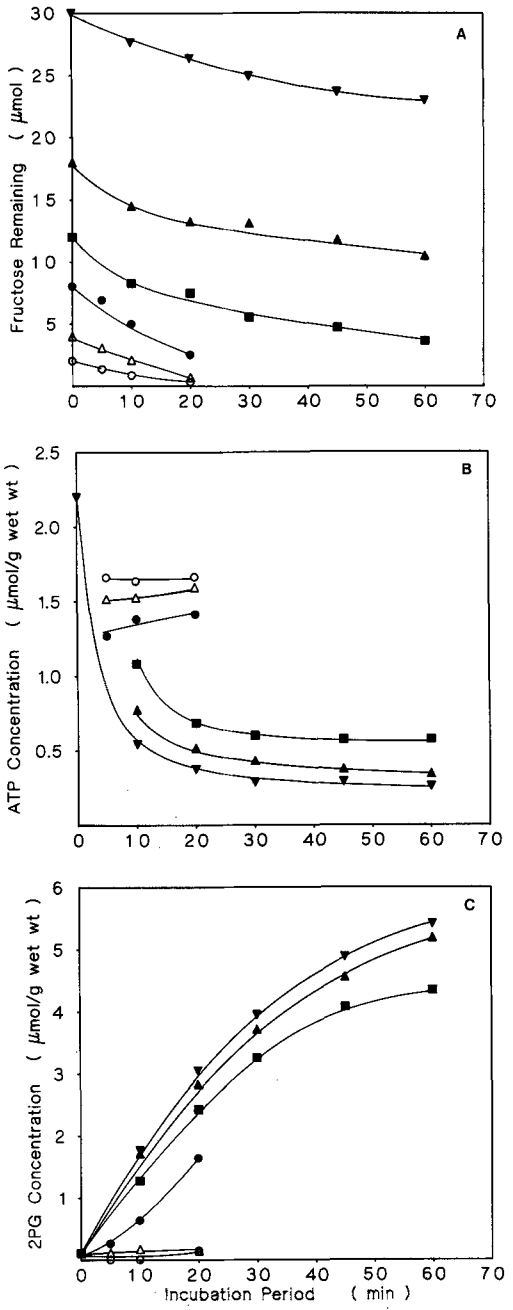

Fig. 2. Time-course of fructose metabolism at varying concentrations of fructose. Hepatocytes were incubated for variable times in phosphate-buffered medium containing 25 μM ATR and the following initial concentrations of fructose: ○, 1 mM; △, 2 mM; ●, 4 mM; ■, 6 mM; ▲, 9 mM; ▼, 15 mM. Metabolites measured include residual fructose (A), ATP concentration (B) and 2PG concentration (C).

5-fold by 100 μM atractyloside. Essentially the same metabolic effects were observed when equivalent concentrations of carboxyatractyloside (a structurally similar inhibitor of the adenine nucleotide translocase) were used instead of atractyloside (data not shown). The only significant difference noted was that 2PG accumulated at lower concentrations of carboxyatractyloside than of atractyloside. For the majority of subsequent experiments, 25 μM atractyloside was selected as the inhibitor concentration routinely employed, since maximal elevations of 2PG and the 2PG:3PG ratio were approached at around this concentration.

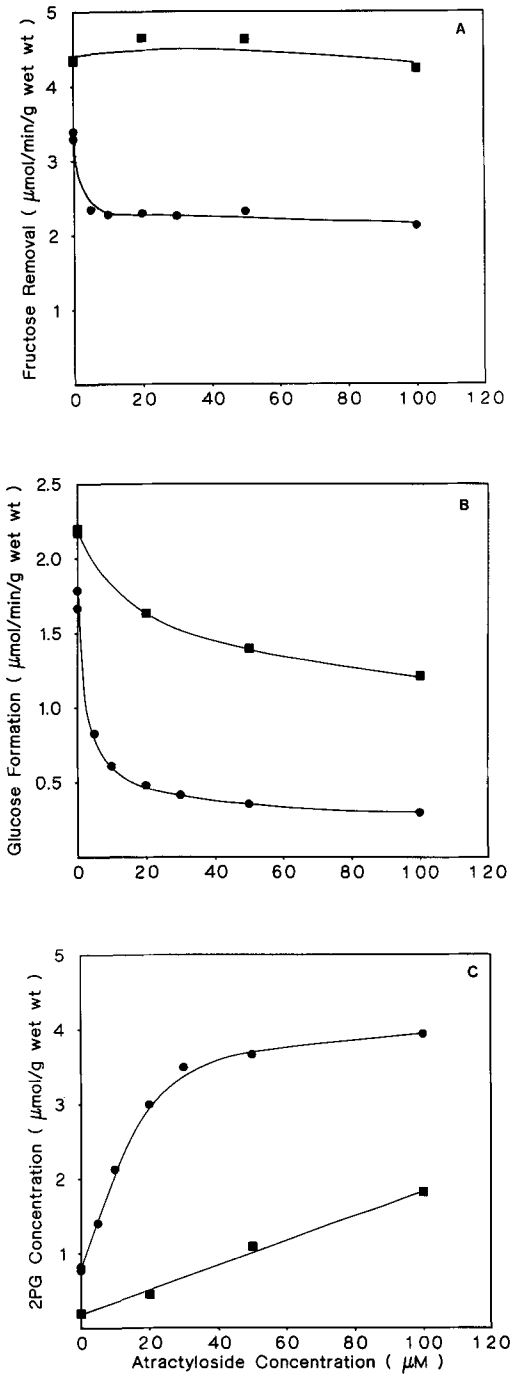

Fig. 3. Effect of atractyloside concentration on fructose metabolism, in the absence or presence of bicarbonate. Hepatocytes were incubated for 30 min in phosphate-buffered medium supplemented with 12 mM fructose and the indicated concentration of ATR. Further additions were: ●, Nil; ■, 25 mM bicarbonate. The figure shows the rate of fructose removal (A), rate of glucose formation (B) and 2PG concentration (C).

Influence of bicarbonate

In further studies, analogous experiments were carried out using an incubation medium which was buffered with 25 mM bicarbonate in equilibrium with an atmosphere of 5% CO_2:95% O_2 (Table I and Fig. 1). In contrast to the results obtained with bicarbonate-free medium, virtually no accumulation of 2PG was observed in bicarbonate-buffered medium even though atractyloside was effective in inhibiting glucose formation. Concentrations of bicarbonate in excess of 10 mM were sufficient to prevent the accumulation of 2PG (Fig. 4A). Moreover, addition of 25 mM bicarbonate after 30 min preincubation of cells with fructose and atractyloside in bicarbonate-free medium, resulted in the removal of previously-accumulated 2PG within about 10 min (Fig. 4B).

Site of 2PG accumulation

By separation of cells from medium, it was determined that virtually all of the measurable 2PG and 3PG was confined to the cell pellet, following 60 min incubation in the presence of fructose and atractyloside. This corresponds to a maximal intracellular concentration of 2PG exceeding 4 mM. Moreover, when the mitochondrial fraction of cells similarly incubated for 30 min was separated by means of the digitonin fractionation technique [19], all of the recovered 2PG, but only about 30% of the 3PG, was associated with the organelle fraction (Table II). The distribution of lactate dehydrogenase and glutamate dehydrogenase, 'marker enzymes' of cytosol and mitochondrial matrix spaces, respectively, was used to confirm the effectiveness of the fractionation technique during each of these experiments. In other experiments involving a wide range of atractyloside and carboxyatractyloside concentrations, virtually all accumulated 2PG was consistently associated with the mitochondrial fraction (data not presented).

Although 2PG concentration did not correlate with either total cellular ATP or mitochondrial ATP levels, it was found to display a strong inverse correlation (Fig. 5) with the cytosolic ATP component as determined by digitonin fractionation.

Glyceraldehyde as a substrate replacing fructose

Because D-glyceraldehyde was thought to be the fructose-derived intermediate serving as the precursor of the accumulated 2PG, glyceraldehyde was tested as a substrate in place of fructose. Glyceraldehyde addition was observed to result in a much more rapid accumulation of 2PG than fructose. In comparison with 12 mM fructose, 20 mM DL-glyceraldehyde, in combination with 25 μM atractyloside, was associated with a 2-fold greater initial rate of accumulation of 2PG and a further reduction in the steady-state concentration of ATP. There was a negligible formation of glucose, but significant accumulation of fructose 1-phosphate and lactate (Table III and Fig. 6). Moreover, in the presence of glyceraldehyde significant levels of 2PG accumulated even in the absence of atractyloside, or with both atractyloside and bicarbonate added together (Fig. 6). However, when bicarbonate was present, the concentration of 2PG reached a maximum value and subsequently decreased rapidly, the observed removal of 2PG presumably corresponding to the period when the bulk of the glyceraldehyde had been metabolised (Fig. 6).

With DL-glyceraldehyde as substrate, it was demonstrated that isolated mitochondria catalysed the formation of 2PG when supplemented with an oxidisable substrate, succinate (Table IV). Significant 2PG was formed irrespective of the presence of ATR, and the

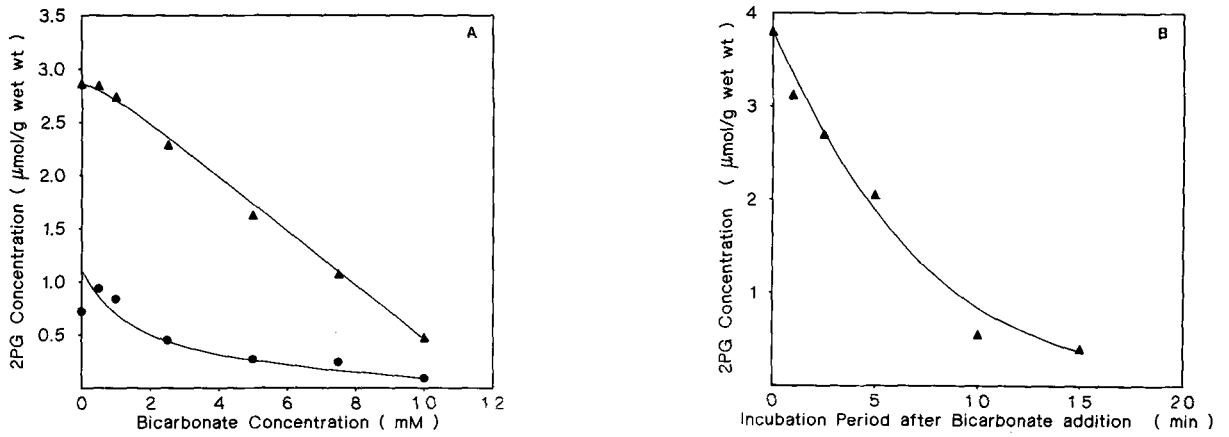

Fig. 4. Influence of bicarbonate on accumulation of 2PG in the presence of fructose. Hepatocytes were incubated in phosphate-buffered medium containing 12 mM fructose and 2PG was measured at the end of the incubation period. (A) Bicarbonate was added to the medium at the initial concentrations indicated, and the cells incubated for 30 min. Further additions were: ●, Nil; ▲, 25 μM ATR. (B) Cells were pre-incubated in the presence of 25 μM ATR for 30 min, at which time 25 mM bicarbonate was added, and the cells were then incubated for the additional times indicated.

TABLE II

Intracellular distribution of metabolites, as revealed by digitonin fractionation of cells

Hepatocytes were incubated for 30 min and then rapidly fractionated into 'intra-mitochondrial' and 'extra-mitochondrial' components as described in Materials and Methods. The incubation medium contained 12 mM fructose, and, where indicated in the table, 25 mM bicarbonate and 25 μM atractyloside. ATP, 3PG and 2PG are expressed as μmol/g wet weight incubated cells. This Table presents data (means ± S.E.) for 3 to 11 determinations. During one experiment, duplicate samples from each incubation were fractionated, so that the distribution of marker enzymes could be compared with that of the analysed metabolites. Marker enzyme activity in each fraction is expressed as a percentage of the recovered total activity. Significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) are between each treatment and the 'fructose' treatment, within each type of fraction.

Additions	Fraction	Marker enzymes		Metabolites (μmol/g wet wt)		
		lactate dehydrogenase	glutamate dehydrogenase	ATP	3PG	2PG
Fructose	extramitochondrial	98%	6%	0.37 ± 0.01	0.09 ± 0.02	0.05 ± 0.03
	intramitochondrial	2%	94%	0.16 ± 0.03	0.18 ± 0.01	0.84 ± 0.07
Fructose, bicarbonate	extramitochondrial	97%	5%	0.58 ± 0.01 ***	0.34 ± 0.06 *	0
	intramitochondrial	3%	95%	0.28 ± 0.01 *	0.08 ± 0.04	0.14 ± 0.01 ***
Fructose, ATR	extramitochondrial	99%	7%	0.21 ± 0.03 **	0.43 ± 0.06 **	0.05 ± 0.02
	intramitochondrial	1%	93%	0.22 ± 0.02 **	0.25 ± 0.18	3.37 ± 0.31 ***
Fructose, ATR, bicarbonate	extramitochondrial	98%	6%	0.49 ± 0.12	0.28 ± 0.02 **	0.04 ± 0.04
	intramitochondrial	2%	94%	0.36 ± 0.09	0.12 ± 0.05	0.57 ± 0.10

TABLE III

Comparison of glyceraldehyde and fructose effects on selected parameters of intermediary metabolism

Freshly prepared hepatocytes were incubated for 10 to 30 min in phosphate-buffered medium as described in Materials and Methods, with the following additions where indicated in the table: 12 mM fructose, 20 mM DL-glyceraldehyde, 25 μM atractyloside. The data presented are means (± S.E. values) for either 3 measurements (without ATR) or 6–14 measurements (with ATR). Significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) are between treatments with and without ATR.

Additions	Rate of change (μmol/min per g wet wt)			Metabolite concentration (μmol/g wet wt)					Ratio 2PG/3PG
	glucose	lactate	pyruvate	fructose 1-phosphate	glycerol 3-phosphate	ATP	3PG	2PG	
Fructose	1.91 ± 0.08	1.38 ± 0.17	0.08 ± 0.03	9.7 ± 0.6	0.75 ± 0.07	0.58 ± 0.01	0.63 ± 0.03	0.77 ± 0.08	1.20 ± 0.08
Fructose, ATR	0.54 ± 0.09 ***	2.15 ± 0.18 **	0.26 ± 0.03 **	10.8 ± 1.2	0.35 ± 0.05 ***	0.47 ± 0.04 *	0.62 ± 0.03	3.20 ± 0.16 ***	4.60 ± 0.25 ***
Glyceraldehyde	0.14 ± 0.01	1.73 ± 0.06	0.15 ± 0.04	8.5 ± 0.3	1.30 ± 0.20	0.40 ± 0.03	0.35 ± 0.04	0.68 ± 0.23	1.85 ± 0.50
Glyceraldehyde, ATR	0.06 ± 0.01 ***	1.94 ± 0.08	0.14 ± 0.02	9.0 ± 1.4	1.06 ± 0.05	0.40 ± 0.02	0.32 ± 0.02	4.61 ± 0.27 ***	14.8 ± 1.2 ***

TABLE IV

Formation of 2-phosphoglycerate by isolated mitochondria

Freshly isolated mitochondria were incubated for 20 min in respiration medium as described in Materials and Methods, including additions of 1 mM ADP, 10 mM succinate, 20 mM DL-glyceraldehyde and atractyloside at the concentrations indicated. Each incubation mixture was separated into a particulate pellet fraction and a supernatant fraction before quenching with acid and subsequent analysis. ATP, 3PG and 2PG are expressed as μmol/g mitochondrial protein, each tabulated mean ± S.E. representing four separate determinations.

ATR (μM)	ATP (μmol/g mitochondrial protein)		3PG (μmol/g mitochondrial protein)		2PG (μmol/g mitochondrial protein)	
	supernatant	pellet	supernatant	pellet	supernatant	pellet
0	469 ± 46	11.0 ± 7.5	3.15 ± 1.8	1.5 ± 0.8	145 ± 34	7.8 ± 4.7
1	273 ± 51	2.0 ± 1.1	2.7 ± 1.6	1.8 ± 1.0	118 ± 43	6.9 ± 2.9
25	77 ± 15	2.0 ± 1.1	2.3 ± 1.3	1.9 ± 1.1	93 ± 33	6.4 ± 2.5

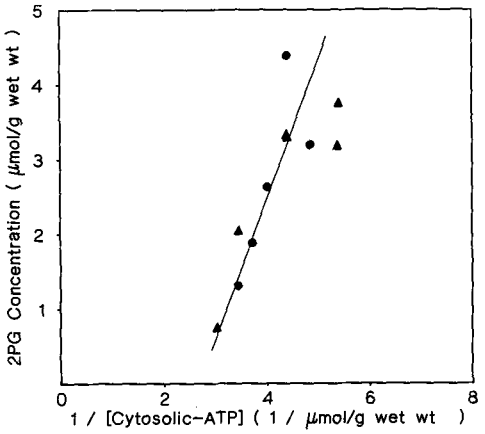

Fig. 5. Relationship between accumulated 2PG and extra-mitochondrial ATP concentration. Hepatocytes were incubated in phosphate-buffered medium containing 12 mM fructose and concentrations of ATR (●) or carboxyatractyloside (▲) that were varied between 2.5 and 100 μM. At the end of an incubation period of 30 min, cells were fractionated with digitonin, as described in Materials and Methods, to yield mitochondrial and extra-mitochondrial fractions, that were separately assayed for 2PG and ATP. Total cellular 2PG is plotted against the inverse of the extra-mitochondrial ATP concentraiton.

addition of ATR did not cause the 2PG to be confined to the particulate fraction. However, it is noteworthy that even a high concentration of ATR did not prevent the formation from ADP of substantial ATP, nor did ATR prevent this ATP from accumulating within the supernatant fraction. No significant quantity of 3PG was formed, indicating an absence of phosphoglycerate mutase. On the basis of an estimate of the hepatic cellular content of mitochondrial protein [22], the formation of 2PG by mitochondria incubated with 25 μM ATR (Table IV) corresponded to about 6 μmol/g wet

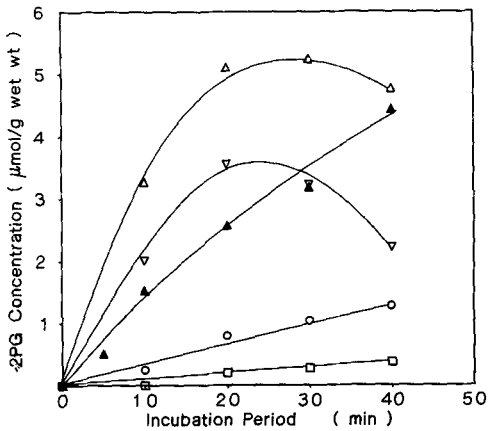

Fig. 6. Glyceraldehyde as a source of 2PG in isolated hepatocytes. Hepatocytes were incubated for the times indicated, as described in Materials and Methods, in phosphate-buffered medium containing either 12 mM fructose and 25 μM ATR (▲) or 24 mM DL-glyceraldehyde and the following additions: ○, Nil; □, 25 mM bicarbonate; △, 25 μM ATR; ▽ 25 mM bicarbonate +25 μM ATR.

weight of hepatocytes, a value comparable to that observed with intact cells (Table III and Fig. 6).

Discussion

There are known to be three routes for the hepatic metabolism of glyceraldehyde derived from fructose [2,8]. While the enzymes of all three pathways occur in the cytosol [22], the enzymes of one of these pathways, that via glycerate, are also present within the mitochondrion of the rat hepatocyte. The product of hepatic glycerate kinase is 2PG [23,24]. The cellular glyceraldehyde dehydrogenase and D-glycerate 2-kinase activities are approximately equally distributed between the cytosolic and mitochondrial compartments [12]. Heinz and Lamprecht [12], used marker enzymes (glyceraldehyde 3-phosphate dehydrogenase and glutamate dehydrogenase) to demonstrate a very low percentage contamination of the mitochondrial fractions with cytosolic material. Moreover, our finding that the glycerate pathway is sensitive to atractyloside confirms that its operation has a dependence on the activity of the adenine nucleotide translocase, and therefore involves the mitochondrion.

In bicarbonate-free phosphate-buffered incubation medium, atractyloside causes a fructose-dependent accumulation of 2PG by intact cells, without a significant change in the concentration of 3PG. Based on estimates of hepatocyte mitochondrial volume [22], the matrix concentration of 2PG can be calculated to be about 40 mM, under the specific conditions of fructose metabolism reported here. The concentration of atractyloside most often used in these studies was 25 μM, although a wide range of concentrations (15 to 100 μM) are effective in causing 2PG accumulation. Because the activity of phosphoglycerate mutase is believed to maintain the cytosolic ratio of 2PG:3PG very close to that corresponding to thermodynamic equilibrium (0.2, Ref. 4), these data suggest that a pool of 2PG, physically isolated from the cytosolic phosphoglycerate mutase, accumulates under these experimental conditions. That this non-cytosolic pool is most likely to be located within the mitochondria of intact cells is indicated by a combination of evidence. (a) The digitonin fractionation experiments revealed that the bulk of the 2PG (but not the 3PG) was physically localised within the particulate (mitochondrial) fraction. (b) The observation that atractyloside (or carboxyatractyloside), highly specific inhibitors of the mitochondrial adenine nucleotide translocase [20,21], is necessary for 2PG accumulation to occur strongly implies a mitochondrial involvement. (c) The existence within the mitochondria of the necessary enzymes (glyceraldehyde dehydrogenase and glycerate 2-kinase [12]) is consistent with this proposed scheme. (d) The observation that isolated mitochondria, in the presence

of an oxidizable substrate and glyceraldehyde, can catalyse the formation of 2PG shows that mitochondria are competent in 2PG formation, at least from glyceraldehyde. That ATR apparently did not cause internal accumulation of 2PG in the case of isolated mitochondria, may be due to the fact that the inner membranes of isolated mitochondrial preparations are somewhat permeable, compared to the mitochondria of intact cells, especially when suspended in hypotonic medium. Certainly the morphology of such mitochondria is different from those of mitochondria in situ [25]. It is pertinent that ATP, although less rapidly synthesised by isolated mitochondria in the presence of ATR, also was not confined to the particulate fraction as expected from the mode of action of the inhibitor. Fig. 7 illustrates this proposed mitochondrial pathway for 2PG formation, a pathway that operates simultaneously with the normal glycolytic sequence in the cytosol.

The presence of the enzymes of the glycerate pathway within the mitochondrial compartment suggests that this pathway is functional in vivo, and that the intramitochondrial formation of 2PG is a normal facet of the metabolism of fructose by rat liver, at least at high substate concentrations. Thus, the influence of atractyloside (and lack of bicarbonate) is merely to exaggerate an existing phenomenon, and allow experimental detection of these intramitochondrial reactions. On the assumption that the mitochondrial pathway is functional in vivo, there is the question of how intramitochondrial 2PG is transferred normally to the cytosolic compartment through the inner mitochondrial membrane. The atractyloside-dependent accumulation of 2PG in intact cells indicates an involvement of the mitochondrial adenine nucleotide translocase in the observed accumulation of 2PG. One possibility is that this translocator is itself capable of the transport of 2PG across the membrane. Another possibility is that the tricarboxylate carrier, responsible for facilitating the mitochondrial egress of phosphoenolpyruvate [26–28], may also transport 2PG [27], although this would not directly explain the sensitivity to atractyloside. One reported example of a 2PG-translocator is that described for the uptake of phosphoenolpyruvate, 2PG and 3PG through the plasma membrane of *Salmonella* [29], although interaction with ADP, ATP or atractyloside was not described. There is evidence that the adenine nucleotide translocase of rabbit kidney cortex mitochondria can transport phosphoenolpyruvate across the mitochondrial membrane [28].

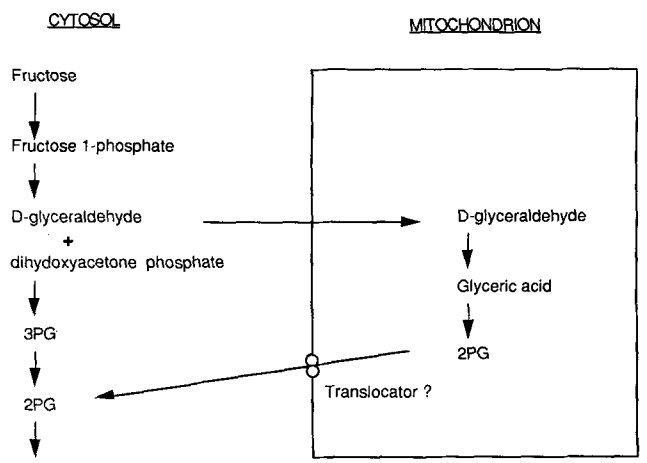

Fig. 7. Proposed mitochondrial involvement in formation of 2PG from fructose.

Pertinent to the potential mechanism of 2PG translocation is the question of the role of bicarbonate. Only in the absence of bicarbonate does atractyloside cause any significant accumulation of 2PG from fructose. Moreover, subsequent addition of bicarbonate causes rapid loss of 2PG already accumulated, an observation which is consistent with the suggested restoration in activity of a transporter that is inactive in the absence of bicarbonate. Indirect involvement of the malate-aspartate shuttle, which has a requirement for bicarbonate [30], is considered unlikely in view of the lack of 2PG accumulation when fluoromalate was added simultaneously with bicarbonate and atractyloside (data not presented). Possibly two translocase mechanisms operate in parallel, one requiring bicarbonate (perhaps as a counter-ion to 2PG) and the other sensitive to atractyloside, since this could explain the simultaneous requirement for absence of bicarbonate and presence of atractyloside for maximal 2PG accumulation. It is of significance that the tricarboxylate carrier from dog kidney mitochondria has an absolute requirement for bicarbonate [31]. Although the precise mechanism of this kidney enzyme has not been determined, it is believed to be not a simple pH effect, but most likely an essential requirement for the bicarbonate anion. The published evidence that both the tricarboxylate anion carrier and the adenine nucleotide translocase can convey phosphoenolpyruvate across the mitochondrial membrane [26–28], and that 2PG may exchange with phosphoenolpyruvate through the tricarboxylate carrier [27], renders it highly likely that accumulated 2PG may be transported normally from the mitochondrial matrix by both these transporter mechanisms operating in parallel. In addition, there is evidence that coenzyme A, as well as ADP and ATP, will bind to the adenine nucleotide translocase [32]. It is possible that 2PG could exchange with phosphoenolpyruvate via the tricarboxylate carrier, with phosphoenolpyruvate returning to the cytosol via the adenine nucleotide translocase. However, the concentration of PEP was not observed to increase in the presence of added bicarbonate (data not presented),

suggesting that greater availability of cytosolic phosphoenopyruvate is not the mechanism by which bicarbonate negates the effect of atractyloside.

If the mitochondrial adenylate translocator is responsible for transporting 2PG as well as ATP out of the mitochondrion, then this may provide an additional explanation for the severe depletion of cellular ATP during fructose metabolism. It is possible that fructose-derived 2PG would compete with ATP for the translocator active-centre on the matrix side, thereby inhibiting the rate of ATP-ADP exchange across the mitochondrial membrane. Because fructose is phosphorylated within the cytosol, the reduced efficiency of replacement of utilised ATP would further exacerbate the cytosolic ATP deficiency at high fructose loads. The observed inverse relationship between 2PG and cytosolic ATP concentration (Fig. 5) is consistent with this hypothesis.

When glyceraldehyde is substituted for fructose, there is a very rapid rate of accumulation of 2PG, possibly as a consequence of the high concentration of glyceraldehyde in comparison to the steady state concentration achieved during fructose utilization. In other respects, the metabolism of added glyceraldehyde reflects that of fructose-derived glyceraldehyde, including the accumulation of significant concentrations of fructose 1-phosphate, as previously reported [33], revealing a reversal of the activity of fructose 1-phosphate aldolase. However, the low rate of formation of glucose suggests the possibility that most of the glyceraldehyde carbon enters the pools of 2PG, 3PG, lactate and pyruvate via the mitochondrial pathway. Should this suggestion be correct, then an upper limit for the flux through the glycerate pathway (at least in the presence of elevated glyceraldehyde) can be calculated by the combined rate of accumulation of 2PG, 3PG, lactate and pyruvate under these conditions. By analogous arguments the lower limit of activity is suggested by the rate of accumulation of 2PG alone (since in the presence of atractyloside, virtually all 2PG is 'trapped' within the mitochondrial compartment). Thus, the limits of activity of this pathway have been calculated as 0.3 μmol min^{-1} g^{-1} to 2.24 μmol min^{-1} g^{-1} of cells, in the presence of high concentrations of glyceraldehyde. This represents 5 to 36% of the corresponding rate of fructose metabolism of about 6 μmol min^{-1} g^{-1} of 3-carbon moiety. Of course, this possible rate represents an upper limit, one not expected to be achieved at the steady-state concentrations of glyceraldehyde existing during the utilisation of even very high concentrations of fructose. Previous attempts at quantitating the relative flux importance of this pathway have indicated that 10% or less of carbon from 10 mM fructose is processed via the glycerate pathway [9]. The data reported here suggest that the activity of this pathway could potentially contribute a much greater proportion to fructose metabolism under some circumstances.

The experimental results reviewed here are of significance in revealing a previously unrecognised aspect of the metabolism of fructose. In particular, this work describes another previously unsuspected compartment of intermediary metabolism, an intramitochondrial pathway operating in parallel with an established cytosolic pathway. However, it may be that the mitochondrial involvement described here for rat hepatocytes is of lesser physiological significance in human beings, since human liver has been reported to possess a low mitochondrial activity of glycerate kinase with a much higher K_m for glycerate than is the case with the rat liver enzyme [2,34,35]. Moreover, glycerate is excreted during fructose metabolism as a consequence of a human inborn error of metabolism that very likely results from a deficiency of triokinase [36].

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