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Quantitative analysis of flux along the gluconeogenic, glycolytic and pentose phosphate pathways under reducing conditions in hepatocytes isolated from fed rats

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Hepatocytes were isolated from the livers of fed rats and incubated with a mixture of glucose (10mM), ribose (1mM), mannose (4mM), glycerol (3mM), acetate (1.25mM), and ethanol (5mM) with one substrate labelled with ^{14}C in any given incubation. Incorporation of label into CO_2, glucose, glycogen, lipid glycerol and fatty acids, acetate and C-1 of glucose was measured at 20 and 40min after the start of the incubation. The data (about 48 measurements for each interval) were used in conjunction with a single-compartment model of the reactions of the gluconeogenic, glycolytic and pentose phosphate pathways and a simplified model of the relevant mitochondrial reactions. An improved method of computer analysis of the equations describing the flow of label through each carbon atom of each metabolite under steady-state conditions was used to compute values for the 34 independent flux parameters in this model. A good fit to the data was obtained, thereby permitting good estimates of most of the fluxes in the pathways under consideration. The data show that: net flux above the level of the triose phosphates is gluconeogenic; label in the hexose phosphates is fully equilibrated by the second 20min interval; the triose phosphate isomerase step does not equilibrate label between the triose phosphates; substrate cycles are operating at the glucose–glucose 6-phosphate, fructose 6-phosphate–fructose 1,6-bisphosphate and phosphoenolpyruvate–pyruvate–oxaloacetate cycles; and, although net flux through the enzymes catalysing the non-oxidative steps of the pentose phosphate pathway is small, bidirectional fluxes are large.

Much work has been done to characterize the behaviour *in vitro* of the enzymes of intermediary metabolism, an ultimate aim being to understand the operation and regulation of metabolism *in vivo*. Extrapolation from behaviour *in vitro* to *in vivo*, however, is hazardous, and studies of metabolic fluxes in living cells are required if one is to integrate the knowledge obtained from the studies made *in vitro* into a more complete understanding of metabolism in the intact cell. In recent years, a method of quantifying the flow of substrates along selected metabolic pathways in living cells has been developed and applied to the analysis of intermediary metabolism in the ciliate *Tetrahymena pyriformis* (Stein & Blum, 1981, and references cited therein). This method involves the measurement of radioactivity incorporated from a mixture of labelled substrates into selected products under steady-state conditions. Through comparison of these measurements with the predictions that can be obtained from a realistic model of the pathways of intermediary metabolism under investigation, the fluxes through the steps of these pathways can be ascertained. In the present study we analysed the metabolism of hepatocytes isolated from the livers of fed rats and incubated in a mixture of glucose, ribose, mannose, glycerol, acetate and ethanol, with only one of these substrates labelled in any given incubation. Measurements were made of label incorporation into glycogen, glucose, CO_2, lipid fatty acid and glycerol, acetate and C-1 of glucose. The number of these measurements was in considerable excess of the number of independent flux parameters to be determined. By using an improved method of computer analysis of the equations describing carbon flow in this system, we have been able to obtain a good fit to this large body of data. This has enabled quantification of: the degree of futile cycling

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at the glucose–glucose 6-phosphate, fructose 6-phosphate–fructose 1,6-bisphosphate and phosphoenolpyruvate–pyruvate–oxaloacetate cycles; flux through the oxidative and non-oxidative portions of the pentose phosphate pathway; the degree of isotopic equilibration between the triose phosphates, hexose phosphates and pentose phosphates; and the bidirectional flux through the reactions of the Embden–Meyerhof pathway. Since these measurements were performed at 20 and 40min after suspension of the hepatocytes in the substrate mixture, the time course of changes in the flux patterns through the above-mentioned pathways during the incubation with this substrate mixture has also been obtained.

Methods

Preparation of hepatocytes

Isolated hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Wood *et al.* (1981*a*) from male Sprague–Dawley rats weighing between 260 and 340g, fed *ad libitum* on Purina chow. Surgery was always begun between 09:30 and 10:00h. Viability was always greater than 91% on the basis of Trypan Blue exclusion, and did not decrease significantly during the incubation period.

Incubation of hepatocytes

Incubations were performed in 50ml-capacity polycarbonate flasks by adding 1.0ml of hepatocyte suspension to 1.5ml of bicarbonate buffer containing the following components such that the final concentrations were: 4–7mg of hepatocyte protein/ml, 0.3% (w/v) defatted bovine serum albumin (Chen, 1967), 10mM-glucose, 4mM-mannose, 1mM-ribose, 3mM-glycerol, 1.25mM-acetate, 5mM-ethanol, 0.2mg of bacitracin/ml, 119.8mM-NaCl, 23.8mM-$NaHCO_3$, 4.8mM-KCl, 1.2mM-KH_2PO_4, 1.5mM-$CaCl_2$, 0.6mM-$MgSO_4$ and 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH7.4. Each flask contained tracer amounts of only one of the following labelled substrates: [1-^{14}C]-, [2-^{14}C]-, [3,4-^{14}C]-, [6-^{14}C]- or [U-^{14}C]-glucose, [1-^{14}C]- or [U-^{14}C]-mannose, [1-^{14}C]ribose, [1,3-^{14}C]- or [2-^{14}C]-glycerol, [1-^{14}C]- or [2-^{14}C]-acetate, or [1-^{14}C]ethanol. Final specific radioactivities were approximately as follows, in d.p.m./nmol: glucose, 150–200; mannose, 400; ribose, 500–800; glycerol, 250; acetate, 1400; ethanol, 460. The flasks were then gassed with O_2/CO_2 (19:1) for 15s at 0°C, capped and incubated with shaking (80 cycles/min) at 37°C. Incubations were terminated at 0, 20 or 40min, and label incorporation into products was determined. For any given hepatocyte preparation, duplicate flasks were run for the measurements of glycogen, glucose and C-1 of glucose, with duplicate assays run on each flask, and triplicate flasks were run for the CO_2 and lipid measurements. Bacitracin was included to enable comparison with future studies. Inclusion in all experiments of flasks incubated for 0min enabled correction for background radioactivity in the various assays.

Glycogen content and radioactivity were measured as described by Wood *et al.* (1981*a*). For measurements of all other products incubations were stopped by addition of 0.3ml of 13% (w/v) $HClO_4$ to the capped flasks. Then 0.2ml of Hyamine was added to the centre wells, and CO_2 was collected during a 1h incubation at 37°C with shaking.

Lipid assay

Lipids were isolated essentially by the Bligh–Dyer procedure (Radin, 1969). A trace amount of [^{3}H]triolein was added to the chloroform/methanol extract from three control flasks incubated in the absence of labelled substrate to monitor yield. In initial studies the dried chloroform/methanol extracts were dissolved in 2ml of acidified methanol (2ml of conc. H_2SO_4/100ml of methanol), tightly capped, and heated for 30min at 75°C to effect transesterification. The glycerol and acyl-methanol moieties were then separated by extracting the methanol phase three times with 3ml volumes of hexane. Since no radioactivity from [1-^{14}C]- or [2-^{14}C]-acetate or from [1-^{14}C]ethanol was found in the methanol phase (i.e. in lipid glycerol), nor any radioactivity from any of the other labelled substrates in the hexane phase (i.e. in the fatty acid moieties), the transesterification steps were subsequently omitted.

Glucose

Radioactivity incorporated into glucose was measured by a modification of procedures used by Wood *et al.* (1981*b*). The neutralized $HClO_4$ extracts were centrifuged, and samples of the supernatant were assayed with the Worthington Automated Glucose Reagent. Duplicate 0.5ml samples were also applied to 0.7cm × 3cm columns of Dowex AG1-X8 (200–400 mesh) anion-exchange resin (HCO_3^- form) and allowed to equilibrate for 30min. Neutral compounds were eluted with 4ml of water. The columns were washed with 8ml of water, and 'total anions' were then eluted with 6ml of 1M-NaCl. Control studies showed that over 98% of [6-^{14}C]glucose was recovered in the first elution with less than 0.37% elution of [1-^{14}C]acetate, and over 95% of [1-^{14}C]acetate was recovered with less than 5% contamination by [6-^{14}C]glucose in the NaCl elution.

A 1.0ml sample of the water eluate, containing radiolabelled glucose and other neutral compounds, was adjusted to pH5.1 and added to 1.0ml of 0.1M-sodium acetate containing 1.6mg of glucose

oxidase and 640 units of catalase. To minimize interference by mannose in the glucose oxidase reaction, the glucose concentration was increased to 1.5mM by the addition of unlabelled glucose. After incubation of the mixture for 30min at 37°C, 0.05ml of 1M-Na_2CO_3 was added and the samples were incubated for 1h at 37°C to convert the gluconolactone into gluconic acid. These samples were then loaded on to a column (0.7cm × 10cm) of Dowex AG1-X8 (50–100 mesh; HCO_3^- form) and washed with 16ml of water for collection of non-glucose neutral compounds. Recovery of labelled ribose, glycerol and mannose with this assay was better than 97%. The gluconic acid was eluted with 1M-NH_4HCO_3 and its radioactivity counted. Recovery of [6-^{14}C]glucose in control samples, used to correct for yield, was always better than 87%.

Partial degradation of glucose

Incorporation of radioactivity into the C-1 position of glucose was determined by a modification of the method of Borowitz *et al.* (1977). Samples (2.5ml) of the neutral-compound-containing water eluate described above, containing approx. 1.5μmol of glucose, were placed into 50ml-capacity polycarbonate flasks containing 6mM-ATP, 2mM-NAD^+, 8mM-$NADP^+$ and 40mM-oxaloacetate in 2ml of 3mM-$MgCl_2$/50mM-Tris/HCl buffer, pH 7.6 (buffer A). The assay was begun by adding 0.1ml of an enzyme mixture in buffer A such that the flask contained at least 20 units of hexokinase/ml, 10 units of glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*)/ml, 10 units of 6-phosphogluconate dehydrogenase/ml and 100 units of malate dehydrogenase/ml. (The 6-phosphogluconate dehydrogenase and malate dehydrogenase were dialysed against buffer A before use.) The flasks were capped, incubated for 2h at 37°C in a shaker bath, and CO_2 was collected in centre wells as described above, except that 0.1ml of 5mM-$NaHCO_3$ was injected into the flasks after the first 30min of CO_2 collection to maximize the recovery of $^{14}CO_2$. The yield of [1-^{14}C]glucose into $^{14}CO_2$ in such assays was in the range 78–83%, with a scatter of less than 1% in any given set.

Ethanol

Ethanol consumption was measured by the method of Bernt & Gutmann (1974). Ethanol evaporation was measured by incubating control flasks with all components of the reaction mixture except hepatocytes, and was found to be 0.5 ± 0.12mM during the first 20min and nil during the second 20min interval.

Glycerol 3-phosphate

Samples of the neutralized $HClO_4$ extracts were analysed for glycerol 3-phosphate by use of the coupled enzyme procedure of Golden & Katz (1980), except that the NADH formed was measured spectrophotometrically at 340nm rather than by the chemiluminescent procedure.

Nucleic acids and amino acids

Radioactivity in nucleic acids was measured by the method of Borowitz *et al.* (1977). No detectable radioactivity from any of the labelled substrates was incorporated into nucleic acids over the 40min period. Label incorporation into amino acids was measured by putting samples of the neutralized $HClO_4$ extract of hepatocyte suspensions on to a column containing Beckman amino acid-exchange resin and eluting the amino acids with 0.2M-sodium citrate buffer, pH 3.25. Radioactivity in alanine, aspartate, glutamate and several other amino acids was negligible for several of the labelled substrates used in this present study.

Protein

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Oxygen

Oxygen consumption was measured polarographically with a Clark oxygen electrode in a thermostatically controlled chamber with magnetic stirring.

Measurements of radioactivity

Hydrofluor scintillant was used for lipid and glucose assays; radioactivities of all other samples were counted in ACS scintillant. Radioactivities of samples were counted in a Packard Tri-Carb spectrometer with automatic external standard for quench correction.

Materials

Collagenase (about 140 units/mg) and Automated Glucose Reagent were from Worthington Biochemical Corp. Hyamine hydroxide and [3,4-^{14}C]-glucose were from New England Nuclear. All other radioactive substrates and ACS scintillant were from Amersham International. Hydrofluor scintillant was from National Diagnostics. Bovine serum albumin (fraction V) and all other incubation-mixture components were from Sigma Chemical Co., as were the enzymes used for metabolite assays. Other chemical were of the highest grade available.

Preliminary considerations

Choice of substrates

The purpose of the present study was to measure the flux of carbon along each step of the gluconeogenic, glycolytic and pentose phosphate pathways in hepatocytes performing gluconeogenesis under

reducing conditions. After preliminary studies, a 'cocktail' consisting of 10mM-glucose, 4mM-mannose, 1mM-ribose, 3mM-glycerol, 1.25mM-acetate and 5mM-ethanol was chosen. This substrate mixture provided adequate input of radioactive tracer from each substrate into most of the products being measured, without marked depletion of any substrate. The concentration of ethanol used is well below toxic values (Goodman & Gilman, 1975) and is adequate to bring the cytoplasmic NAD^+–NADH couple to a reduced condition (Christensen & Higgins, 1979).

Metabolic scheme

The metabolic scheme used in the present study is shown in Scheme 1. All the reactions of the gluconeogenic, glycolytic and pentose phosphate pathways are included. A simplified model of the tricarboxylic acid cycle and several associated reactions is included to allow the interpretation of data on the flow of label from acetate and ethanol into the gluconeogenic and pentose phosphate pathways and from the carbohydrate substrates into CO_2.

Analysis of data

The metabolic network shown in Scheme 1 has 54 flux parameters, of which 34 are independent. Equations were written (Blum & Stein, 1982) describing the flow of radioactivity under steady-state conditions into and out of each carbon atom of each metabolite. These 127 simultaneous linear equations, with the specific radioactivities of each carbon atom as the unknowns, were put into matrix form, and the matrix was inverted by a computer program written in FORTRAN by using a linked lists method to take advantage of the fact that the matrix is sparse. The program was written in such a way that variations on the structure of the metabolic scheme could be incorporated quickly, in contrast with the time-consuming algebraic labour previously required to test alternative metabolic schemes. The new program was tested by using a set of flux values taken from the work of Borowitz *et al.* (1977). The two programs gave results identical to within six decimal places. A full description of the matrix methods and a print-out of the computer program are available (Crawford, 1981).

Results

Presentation of data

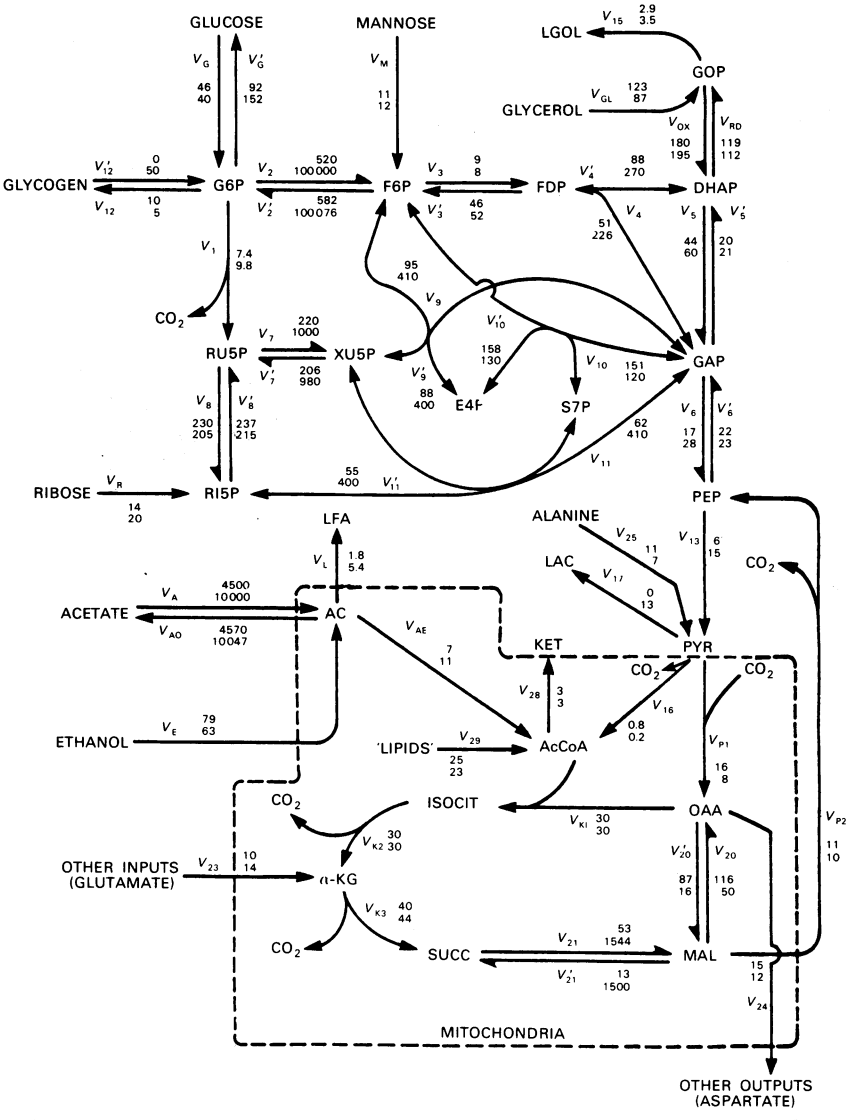
Tables 1 and 2 present the results obtained from experiments in which hepatocytes from fed rats were incubated with the standard substrate mixture (see the Methods section) with one substrate labelled as indicated. The rates of substrate consumption and of product evolution are in all cases consistent with values reported in the literature.

Six measurements were obtained in addition to those presented in Tables 1 and 2. Glucose production was 126 ± 75 and 97 ± 54 nmol/20min per mg of protein for the 0–20 and 20–40min intervals respectively ($n = 17$). These high rates of gluconeogenesis indicate that the hepatocytes are in good metabolic condition throughout the incubation, and confirm the net gluconeogenic behaviour deduced from the fit to the radioisotopic data (see below). The initial glycogen content was 1095 ± 331 nmol of glucose equivalents/mg of protein ($n = 18$). It remained essentially constant during the first 20min, but decreased by 48 ± 42 nmol/20min per mg of protein during the 20–40min interval.

Initial ethanol concentration was 4.8 ± 1.2mM ($n = 7$), in agreement with the nominal concentration of 5mM. Ethanol concentration decreased by 101 ± 16 and 88 ± 22 nmol/20min per mg of protein during the 0–20 and 20–40min intervals respectively, equivalent to decreases of 0.42 ± 0.10 and 0.36 ± 0.16mM. The enzymic determination of ethanol consumption agrees well with the measurements of [1-^{14}C]ethanol conversion into acetate, 78.9 ± 0.7 and 63.5 ± 5.0nmol/20min per mg of protein ($n = 2$), for the two intervals, given the uncertainties due to evaporation of ethanol during the first interval.

The O_2 consumption averaged 423 ± 17 and 408 ± 16 ng-atoms/20min per mg of protein during the first and second 20min intervals respectively ($n = 5$). Because computations indicated that metabolism of the added substrates accounted for only 245ng-atoms/20min per mg of protein during each interval, inputs from protein catabolism (V_{23}, V_{25} and V_{29}) were included in the scheme (Scheme 1). V_{23} and V_{25} represent input from gluconeogenic amino acids. V_{29} represents input from ketogenic amino acids and from endogenous lipids, reported (McGarry *et al.*, 1978) as 5nmol of acetate equivalents/20min per mg of protein in the absence of exogenous substrates. The total amino acid input ($V_{23} + V_{25} + V_{29}$) is about half of the rate of protein degradation (3–4%/h) in hepatocytes from fed rats (Seglen, 1976). [Conversion factors used in the present paper for computing tissue concentrations of intermediates or for converting reported literature flux values into the units of flux used in the present study are as follows: 200mg of hepatocyte protein/g liver wet wt. (Okajima & Katz, 1979), 3.5g liver wet wt./100g body wt. (Scholz & Buche, 1965), 0.42g dry wt./g hepatocyte wet wt. (Siess *et al.*, 1976), 0.8g hepatocyte wet wt./g liver wet wt. (Manery & Hasting, 1939), 4.2mg of DNA/g liver wet wt. (Berry *et al.*, 1973), 0.54g of cytosolic protein/g liver wet wt. (Baquer & McLean, 1972) and 60mg of mitochondrial protein/g liver wet wt. (Scholz &

Buche, 1965).] It should be emphasized that, although inclusion of endogenous substrate utilization allows an excellent fit to the data for oxygen consumption (420 and 412 nmol/20 min per mg of protein are the computed values for the first and second 20 min intervals respectively), it has practically no effect on the flux values above the level of the triose phosphates; essentially the same values for all fluxes above V_6 and V'_6 are computed by fitting the data in Tables 1 and 2 to a scheme with a



Scheme 1. *Metabolic scheme for metabolism of glucose, mannose, ribose, glycerol, acetate and ethanol in hepatocytes*
Each V represents the reaction or sequence of reactions defined in Table 1 of the Appendix. Each pair of smaller numbers represents the values for the fluxes, in nmol/20 min per mg of protein, that give the best fit to the data under the experimental conditions used. The upper number is the best-fit flux value for the 0–20 min incubation interval, and the lower number the best-fit value for the 20–40 min interval. For convenience of presentation some of the flux values have been rounded off. Abbreviations: AC, acetate; AcCoA, acetyl-CoA; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; FDP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GOP, glycerol 3-phosphate; G6P, glucose 6-phosphate; ISOCIT, isocitrate; KET, ketone bodies; α-KG, 2-oxoglutarate; LAC, lactate; LFA, lipid fatty acids; LGOL, lipid glycerol; 'LIPIDS', acetyl groups from endogenous ketogenic amino acids and triacylglycerols; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; RI5P, ribose 5-phosphate; RU5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SUCC, succinate; XU5P, xylulose 5-phosphate.

Table 1. *Experimental and computed incorporation of ^{14}C-labelled substrates into glucose, glycogen, CO_2 and lipid glycerol*

Hepatocytes from fed rats were prepared and incubated in a standard mixture of six substrates as described in the Methods section, with radioactive label present as indicated in the left-most column. n denotes the number of different cell preparations (rats) for which measurements were made. For every labelled product measured, an overall mean ± S.D. was computed from the mean results of different cell preparations: these are presented as the upper numbers in each row. When $n = 2$, the overall mean plus one-half the difference between the cell-preparation means is shown; when $n = 1$, the cell-preparation mean ± S.D. for that particular preparation is shown. When a datum = 0.00, a conservative estimate of the limits of detection for that assay is shown. The first of these experimental numbers was then used as μ_j in eqn. (1), the second as S.D.$_j$. The lower number of each pair is the value of label incorporation predicted from the best-fit flux values shown in Scheme 1; this number was used as C_j in eqn. (1). Interval I is the 0–20 min interval, interval II is the 20–40 min interval. N.M., not measured.

	Incorporation (nmol/20 min per mg of protein)							
	Glucose		Glycogen		CO_2		Lipid glycerol	
Substrate	Interval I	Interval II	Interval I	Interval II	Interval I	Interval II	Interval I	Interval II
[1-^{14}C]Glucose	N.M.	N.M.	3.57 ± 1.53	1.49 ± 0.94 ($n = 4$)	2.70 ± 0.05	1.67 ± 0.33 ($n = 3$)	0.053 ± 0.021	0.054 ± 0.022 ($n = 4$)
	38.9	36.9	4.04	1.14	2.70	1.88	0.023	0.029
[2-^{14}C]Glucose	N.M.	N.M.	3.15 ± 0.92	0.99 ± 0.16 ($n = 3$)	0.45 ± 0.10	0.34 ± 0.05 ($n = 4$)	0.024 ± 0.008	0.025 ± 0.008 ($n = 4$)
	41.2	38.6	4.27	1.19	0.18	0.17	0.025	0.030
[3,4-^{14}C]Glucose	N.M.	N.M.	N.M.	N.M.	0.45 ± 0.20	0.32 ± 0.15 ($n = 1$)	N.M.	N.M.
	40.3	37.6	4.18	1.16	0.52	0.46	0.047	0.046
[6-^{14}C]Glucose	N.M.	N.M.	3.48 ± 1.91	1.59 ± 0.41 ($n = 4$)	0.55 ± 0.10	0.36 ± 0.12 ($n = 5$)	0.053 ± 0.011	0.054 ± 0.011 ($n = 4$)
	39.6	37.0	4.11	1.14	0.48	0.37	0.069	0.063
[U-^{14}C]Glucose	N.M.	N.M.	2.99 ± 1.26	N.M. ($n = 4$)	0.99 ± 0.12	0.78 ± 0.22 ($n = 4$)	0.057 ± 0.010	0.059 ± 0.010 ($n = 4$)
	40.0	37.5	4.15	1.16	0.76	0.59	0.047	0.046
[1-^{14}C]Mannose	8.76 ± 1.89	10.0 ± 4.3 ($n = 4$)	0.61 ± 0.52	N.M. ($n = 4$)	0.71 ± 0.24	0.61 ± 0.10 ($n = 3$)	0.013 ± 0.010	0.013 ± 0.010 ($n = 3$)
	9.61	11.3	1.00	0.35	0.64	0.57	0.007	0.009
[U-^{14}C]Mannose	9.82 ± 0.21	12.3 ± 2.6 ($n = 3$)	0.89 ± 0.68	0.28 ± 0.33 ($n = 4$)	0.27 ± 0.04	0.16 ± 0.03 ($n = 4$)	0.015 ± 0.006	0.015 ± 0.006 ($n = 3$)
	9.82	11.4	1.02	0.35	0.20	0.18	0.014	0.014
[1-^{14}C]Ribose	13.3 ± 1.9	19.8 ± 5.0 ($n = 6$)	1.63 ± 0.59	1.35 ± 0.22 ($n = 4$)	0.46 ± 0.17	0.41 ± 0.13 ($n = 8$)	0.010 ± 0.010	0.010 ± 0.010 ($n = 3$)
	12.0	18.9	1.25	0.59	0.53	0.63	0.009	0.015
[1,3-^{14}C]Glycerol	60.8 ± 8.8	82.7 ± 7.9 ($n = 3$)	6.21 ± 0.81	1.44 ± 1.45 ($n = 4$)	1.85 ± 0.03	2.13 ± 0.41 ($n = 3$)	2.76 ± 0.67	3.16 ± 0.64 ($n = 3$)
	56.7	76.4	5.88	2.36	1.86	2.04	2.69	3.19
[2-^{14}C]Glycerol	57.9 ± 5.7	78.8 ± 12.8 ($n = 4$)	6.57 ± 0.76	1.01 ± 1.33 ($n = 5$)	0.46 ± 0.13	0.53 ± 0.26 ($n = 5$)	2.79 ± 0.70	3.23 ± 0.52 ($n = 3$)
	57.8	77.5	5.99	2.40	0.39	0.52	2.69	3.19
[1-^{14}C]Acetate	0.57 ± 0.06	0.51 ± 0.20 ($n = 3$)	0.057 ± 0.010	0.022 ± 0.018 ($n = 5$)	7.46 ± 1.86	6.51 ± 0.23 ($n = 4$)	0.00 ± 0.10	0.00 ± 0.10 ($n = 2$)
	0.48	0.56	0.049	0.017	4.16	6.47	0.00	0.01
[2-^{14}C]Acetate	1.43 ± 0.07	1.98 ± 0.42 ($n = 3$)	0.15 ± 0.15	0.049 ± 0.028 ($n = 3$)	1.47 ± 0.68	1.3 ± 0.68 ($n = 5$)	0.00 ± 0.10	0.00 ± 0.10 ($n = 2$)
	1.49	1.83	0.02	0.057	1.57	2.69	0.01	0.00
[1-^{14}C]Ethanol	0.09 ± 0.09	0.09 ± 0.09 ($n = 2$)	0.00 ± 0.10	0.00 ± 0.10 ($n = 2$)	0.00 ± 1.00	0.00 ± 1.00 ($n = 2$)	0.00 ± 0.10	0.00 ± 0.10 ($n = 1$)
	0.01	0.00	0.00	0.00	0.07	0.04	0.00	0.00

Table 2. *Experimental and computed incorporation of ^{14}C-labelled substrates into 'C-1 of glucose', lipid fatty acids and acetate*

For explanation see the legend to Table 1.

Substrate	Incorporation into C-1 of glucose (nmol/20min per mg of protein)	
	Interval I	Interval II
[2-^{14}C]Glucose	1.70±0.50 (*n* = 2) 2.10	2.16±1.01 (*n* = 2) 2.45
[6-^{14}C]Glucose	0.95±0.34 (*n* = 2) 1.38	0.96±0.60 (*n* = 2) 1.15
	Radioactivity (d.p.m.) in C-1 of glucose/total radioactivity (d.p.m.) in glucose	
	Interval I	Interval II
[1-^{14}C]Ribose	0.536±0.016 (*n* = 3) 0.536	0.528±0.031 (*n* = 3) 0.511
[1,3-^{14}C]Glycerol	0.243±0.007 (*n* = 3) 0.255	0.238±0.015 (*n* = 3) 0.240
[2-^{14}C]Glycerol	0.020±0.008 (*n* = 3) 0.029	0.022±0.006 (*n* = 3) 0.033
	Incorporation into fatty acids of lipid (nmol/20min per mg of protein)	
	Interval I	Interval II
[1-^{14}C]Acetate	1.80±0.11 (*n* = 2) 1.80	5.42±1.16 (*n* = 2) 5.37
[2-^{14}C]Acetate	1.78±0.14 (*n* = 2) 1.80	5.15±1.79 (*n* = 2) 5.37
[1-^{14}C]Ethanol	0.03±0.03 (*n* = 2) 0.03	0.03±0.03 (*n* = 2) 0.03
	Incorporation into acetate (nmol/20min per mg of protein)	
	Interval I	Interval II
[1-^{14}C]Ethanol	78.9±0.7 (*n* = 2) 78.9	63.5±5.0 (*n* = 2) 63.3

simplified model of mitochondrial and associated reactions containing only 27 independent flux variables and ignoring the data for oxygen consumption (for further details see Crawford, 1981).

During the first 20min interval the depletion of [1,3-^{14}C]- and [2-^{14}C]-glycerol from the medium was 146±9 (*n* = 3) and 148±28 (*n* = 5) nmol/20min per mg of protein respectively. Of this 55±8 (*n* = 4) and 58±6 (*n* = 5) nmol/20min per mg of protein respectively were found to accumulate in 'total anions'. It seemed likely that most of this label remained in glycerol 3-phosphate, since large accumulations of this metabolite have been reported in hepatocytes incubated with glycerol (Burch *et al.*, 1970; Kneer *et al.*, 1979; Hensgens *et al.*, 1980). Confirmation of this hypothesis was provided by measurements of glycerol 3-phosphate; its concentration, which was negligible at the start of the incubation, rose to 40.3±6.4nmol/mg of protein (*n* = 3) by 20min, and did not change significantly thereafter, the concentration at 40min being 38.7±7.4nmol/mg of protein. Depletion of [1,3-^{14}C]- and [2-^{14}C]-glycerol during the second interval was 107±41 (*n* = 3) and 94±25 (*n* = 5) nmol/20min per mg of protein, and label accumulation in 'total anions' was 9±5 (*n* = 4) and 7±4 (*n* = 5) nmol/20min per mg of protein respectively. This accumulation was accounted for by the lactate and aspartate label incorporation computed from the flux values in Scheme 1 (see below), as was the remaining 'total anion' accumulation in the first interval not accounted for by the increase in glycerol 3-phosphate. Both direct and indirect estimates were thus consistent with an increase in glycerol 3-phosphate pool size during the first interval only. This increase in pool size is included in Scheme 1 by the fact that total input to this pool in the first 20min is 242nmol/mg of protein, rather than the 183nmol/mg of protein required to balance total output.

Three of the substrate inputs to the system, namely glucose, glycogen and acetate, are also outputs. When glucose was the labelled substrate, gluconeogenesis during the first 20min interval caused an approx. 2.8% decrease in its specific radioactivity. The corrected value of specific radioactivity was used for the 20–40min-interval computations. Recently synthesized glycogen is preferentially degraded by hepatocytes (Devos & Hers, 1980). Calculations show that, even if there were complete preferential degradation, the best-fit flux values presented in Scheme 1 would not be significantly affected. Tables 1 and 2 reveal that [^{14}C]-acetate is virtually the sole product of [1-^{14}C]-ethanol metabolism. This results in a decrease in the specific radioactivity of labelled acetate of 15% during the first 20min interval and an additional 18% during the second 20min interval; the specific radioactivity of acetate for each interval was corrected accordingly. Finally, any of these three outputs that were initially unlabelled in a given experiment might have become labelled subsequent to the metabolism of another labelled substrate, as in the case of acetate. Calculations showed that any errors that might arise by ignoring the effects of label re-uptake from initially unlabelled substrates were negligible.

Analysis of data

The data in Tables 1 and 2 were analysed by assuming a trial set of the independent flux parameters and comparing the computed values for incorporation with the measurements. A weighted least-squares parameter, *E* (Deming, 1943):

$$E = \sum_{j=1}^{N} \left(\frac{\mu_j - C_j}{\text{S.D.}_j} \right)^2 \qquad (1)$$

was used as a guide to improving the fit of the trial set. In eqn. (1) μ_j is the measured value of label incorporation for the jth measurement, obtained from Tables 1 and 2, S.D.$_j$ is the standard deviation of the mean for that measurement, and C_j is the calculated value of label incorporation for that measurement for the trial set of parameters under consideration. The flux values that gave the minimum value for E for each interval are presented in Scheme 1. As noted by Blum & Stein (1982), an excellent fit would have each calculated value within 1 S.D. for the corresponding datum, so that the value of E should be of the same order as the number of measurements, N. For the 0–20 min interval, $N = 48$ and $E = 41$. For the 20–40 min interval, $N = 46$ and $E = 50$. To assess the goodness of fit further we first address the question as to whether random errors of measurement could account for the fit. Use of an appropriate test of significance such as a χ^2 test shows ($P < 0.0001$) that such errors could not explain the goodness of fit for either interval. A second question is whether there remains a substantial amount of unexplained variation. The ratio of explained variation to total variation (0.993 and 0.989 for the first and second intervals respectively) indicates that only a very small amount of variation remains unexplained by the model.

The effect of systematic variation of individual flux parameters keeping all others at their best-fit value was investigated to assess the sensitivity of the fit to that flux value and hence to obtain an estimate of the possible range of each flux. The results of this analysis (see the Appendix) show that most of the fluxes have rather narrow ranges. The fluxes shown in Scheme 1 thus constitute a fairly precise description of metabolite flow in hepatocytes, from fed rats, incubated with the substrate mixture used in the present study.

Validity of steady-state assumption

The above analysis is based on the assumption that the system reached metabolic and isotopic steady state soon after the initiation of the incubation. The time constant, τ, for achieving the isotopic steady state can be computed (see Borowitz *et al.*, 1977) by $\tau = M_k / \sum_k V_{0,k}$, where M_k is the pool size of the kth intermediate and $\sum_k V_{0,k}$ is the sum of all output fluxes from that pool. Glucose 6-phosphate is likely to be one of the most rapidly changing metabolite pools after exposure of the cells to the substrates. According to Nordlie *et al.* (1980), the steady-state concentration of glucose 6-phosphate in livers perfused with 11.9 mM-glucose is 63 nmol/g wet wt. Assuming 0.2 g of protein/g wet wt. and by using the flux values shown in Scheme 1 for the 0–20 min interval, τ is computed to be less than 10 s. Thus isotopic equilibration of this pool will be achieved very shortly after addition of the substrates.

If subsequent changes in pool size occur slowly relative to the total carbon flux through that pool, then the system can be regarded as being in a quasi-steady state and the above flux value calculations are valid (Borowitz *et al.*, 1977). Nordlie *et al.* (1980) found that on exposure of a liver from a starved rat to 16 mM-glucose the glucose 6-phosphate concentration rose from 51 to 123 nmol/g wet wt. in 15 min. Although this is likely to be a larger increase than would occur in hepatocytes from fed rats exposed to 10 mM-glucose, it is nevertheless a very slow change compared with a throughput of over 100 000 nmol/20 min per g wet wt. during the first interval (Scheme 1). Kinetic simulations of liver glycogen metabolism (El Refai & Bergman, 1976) show that, after a sharp rise in glucose concentration from 5.5 to 16 mM, glucose 6-phosphate content would increase by less than 2-fold and that this increase would be complete within 3 min. These two independent approaches indicate that the glucose 6-phosphate pool should reach a quasi-steady state within a few minutes, and maintain that state for the remainder of the incubation. Examination of the computed values for the specific radioactivities of each carbon atom of each intermediate in the metabolic scheme for each labelled substrate (including [^{14}C]glycerol) confirms that by far the largest change in specific radioactivity between the first and second 20 min intervals occurs at the glucose 6-phosphate node (see Crawford, 1981). That the assumption of quasi-steady state also applies to other pools that are likely to be changing is indicated by measurements of radioactivity incorporation into 'total anions' (see the Methods section). For labelled glucose, mannose and ribose such accumulation of label was less than 2% of the 'throughput' at the glucose 6-phosphate, fructose 6-phosphate and ribose 5-phosphate nodes respectively.

Further evidence supporting the validity of the assumption of a metabolic steady state throughout most of the first interval and all of the second interval comes from measurements of O_2 consumption and of the fluorescence of the reduced nicotinamide nucleotides in hepatocytes. Both O_2 consumption and the fluorescent signal stabilize in less than 4 min after the addition of cells to the substrate mixture under nearly identical conditions (Balaban & Blum, 1982). Experiments with the present substrate mixture confirmed (results not shown) that the rate of O_2 consumption reached its steady-state value within 3 min after addition of the cells to the flasks. The flux values presented in Scheme 1 will thus be a good approximation to the average behaviour of the system during each interval studied, except for some error introduced because the system

is changing rapidly during the first few minutes of the incubation.

Alternative models

Because Longenecker & Williams (1980) have presented an alternative formulation for the reactions of the pentose phosphate pathway, it was important to attempt to fit our data to their scheme. The model used in Scheme 1 was therefore altered to include their formulation of the pentose phosphate pathway, and attempts were made to find a good fit to our data. Such a fit could not be achieved, even though there are more parameters in their scheme than in Scheme 1. The scheme of Longenecker & Williams (1980) predicts a high rate of flux of ^{14}C from [1-^{14}C]ribose (and other labelled substrates) through dihydroxyacetone phosphate, whereas the conventional scheme (Scheme 1) predicts a high flux of label through glyceraldehyde 3-phosphate. The best possible fit to the data for label flow into products from [1-^{14}C]ribose and into C-1 of glucose from [2-^{14}C]- and [6-^{14}C]-glucose and [1,3-^{14}C]- and [2-^{14}C]-glycerol resulted in a predicted label flow from [^{14}C]ribose, [^{14}C]mannose and [^{14}C]glucose into lipid glycerol 10–100-fold greater than that measured. Conversely, adjusting the pentose phosphate pathway fluxes to fit the data for lipid glycerol made it impossible to fit the remainder of the incorporation data. It is therefore unlikely that the alternative reactions proposed by Williams *et al.* (1978) operate to an appreciable extent in isolated hepatocytes under our conditions.

To improve the interpretations of findings not consistent with a unidirectional pentose cycle, Landau & Bartsch (1966) introduced a transaldolase-catalysed exchange of C_3 units between glyceraldehyde 3-phosphate and fructose 6-phosphate. We found that, on introduction of their V_T (see Fig. 1 of Landau & Bartsch, 1966) into the present scheme, the only permissible value of V_T was zero. Finally, we found it unnecessary to introduce more than one pool of any metabolite in the pentose phosphate pathway to obtain good fits to the experimentally determined data, even though several enzymes of the pentose phosphate pathway are present in particulate fractions of rat liver (Baquer & McLean, 1972; Baquer *et al.*, 1972).

Discussion

The present work provides a detailed analysis of the flux of carbon through the reactions of gluconeogenesis, glycolysis and the pentose phosphate pathways under conditions of net gluconeogenesis under reducing conditions in hepatocytes isolated from fed rats. Having obtained a realistic estimate of the pattern of metabolite flow in hepatocytes with these conditions, we now explore the metabolic implications of our results.

Futile cycles

Although many of the reactions of glycolysis and gluconeogenesis are reversible, there are three sites where energetically 'futile' cycling can occur. Previous attempts to quantify the rate of futile cycling in hepatocytes have required assumptions about the rates of isotopic exchange in the reactions catalysed by hexose phosphate isomerase, fructose bisphosphate aldolase, triose phosphate isomerase and the reversible reactions of the tricarboxylic acid cycle, in addition to estimates of ^3H-isotope discrimination in some of these reactions and of exchange reactions catalysed by transaldolase. The serious problems inherent in these and related methods have been discussed in detail by Katz & Rognstad (1976), Hue (1981) and Crawford & Blum (1982). The present data are well suited to analysis of the substrate cycles. No further assumptions need be made than those at the onset of the investigation, i.e. that the system is effectively in a metabolic steady state and that the reaction scheme used (Scheme 1) is an adequate representation of the gluconeogenic, glycolytic and pentose phosphate pathways. Quantification of substrate cycling is an automatic consequence of a successful fit to the data.

Glucose–glucose 6-phosphate cycle. Although the hepatocytes were in a gluconeogenic mode, the rate of glucose phosphorylation (V_G) declined only slightly during the incubation (from 46 to 40nmol/20min per mg of protein from the first to the second interval). Glucose 6-phosphate dephosphorylation (V'_G), however, increased from 92 to 152nmol/20min per mg of protein (largely as the result of increased glycogen degradation). These values fall within the wide range reported in the literature (Okajima & Ui, 1979; Huang & Lardy, 1981; Clark *et al.*, 1975, 1980; Katz *et al.*, 1975, 1978). The relative constancy of V_G in the face of increasing gluconeogenesis in the present study is consistent with the sole dependence of glucokinase activity on glucose concentration (Storer & Cornish-Bowden, 1976). By using the Michaelis–Menten equation with $K_m = 5$mM (Storer & Cornish-Bowden, 1976), $V = 43$nmol/20min per mg of protein and [S] = 10mM, the $V_{max.}$ for this enzyme *in situ* is computed to be 65nmol/20min per mg of protein, in good agreement with a reported $V_{max.}$ of 77nmol/20min per mg of protein in hepatocytes from fed rats (Bontemps *et al.*, 1978).

Since the known inhibitors of glucose 6-phosphatase have K_i values considerably above their physiological concentrations (Arion *et al.*, 1972, 1980), it is likely that flux through this entire system is determined primarily by substrate concentration (Hue, 1981). One can thus use the flux values in

Scheme 1, a K_m of 2.6mM (Arion *et al.*, 1972; Nilsson *et al.*, 1978) and a $V_{max.}$ of approx. 1450nmol/20min per mg of protein to compute intracellular glucose 6-phosphate concentrations of 0.18 and 0.31mM during the first and second intervals respectively. [The reported $V_{max.}$ for glucose 6-phosphatase in intact microsomes is 0.261μmol of phosphate hydrolysed/min per mg of microsomal protein (Arion *et al.*, 1980). The endoplasmic reticulum constitutes approx. 0.28mg of protein/mg of cell protein. This value is derived from two independent measurements (Jelsema & Marré, 1978): (1) total protein of endoplasmic reticulum per 10g of rat liver is 383mg, and total liver protein is 1300mg/100g of liver, giving a ratio of 0.29mg of endoplasmic-reticulum protein/mg of cell protein, and (2) purification of glucose 6-phosphatase from total liver homogenate to the endoplasmic-reticulum fractions results in a 3.73-fold increase in the specific activity (units/mg of protein), indicating 0.27mg of endoplasmic-reticulum protein/mg of cell protein.] These glucose 6-phosphate concentrations are in agreement with values reported in the literature (Greenbaum *et al.*, 1971; Hue & Hers, 1974; Nordlie *et al.*, 1980; Van Schaftingen *et al.*, 1980) (reported tissue concentrations for this metabolite, in mM, were computed by using the conversion factors presented by Arion *et al.*, 1980). The 1.7-fold rise in glucose 6-phosphate concentration and thus glucose 6-phosphatase activity results in a 2.5-fold rise in net flux through the glucose–glucose 6-phosphate cycle (Scheme 1). Katz *et al.* (1978) observed a tripling of net glucose formation associated with a 1.5-fold increase in glucose 6-phosphate concentration.

Fructose 6-phosphate–fructose 1,6-bisphosphate cycle. Although V_3' increased from 46 to 52nmol/20min per mg of protein during the incubation, V_3 decreased only slightly from 9.0 to 7.9nmol/20min per mg of protein (Scheme 1). Thus, despite the high potential for regulation of phosphofructokinase and of fructose bisphosphatase (Hers & Van Schaftingen, 1982), considerable futile cycling between fructose 6-phosphate and fructose 1,6-bisphosphate occurs under these conditions.

Phosphoenolpyruvate – pyruvate – oxaloacetate cycle. Although the main focus of the present investigation was on reaction fluxes in the 'upper' portion of the metabolic scheme, some important qualitative conclusions can be drawn about the operation of this substrate cycle. During both intervals the flux through phosphoenolpyruvate carboxykinase (V_{P2}) was of magnitude comparable with that of the fluxes through pyruvate kinase (V_{13}) and pyruvate carboxylase (V_{P1}). The net flux through this cycle ($V_6 - V_6'$) changed from about 5nmol/20min per mg of protein in the gluconeogenic direction during the first 20min interval to 5nmol/20min per mg of protein in the glycolytic direction in the second interval. Thus throughout the 40min incubation flux through the individual enzymes of this cycle exceeded the net flux, i.e. considerable futile cycling occurred.

Glycogenic–glycogenolytic cycle. Several investigators (Hue *et al.*, 1975; Katz *et al.*, 1975, 1979) have detected label incorporation from glucose into glycogen, despite a maintenance or loss of glycogen during the interval measured. In the first 20min of incubation the cells in the present experiment were modestly glycogenic, with V_{12} (synthesis) equal to 9.5 and V_{12}' (degradation) equal to 0nmol/20min per mg of protein. Net glycogen degradation clearly occurred in the 20–40min interval ($V_{12}' = 50$nmol/20min per mg of protein), despite a continued influx of label into glycogen ($V_{12} = 4.7$nmol/20min per mg of protein). Since these fluxes represent the average values for a system that was changing from net glycogenesis to net glycogenolysis, it is possible that glycogen synthase was active for a limited time during the 20–40min interval, followed by its complete inactivation and the activation of glycogen phosphorylase. The present data therefore permit no conclusions as to whether futile cycling occurred at this pair of opposing reactions.

Embden–Meyerhof pathway and associated reactions

Glucose 6-phosphate isomerase. Although this enzyme has a strategic location in glucose metabolism, it is generally regarded as having little regulatory role because its equilibrium is close to unity (Dyson & Noltmann, 1968; Plesser *et al.*, 1979), it is present at relatively high concentrations (Weber, 1963) and it has a high catalytic efficiency (Plesser *et al.*, 1979). Under the conditions of the present study the bidirectional flux through this enzyme was considerably larger than any of the associated reactions. The exceedingly large values for V_2 and V_2' for the 20–40min interval merely indicate that glucose 6-phosphate and fructose 6-phosphate are effectively in isotopic equilibrium during this interval, whereas there is a slight difference in their specific radioactivities during the first 20min interval (for details see Crawford, 1981).

Fructose bisphosphate aldolase and triose phosphate isomerase. Despite the high activities of these enzymes in hepatic tissue (see Knox, 1976), incomplete isotopic equilibration of the triose phosphates has been shown to occur in a variety of experimental conditions (Schambye *et al.*, 1954; Hers, 1955; Rose *et al.*, 1962). In the present experiments, the triose phosphates were far from isotopic equilibrium during both intervals. For example, with [2-^{14}C]glycerol as the label source, the computed steady-state relative specific radioactivity of C-2 in

dihydroxyacetone phosphate during the second 20min interval was 0.84, whereas that of C-2 in glyceraldehyde 3-phosphate was 0.37. Since equilibration of the triose phosphates is assumed for conventional estimates, not only of futile cycling between fructose 6-phosphate and fructose 1,6-bisphosphate (Okajima & Ui, 1979; Huang & Lardy, 1981) but also of pentose phosphate cycle activity (e.g. Wood *et al.*, 1963; Sochor *et al.*, 1979), methods based on this assumption could have yielded erroneous estimates had they been used with hepatocytes incubated with this substrate mixture (Crawford & Blum, 1982).

Glycerol metabolism. Glycerol was the primary gluconeogenic substrate under these conditions, as demonstrated by a comparison of the net gluconeogenic flux through fructose bisphosphate aldolase ($V_4'-V_4$, equal to 37 and 44nmol/20min per mg of protein for the first and second intervals respectively) with the flow of glucose equivalents through glycerol phosphate dehydrogenase [$(V_{OX}-V_{RD})/2$, equal to 30 and 41nmol/20min per mg of protein respectively]. The glycerol and ethanol oxidation rates were comparable, suggesting that their rates may be limited by the availability of NAD^+.

Lower Embden–Meyerhof pathway. During both intervals the carbon flux through these reactions is two to three orders of magnitude less than the reported $V_{max.}$ values of these four enzymes (Baquer *et al.*, 1976). This result is consistent with the reported inhibition of glyceraldehyde 3-phosphate dehydrogenase by a high cytosolic [NADH]/[NAD^+] ratio (Williamson *et al.*, 1969; Pilkis *et al.*, 1976; Harris & Walters, 1976). The low values for V_6 and V_6' effectively limit exchange of carbon between the 'upper' and 'lower' portions of intermediary metabolism, thereby accounting for the observed insensitivity of flux values above the triose phosphate level to the inclusion or neglect of endogenous contributions to mitochondrial metabolism.

Pentose phosphate pathway

The flux through the oxidative steps of the pentose phosphate pathway (V_1) was 7.4 and 9.8nmol/20min per mg of protein for the first and second time intervals respectively. Although these values are far below the capacities of these enzymes (Baquer *et al.*, 1976; Knox, 1976), they constitute a significant fraction of the net glucose output. Flux through ribokinase (V_R) was 14 and 20nmol/20min per mg of protein for the first and second intervals respectively, thus demonstrating that ribose can serve as a significant gluconeogenic substrate for hepatocytes. The bidirectional fluxes through the reversible reactions of the pentose phosphate pathway, V_7, V_7' ... V_{11}, V_{11}', were near the reported $V_{max.}$ values for these enzymes (Novello & McLean, 1968; Baquer *et al.*, 1976) and exceeded the net fluxes through these reactions by an order of magnitude. These high rates of bidirectional carbon flow were sufficient to effect considerable randomization of label between pentose phosphate-cycle intermediates (for details see Crawford, 1981).

Conclusion

The present investigation constitutes the first application to mammalian cells of techniques originally developed in studies on protozoa. It provides a detailed description of metabolite flow along the gluconeogenic, glycolytic and pentose phosphate pathways in fed hepatocytes incubated with a particular substrate mixture. For this metabolic state (net gluconeogenesis under reducing conditions in the presence of 10mM-glucose), these hepatocytes show considerable futile cycling at several important regulatory steps of the gluconeogenic pathway, indicating that intrinsic regulation (i.e. in the absence of hormonal or nervous signals), though quite good, is inadequate to prevent some energy wastage. It is also evident that many of the enzymes are operating at rates well below the $V_{max.}$ values measured *in vitro*, indicating that substrate availability and modulating factors play a predominant role in determining flux patterns in the intact cell. The ability to fit the large amount of data gathered in the present study by the metabolic scheme shown in Scheme 1 indicates that this is a satisfactory representation of the structure of the 'upper' portion of intermediary metabolism in hepatocytes, and thus opens the way for further quantitative studies of hepatocyte metabolism in other physiological states.

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APPENDIX

To obtain an estimate of the possible range of the flux values, each was varied from its best-fit value (keeping all others constant; see Table 1 of Appendix) until the fit parameter, E, increased by 5%; this was considered to reflect a significant worsening of the fit. Table 1 of Appendix presents the reaction name and best-fit values for each flux of Scheme 1, along with the possible range for each flux. High upper limits for flux values, when shown, indicate that the substrates involved in these reactions are sufficiently close to isotopic equilibrium that there is no worsening of the fit if they are assumed to be in isotopic equilibrium. Although the ranges of acceptable values for reversible reactions (including those in the pentose phosphate pathway) reflect uncertainties in the bidirectional fluxes through these steps, the net flux is as tightly determined as the overall stoicheiometry of the system. Finally, our data do not permit a thorough characterization of mitochondrial metabolism: the flux values and ranges shown for the 'lower' portion of Scheme 1 reflect the best configurations for describing label flow from [^{14}C]acetate into glucose and glycogen and from labelled carbohydrate substrates into $^{14}CO_2$, while maintaining a good fit to the data for O_2 consumption.

Table 1. *Best-fit flux values and their ranges for a 5% increase in fit parameter*

The symbols for the reactions in Scheme 1 are shown in the left-most column, the relevant enzyme names or pathways are listed in the second column, and the best-fit values from Scheme 1 are given in the third and fifth columns. Flux values were varied individually (or in pairs for bidirectional reactions) until an increase in E of greater than 5% was obtained. The extent of this permissible variation is the range presented in the fourth and sixth columns.

		Flux (nmol/20 min per mg of protein)			
		0–20 min interval		20–40 min interval	
Flux	Enzyme(s) or reaction	Best-fit value	Range	Best-fit value	Range
V_G	Glucokinase	46	45–47	40	35–46
V'_G	Glucose 6-phosphatase	92	91–93	152	146–158
V_2	Glucose phosphate isomerase	520	290–2000	100000	>1000
V'_2	Glucose phosphate isomerase	582	352–2062	100076	>1076
V_{12}	Glycogen synthesis	9.5	8.8–10.2	4.7	3.9–5.5
V'_{12}	Glycogen degradation	0	0–2	50	38–65
V_1	Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase	7.4	7.2–7.6	9.8	8.5–11.1
V_M	Hexokinase and mannose phosphate isomerase	11.4	11–12	12	10–14
V_3	6-Phosphofructokinase	9	6–12	8	5–11
V'_3	Fructose bisphosphatase	46	43–49	52	50–56
V_4	Fructose bisphosphate aldolase	51	26–133	226	>110
V'_4	Fructose bisphosphate aldolase	88	63–170	270	>154
V_5	Triose phosphate isomerase	44	41–48	60	55–56
V'_5	Triose phosphate isomerase	20	17–24	21	16–27
V_{OX}	Glycerol 3-phosphate oxidation	180	142–240	195	160–255
V_{RD}	Dihydroxyacetone phosphate reduction	119	81–179	112	77–172
V_{GL}	Glycerol kinase	123	109–148	87	84–105
V_{15}	Lipid glycerol synthesis	2.9	2.5–3.2	3.5	3.1–3.9
V_7	Ribulose phosphate 3-epimerase	220	155–480	1000	>300
V'_7	Ribulose phosphate 3-epimerase	206	141–466	980	>280
V_8	Ribose phosphate isomerase	230	160–560	205	130–460
V'_8	Ribose phosphate isomerase	237	167–567	215	140–470
V_9	Transketolase	95	65–180	410	>150
V'_9	Transketolase	88	58–173	400	>140
V_{10}	Transaldolase	151	120–230	120	80–170

Table 1—(*cont.*)

Flux	Enzyme(s) or reaction	Flux (nmol/20 min per mg of protein)			
		0–20 min interval		20–40 min interval	
		Best-fit value	Range	Best-fit value	Range
V'_{10}	Transaldolase	158	127–237	130	90–180
V_{11}	Transketolase	62	52–75	410	>175
V'_{11}	Transketolase	55	45–68	400	>165
V_R	Ribokinase	14	12–16	20	16–25
V_6	Lower Embden–Meyerhof pathway	17	15–20	28	16–56
V'_6	Lower Embden–Meyerhof pathway	22	20–25	23	11–51
V_{13}	Pyruvate kinase	6.3	5.9–6.8	15	13–18
V_{16}	Pyruvate dehydrogense	0.8	0.6–1.0	0.2	0–0.6
V_{25}	Alanine aminotransferase	11.3	11–12	7	3–24
V_{17}	Lactate dehydrogenase	0.4	0–1.4	13	9–30
V_{P1}	Pyruvate carboxylase	16.4	16.0–16.9	8	6–11
V_{P2}	Phosphoenolpyruvate carboxykinase	11.4	11.0–11.9	10	8–13
V_A	Acetate into mitochondria	4500	>2000	10000	>4500
V_{AO}	Acetate out of mitochondria	4570	>2070	10047	>4547
V_E	Alcohol dehydrogenase and aldehyde dehydrogenase	79	78–80	63.4	56–72
V_{AE}	Mitochondrial acetyl-CoA synthetase	7.3	6.9–7.7	10.7	10–12
V_L	Cytosolic lipid fatty acid synthesis	1.8	1.7–1.9	5.4	4.1–6.7
V_{29}	Input from ketogenic amino acids and endogenous lipids	25	23–27	22.5	21–25
V_{28}	Ketogenesis	3.4	1.5–5.0	3.2	2–5
V_{K1}	Citrate synthase and aconitate hydratase	30	27–33	30	29.5–30.4
V_{K2}	Isocitrate dehydrogenase	30	27–33	30	29.5–30.4
V_{K3}	Oxoglutarate dehydrogenase and succinyl-CoA synthetase	40	37–43	44	42–47
V_{21}	Succinate dehydrogenase and fumarate hydratase	53	>37	1544	>44
V'_{21}	Succinate dehydrogenase and fumarate hydratase	13	>0	1500	>0
V_{20}	Malate dehydrogenase	116	>70	50	>34
V'_{20}	Malate dehydrogenase	87	>40	16	>0
V_{23}	Glutamate aminotransferase	10	8–12	14	12–17
V_{24}	Aspartate aminotransferase	15	14–17	12	10–15