Biochem. J. (1985) **225**, 761–786
Printed in Great Britain

Quantitative analysis of intermediary metabolism in hepatocytes incubated in the presence and absence of glucagon with a substrate mixture containing glucose, ribose, fructose, alanine and acetate

Michael RABKIN* and J. J. BLUM†
Department of Physiology, Duke University Medical Center, Durham, NC 27710, U.S.A.

(*Received 10 July 1984/Accepted 16 October 1984*)

Hepatocytes were isolated from the livers of fed rats and incubated, in the presence and absence of 100nM-glucagon, with a substrate mixture containing glucose (10mM), fructose (4mM), alanine (3.5mM), acetate (1.25mM), and ribose (1mM). In any given incubation one substrate was labelled with ^{14}C. Incorporation of ^{14}C into glucose, glycogen, CO_2, lactate, alanine, glutamate, lipid glycerol and fatty acids was measured after 20 and 40min of incubation under quasi-steady-state conditions [Borowitz, Stein & Blum (1977) *J. Biol. Chem.* **252**, 1589–1605]. These data and the measured O_2 consumption were analysed with the aid of a structural metabolic model incorporating all reactions of the glycolytic, gluconeogenic, and pentose phosphate pathways, and associated mitochondrial and cytosolic reactions. A considerable excess of experimental measurements over independent flux parameters and a number of independent measurements of changes in metabolite concentrations allowed for a stringent test of the model. A satisfactory fit to the data was obtained for each condition. Significant findings included: control cells were glycogenic and glucagon-treated cells glycogenolytic during the second interval; an ordered (last in, first out) model of glycogen degradation [Devos & Hers (1979) *Eur. J. Biochem.* **99**, 161–167] was required in order to fit the experimental data; the pentose shunt contributed approx. 15% of the carbon for gluconeogenesis in both control and glucagon-treated cells; net flux through the lower Embden–Meyerhof pathway was in the glycolytic direction except during the 20–40min interval in glucagon-treated cells; the increased gluconeogenesis in response to glucagon was correlated with a decreased pyruvate kinase flux and lactate output; fluxes through pyruvate kinase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase were not co-ordinately controlled; Krebs cycle activity did not change with glucagon treatment; flux through the malic enzyme was towards pyruvate formation except for control cells during interval II; and 'futile' cycling at each of the five substrate cycles examined (including a previously undescribed cycle at acetate/acetyl-CoA) consumed about 26% of cellular ATP production in control hepatocytes and 21% in glucagon-treated cells.

In a preceding paper (Crawford & Blum, 1983), hepatocytes isolated from fed rats were incubated with a mixture of glucose (10mM), ribose (1mM), mannose (4mM), glycerol (3mM), acetate (1.25mM), and ethanol (5mM), and label incorporation into metabolic products was analysed with the aid of a complete model of the 'upper' metabolic pathways (gluconeogenesis, glycolysis, pentose phosphate pathway) and a minimal model of the 'lower' pathways (mitochondrial and associated reactions). The technique of analysis rests on only two assumptions: that the metabolic model adequately represents pathways relevant to metabolism of the substrates employed, and that the system is close to the isotopic and metabolic steady

* Present address: Department of Pathology, University of Utah, Salt Lake City, UT 84132, U.S.A.
† To whom correspondence and reprint requests should be addressed.

state by criteria defined elsewhere (see Blum & Stein, 1982, for a review of the theoretical aspects). The study of Crawford & Blum (1983) confirmed the classical formulation of the pentose phosphate pathway, and provided estimates of bidirectional fluxes in the pathways under investigation as well as quantification of futile cycling at several major metabolic crossroads. In an effort to extend our insight into the structural organization of metabolism in intact hepatocytes, we have incubated hepatocytes from fed rats with a mixture of glucose, ribose, fructose, alanine, and acetate, a substrate mixture which more closely resembles portal blood after a rich meal and also has a pattern of substrate input into the metabolic pathways that permitted a more comprehensive model of the 'lower' portion of metabolism to be developed. The experiments to be described were done as paired experiments, in the presence and absence of 100nM-glucagon, so that both the time course of changes in flux patterns and the ways in which glucagon altered metabolic flow under these conditions could be determined. Data obtained from control and glucagon-treated cells were analysed with a more complete metabolic model than that employed earlier, and a detailed view of the metabolism of these substrates was obtained. In addition to measurements of label flow and of oxygen consumption, measurements were made at a number of time points of the concentrations of glucose, glycogen, fructose, alanine, lactate, and urea, as well as of glucose 6-phosphate and fructose 1,6-bisphosphate. The agreement between the predictions of the model and these measurements constitutes evidence, besides the fit to the isotopic flow and oxygen consumption data, as to the model's validity. The pattern of metabolic flow in the glucagon-treated cells is shown to differ appreciably from that of control cells in a number of areas. These differences are discussed in relation to the structural organization of hepatic metabolism and the known regulatory actions of glucagon. A discussion of some energetic implications of this work was presented at the Second International Congress on Myocardial and Cellular Bioenergetics and Compartmentation (Blum & Rabkin, 1984).

Methods

Isolated hepatocytes were prepared by the method of Berry & Friend (1969) as modified by Wood & Blum (1982) from male Sprague–Dawley rats weighing between 260 and 380g, fed *ad libitum* on Purina chow. Surgery was performed between 09:30 and 10:00h. Viability as assessed by Trypan Blue exclusion was always greater than 83% and did not decrease during the incubation period.

Incubation of hepatocytes

All incubations were performed in triplicate in 50ml polycarbonate flasks, and were initiated by adding 1.0ml of washed hepatocyte suspension to 1.5ml of a buffered substrate mixture. In each experiment paired flasks were run with and without 100nM-glucagon. Final concentrations were: approx. 6–12mg of hepatocyte protein/ml, 0.3% (w/v) defatted bovine serum albumin (Chen, 1967), 10mM-glucose, 4mM-fructose, 3.5mM-alanine, 1.25mM-acetate, 1mM-ribose, 0.2mg of bacitracin/ml (included to retard degradation of glucagon; cf. Desbuqois *et al.*, 1974), 119.8mM-NaCl, 23.8mM-$NaHCO_3$, 4.8mM-KCl, 1.2mM-KH_2PO_4, 1.5mM-$CaCl_2$, 0.6mM-$MgSO_4$, and 5.0mM-Hepes/NaOH buffer, pH7.4. The following labelled substrates were used: [1-^{14}C]-, [6-^{14}C]-, or [U-^{14}C]-glucose, [U-^{14}C]fructose, [1-^{14}C]- or [U-^{14}C]-alanine, [1-^{14}C]- or [2-^{14}C]acetate, or [1-^{14}C]ribose. Final specific radioactivities were (in d.p.m./nmol): glucose, 30–90; fructose, 210–545; alanine, 165–590; acetate, 500–1400; ribose, 120.

Flasks were gassed with O_2/CO_2 (19:1) for 15s at 0°C, then capped and incubated with shaking (80 cycles/min) at 37°C for 0, 20 or 40min (except for some studies performed at 5min intervals). For measurement of glycogen content and radioactivity, incubations were terminated by adding 1ml of incubation suspension to 6ml of ice-cold 83% (v/v) ethanol (Wood & Blum, 1982). For all other measurements, incubations were terminated by adding 0.3ml of 13% (w/v) $HClO_4$ to the capped flasks and cooling to 0°C. $^{14}CO_2$ was collected by adding 0.2ml of Hyamine to the centre wells and incubating for 1h at 37°C with shaking. The $HClO_4$ suspensions were then neutralized with KOH and the centrifuged supernatants used for all assays except those involving glycogen, which were terminated as above.

Lactate

To measure the flow of ^{14}C from multiple labelled substrates into lactate, it was desirable to develop a method of lactate isolation which could be conveniently applied to a large number of samples. The method employs the conversion of lactate to pyruvate in the presence of lactate dehydrogenase and NAD^+, followed by conversion of pyruvate to alanine by glutamate–pyruvate transaminase and high concentrations of glutamate (cf. Noll, 1974). Pre-existing cations are first removed by passage through columns containing cation-exchange resin. After incubation with lactate dehydrogenase, NAD^+, glutamate–pyruvate transaminase, and glutamate, samples are applied to a second set of cation-exchange columns, which are then eluted and the radioactivity determined.

Parallel incubations in the absence of lactate dehydrogenase and NAD^+ allow subtraction of any radioactivity that may be present in pre-existing pyruvate or other transaminase substrates and confer on the assay the high specificity of lactate dehydrogenase. Results are corrected for yield as determined by the recovery of triplicate control samples containing 2–3mM-lactate and tracer [^{14}C]lactate. A demonstration of the linearity of this method and a detailed protocol are given in Fig. 1.

Other measurements

Incorporation of radioactivity into lipid glycerol, fatty acids, glucose, and amino acids was measured essentially as described in Crawford & Blum (1983). Total content of fructose plus fructose phosphate esters was measured by the method of Roe & Papadopoulos (1954), with appropriate standards for glucose and fructose. Details of these assays are available (Rabkin, 1984). Glycogen content and radioactivity were measured as described by Wood & Blum (1982). Alanine content was determined with alanine dehydrogenase by the method of Williamson (1974), lactate content according to Gutmann & Wahlefeld (1974), and glucose content was assayed with Worthington Automated Glucose Reagent. Glucose 6-phosphate and fructose 1,6-bisphosphate were measured by the chemiluminescence procedure of Golden & Katz (1980), and urea according to Sigma Technical Bulletin no. 535 (1974). Oxygen consumption was measured polarographically with a Clark oxygen electrode in a thermostatically controlled chamber with magnetic stirring. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Measurements of radioactivity

Radioactivity was counted in a Packard Tri-Carb Spectrometer with automatic external standard for quench correction. Hydrofluor scintillant (National Diagnostics) was used for determination of glucose, lipid, and lactate; other samples were counted in ACS scintillant (Amersham International).

Materials

Collagenase (about 40 units/mg) and Automated Glucose Reagent were from Worthington Biochemical Corp. Hyamine hydroxide and [1-^{14}C]-ribose were from New England Nuclear. [U-^{14}C]-fructose, [1-^{14}C]- and [6-^{14}C]glucose, and [U-^{14}C]-alanine were from Research Products International. All other radioactive substances were from Amersham Corp. Bovine serum albumin (fraction V), all incubation-mixture components, and all enzymes used for metabolite assays were from Sigma Chemical Co. Insulin-free glucagon was a gift from Dr. R. J. Hosele (Eli Lilly, Indianapolis, IN, U.S.A.). It was dissolved in 1mM-HCl and stored frozen in suitable portions.

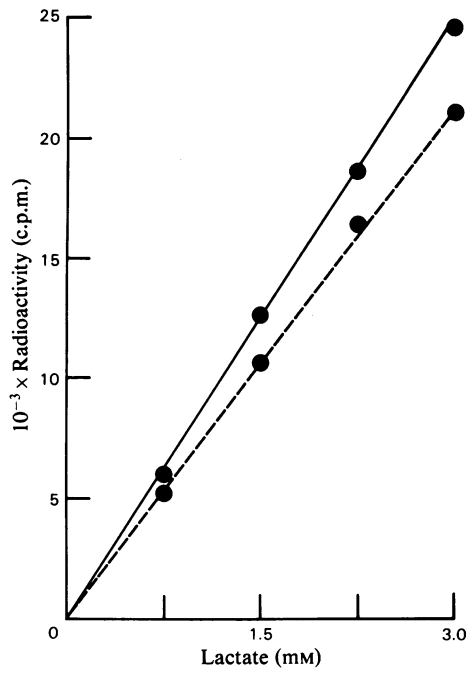

Fig. 1. *Linearity of lactate isolation procedure*
Aliquots of the standard incubation mixture containing 0–3mM-L-[U-^{14}C]lactate (50d.p.m./nmol) but without hepatocytes were treated with $HClO_4$ and then neutralized with KOH. Samples (0.5ml) were applied to columns (0.7cm × 7cm) of Dowex AG50W-X8 (50–100 mesh; H^+ form) and eluted with 6.0ml of water. Eluant (2ml) was combined with 0.65ml of 0.52M-glutamate (pH8.9), 0.1ml of 48mM-β-NAD^+, 0.04ml of lactate dehydrogenase [1100 units/ml in 3.2M-$(NH_4)_2SO_4$], and 0.03ml of glutamate–pyruvate transaminase [270 units/ml in 3.2M-$(NH_4)_2SO_4$]. After incubation for 2h at 37°C, the samples were put onto similar cation-exchange columns which were washed with 14ml of water and then eluted with two 7ml portions of 1M-NH_4OH. The radioactivity of both NH_4OH eluates was then determined. ——, Radioactivity in the original 0.5ml samples; – – –,radioactivity recovered from the second set of cation-exchange columns (corrected for dilution). Some assays were performed with smaller or larger quantities of cation-exchange eluant and proportional changes in other components; lactate recovery was in each case shown to be linear over the range 0–2mM. Background radioactivity in the presence or absence of lactate dehydrogenase and NAD^+ was very low when [^{14}C]acetate, -alanine, -glucose, or -fructose were included in control experiments.

Other chemicals were of the highest grade commercially available.

Preliminary considerations

Choice of substrates

After many preliminary experiments, we chose a mixture of 10mM-glucose, 4mM-fructose, 3.5mM-alanine, 1.25mM-acetate, and 1mM-ribose. This substrate mixture, which provides inputs and outputs which are well distributed about the metabolic pathways of interest, allows for a more detailed view of mitochondrial metabolism and the phosphoenolpyruvate/pyruvate/oxaloacetate crossroads than was possible in the study of Crawford & Blum (1983). It is considerably less reducing and more gluconeogenic than the mixture employed by them.

Metabolic scheme

The metabolic model used to analyse data gathered for control and glucagon-treated hepatocytes is shown in Fig. 2. Except for the deletion of mannose input and the addition of the fructokinase pathway (Sillero *et al.*, 1969; Van den Berghe, 1978), the upper portion of the model is identical to that employed by Crawford & Blum (1983). While fructose can also be phosphorylated by glucokinase, calculations using K_m values from Parry & Walker (1966) and assuming competitive binding indicate that fructose phosphorylation by this pathway would occur at less than 2% of the rate of glucose phosphorylation. Although some fructose 1-phosphate may accumulate intracellularly (Van den Berghe, 1978), V_F represents only fructose uptake resulting in label flow beyond fructose 1-phosphate.

The lower portion of the model is considerably more detailed than that employed by Crawford & Blum (1983). Amino acid carbon atoms may enter or leave at the level of pyruvate, oxaloacetate or 2-oxoglutarate. Input (from proteolysis) and output of other amino acids is subsumed under that of glutamate, aspartate, and alanine. Aspartate aminotransferase is present in both the mitochondrial and cytosolic compartments (DeRosa & Swick, 1975). Cytosolic oxaloacetate was assumed to be the source of carbon for aspartate formation, because the urea cycle utilizes cytosolic aspartate; any shuttling of carbon between mitochondria and cytosol in the form of aspartate is subsumed in the model under the movement of malate. Although alanine catabolism has been thought to be primarily mitochondrial (Mendes-Mourao *et al.*, 1975; Dieterle *et al.*, 1978; Chan *et al.*, 1979), a recent study suggests that mitochondrial alanine aminotransferase is poised toward alanine formation in hepatocytes incubated with 0.15–6.0mM-alanine (Groen *et al.*, 1982). Since we assume single pools of alanine and of pyruvate, the location of alanine aminotransferase in the model is arbitrary, and done only for convenience in drawing. To the extent that pyruvate is generated in the cytosol, changes in the flux through pyruvate carboxylase (V_{PC}) or pyruvate dehydrogenase (V_{PDH}) may be due in part or even entirely to changes in pyruvate transport into the mitochondria. Because the K_m of pyruvate dehydrogenase for pyruvate is much lower than that of pyruvate carboxylase (Siess *et al.*, 1982), flux through the dehydrogenase should be much less sensitive to changes in mitochondrial pyruvate availability.

Although the structural organization of the 'lower' portion of the present model contains most of the appropriate reactions and includes a degree of compartmentation, some caution must be exercised in interpreting the flux values of this portion of metabolism. Because alanine aminotransferase and aspartate aminotransferase are present in both mitochondria and cytosol, the location and size of these intracellular amino acid pools are not well defined, and it is difficult to give a precise physical interpretation of the bidirectional transaminase fluxes and of the aspartate and glutamate input and output fluxes. Furthermore, measured inputs and outputs in this region of the model are somewhat sparse, and estimates of such parameters as glutamate input and aspartate input and output are based primarily on the effects of isotope dilution. Therefore, estimates of these quantities are inherently less certain than those in other portions of the model.

In setting up the equations to analyse the model we have ignored possible reincorporation of $^{14}CO_2$, which is equivalent to assuming that metabolically generated $^{14}CO_2$ is equilibrated with the large amounts of unlabelled bicarbonate present. Under certain experimental conditions, reincorporation of $^{14}CO_2$ is not negligible (Tomera *et al.*, 1982; Hems & Saez, 1983). Since [^{14}C]-bicarbonate was not used as a substrate, it was not possible to estimate back flux in the tricarboxylic acid cycle, which was satisfactorily modelled as being unidirectional from citrate to malate.

Although rat liver mitochondria do not have a true acetyl-CoA synthase, some 23–33% of acetate activation (at 1mM-acetate) occurs in the mitochondrial fraction and has been attributed to a propionyl-CoA synthase (Scholte & Groot, 1975; Groot *et al.*, 1976). Goldberg & Brunengraber (1979) have estimated that approx. 36% of the acetate incorporated into hepatic lipids *in vivo* derives from mitochondrially activated acetate. Provision was therefore made for both mitochon-

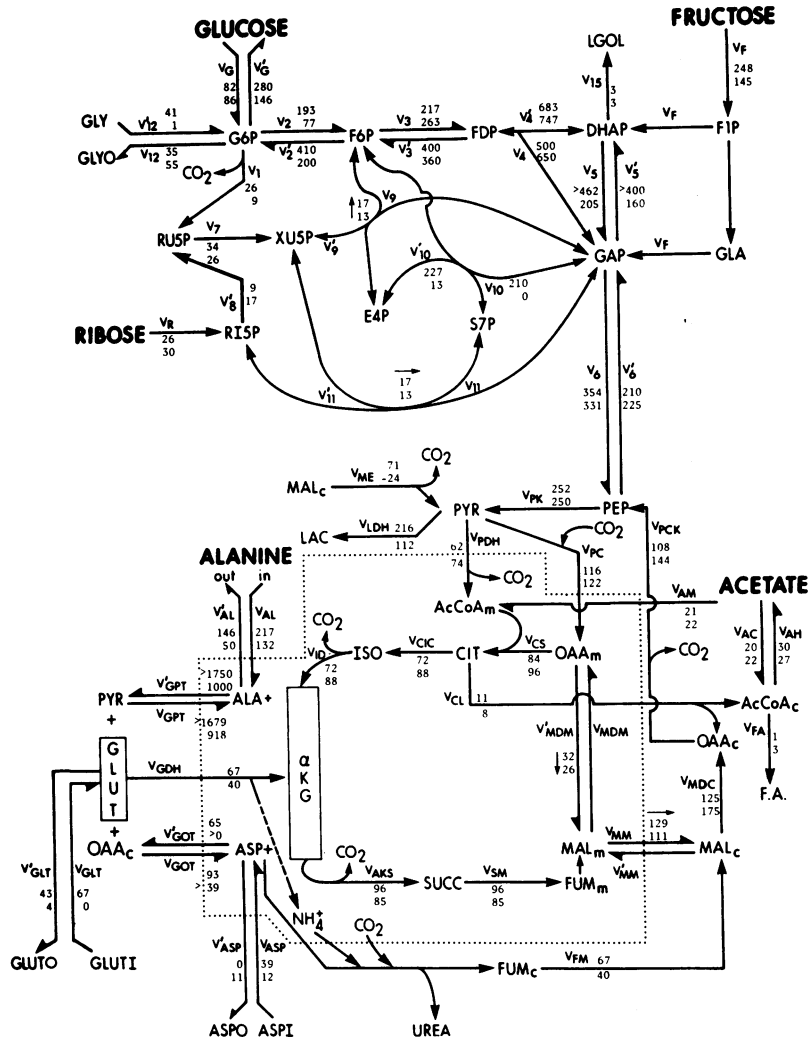

Fig. 2. *Best-fit model for data obtained from control hepatocytes*

Data presented in Tables 1 and 2 and oxygen consumptions given in the text were used to obtain best-fit estimates for the metabolic scheme shown. Each V represents the flux through the reaction or sequence of reactions defined in Appendix II. Each pair of numbers represents the values for fluxes, in nmol/20min per mg of cell 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 0–20min incubation interval, and the lower number for the 20–40min interval. In cases where only the net flux through a bidirectional reaction could be determined, a single pair of numbers represents the best-fit net flux values and a small arrow designates the direction of net flux. Where no upper limit could be determined for a bidirectional reaction, the lower limit values are preceded by a '>' sign, and the net flux is equal to the difference between the lower limit values of the unidirectional fluxes. For convenience of presentation, some of the flux values have been rounded off. Abbreviations used: $AcCoA_c$ and $AcCoA_m$, cytosolic and mitochondrial acetyl-CoA; ALA, intracellular alanine; ASP, intracellular aspartate; ASPI and ASPO, aspartate input and output; CIT, citrate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F. A., fatty acid; FDP, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GLA, glyceraldehyde; GLUT, intracellular glutamate; GLUTI and GLUTO, glutamate input and output; GLY and GLYO, glycogen input and output; G6P, glucose 6-phosphate; ISO, isocitrate; α-KG, 2-oxoglutarate; LAC, lactate; LGOL, lipid glycerol; MAL_c and MAL_m, cytosolic and mitochondrial malate; OAA_c and OAA_m, cytosolic and mitochondrial oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; RI5P, ribose 5-phosphate; RU5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SUCC, succinate; XU5P, xylulose 5-phosphate.

drial (V_{AM}) and cytosolic (V_{AC}) activation of acetate.

Mitochondrially generated acetyl-CoA is generally believed to participate in lipogenesis via transport of citrate to the cytosol and its cleavage there to oxaloacetate and acetyl-CoA (Kornacker & Lowenstein, 1965). A possible route of bidirectional acetyl-CoA transport between mitochondria and cytosol would be via carnitine acetyltransferase. However, the activity of this enzyme in mitochondria from the liver of fed rats is extremely low (Lund & Bremer, 1983), and activity in isolated hepatocytes may be further reduced because of carnitine depletion (McGarry *et al.*, 1978). This pathway was therefore omitted; to the extent that it may operate, V_{CL} and V_{AM} will overestimate the flux through citrate lyase and mitochondrial acetate activation, respectively.

Although the existence of an intramitochondrial acetyl-CoA hydrolase has been controversial, an extramitochondrial acetyl-CoA hydrolase has been demonstrated (Prass *et al.*, 1980). We did not originally appreciate the importance of this enzyme, but were unable to fit the data on acetate utilization and fatty acid synthesis without its inclusion in the model. Although Crawford & Blum (1983) were able to fit their data without provision for acetyl-CoA hydrolase, their model did not include separate mitochondrial and cytosolic pools of acetyl-CoA. It was found in the current study that the inclusion of intramitochondrial acetyl-CoA hydrolase or of non-zero values for oxidation of endogenous lipids or ketogenesis did not contribute to an improved fit, so these pathways were omitted from the metabolic scheme.

Analysis of data

The method for computer analysis of the data has been described in detail (Blum & Stein, 1982; Crawford & Blum, 1983). Briefly, the metabolic scheme was described by a set of simultaneous linear equations depicting the flow of radioactivity into and out of each carbon atom of each metabolite under steady-state conditions. A computer program allowed computation of oxygen consumption and of flow of ^{14}C from each labelled substrate into each metabolic product for any consistent set of flux values. Trial sets of flux values were repeatedly adjusted until an optimal fit (see below) was obtained. The model was completely determined by at most 33 independent flux values; in each fit there was sufficient information to quantify only the net flux through some bidirectional flux pairs, thereby reducing the number of independent fluxes to an average of 29. The considerable excess of experimental measurements (40–42/fit) over independent parameters ensured a stringent test of the model.

Results

Tables 1–4 present the data for incorporation of ^{14}C into the metabolites shown when hepatocytes were incubated with the standard substrate mixture with a single substrate labelled as indicated. Flow of ^{14}C from fructose into both lactate and glucose indicates simultaneous glycolytic and gluconeogenic fluxes. Evolution of $^{14}CO_2$ from [6-^{14}C]glucose requires that carbons from glucose flow in a glycolytic direction at least as far as the triose phosphates. Since carbons from fructose, alanine, and acetate appear in glucose over this same time period, futile cycling must occur at both the glucose/glucose 6-phosphate and fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycles. The flow of label from fructose to CO_2 and lactate while ^{14}C from alanine and acetate enters glucose indicates futile cycling at the phosphoenolpyruvate/pyruvate/oxaloacetate crossroads. The substantially greater evolution of $^{14}CO_2$ from [1-^{14}C]- than [6-^{14}C]glucose shows operation of the oxidative arm of the pentose phosphate pathway despite the low rate of lipogenesis.

Although these considerations apply equally to control and glucagon-treated cells, certain quantitative differences are apparent (Tables 1–4). Glucagon-treated cells incorporated more label from fructose, alanine, and acetate into glucose and less label into lactate, alanine, and glutamate than did control cells, especially in interval II. The incorporation of label from acetate into fatty acid was considerably lower in glucagon-treated than in control cells. Net glucose release was greater in glucagon-treated than in control cells (Table 5), as expected from the gluconeogenic and glycogenolytic effects of glucagon. The decrease in lactate production and increase in urea production in glucagon-treated cells is in accord with earlier studies (McGarry *et al.*, 1978; Meijer & Hensgens, 1982).

Oxygen consumption of control cells was 529 ± 34 and 508 ± 42 nmol of O atoms/20min per mg of protein during the first and second intervals, respectively ($n = 4$), as compared with fitted values of 534 and 504nmol of O atoms/20min per mg of protein. This was about 25% higher than the consumption observed by Crawford & Blum (1983) with a different substrate mixture. Measured and fitted oxygen consumptions for the glucagon-treated hepatocytes were 588 ± 32 and 579nmol of O atoms/20min per mg of protein, respectively, for interval I and 587 ± 40 and 574, respectively, for interval II. This represents an approx. 14% stimulation of respiration by glucagon.

Initial glycogen content was 1054 ± 255nmol of glucose equivalents/mg of protein ($n = 10$) and did not change appreciably over the 40min incuba-

Table 1. *Experimental and computed incorporation of ^{14}C-labelled substrates into glucose, glycogen, CO_2 and lactate in control hepatocytes*

Hepatocytes from fed rats were prepared and incubated in standard substrate mixture as described in the Methods section, with a single substrate labelled as indicated in the left-hand column. Interval I designates the period from 0 to 20min of incubation; interval II the period from 20 to 40min of incubation. The mean values for incorporation of radioactivity into the indicated products are given, along with their confidence limits, as the upper numbers in each row. For $n>2$, the confidence limit is the standard deviation of the mean. For $n=2$, the confidence limit is one-half the difference between the two experimental values. When incorporation was not detectable, the estimated lower limit of detection for the assay is presented. The lower number of each pair is the value of incorporation predicted from the best-fit flux values shown in Fig. 2. Negative values designate net loss of label over the interval. Values in parentheses designate the number of rats upon which that experimental measurement was made. Abbreviations used: N.M., not measured; *, not used in calculating Fit Parameter.

	Incorporation (nmol/20min per mg of protein)							
	Glucose		Glycogen		CO_2		Lactate	
Substrate	Interval I	Interval II	Interval I	Interval II	Interval I	Interval II	Interval I	Interval II
[1-^{14}C]Glucose	N.M. 65.39	N.M. 57.72	6.71±3.42 (4) 8.19	21.81±7.62 (4) 21.51	5.90±0.82 (4) 5.84	4.06±0.81 (4) 4.08	<5.71 (2) 1.26	<5.71 (2) 1.57
[6-^{14}C]Glucose	N.M. 66.40	N.M. 57.79	8.12±3.04 (4) 8.32	23.04±8.61 (4) 21.53	0.85±0.19 (4) 0.86	1.59±0.31 (4) 1.47	<5.71 (2) 3.40	<5.71 (2) 3.20
[U-^{14}C]Glucose	N.M. 67.47	N.M. 58.34	8.43±1.87 (4) 8.45	19.27±3.61 (4) 21.74	N.M. 1.67	N.M. 2.18	N.M. 2.41	N.M. 2.33
[U-^{14}C]Fructose	147.56±8.72 (5) 150.98	59.10±23.63 (4) 67.26	18.29±2.53 (4) 18.91	25.42±1.57 (3) 24.80	9.70±1.20 (8) 11.01	15.67±1.98 (9) 16.23	37.84±2.71 (4) 36.66	21.10±6.99 (3) 22.78
[1-^{14}C]Ribose	21.02±6.17 (4) 20.30	22.11±6.66 (4) 18.12	N.M. 2.54	N.M. 6.75	0.66±0.23 (4) 0.66	1.48±0.52 (4) 1.93	N.M. 1.15	N.M. 1.93
[1-^{14}C]Alanine	4.21±0.88 (7) 4.66	5.12±1.69 (6) 4.73	0.58±0.10 (2) 0.58	0.62±0.00* (2) 1.77	58.56±7.14 (10) 49.31	59.21±6.66 (10) 54.55	N.M. 95.76	N.M. 44.94
[U-^{14}C]Alanine	8.12±1.70 (4) 7.08	9.83±1.85 (4) 9.70	N.M. 0.89	N.M. 3.63	26.54±5.02 (11) 24.24	33.06±3.45 (11) 34.28	92.70±15.76 (4) 101.16	39.20±10.74 (4) 50.18
[1-^{14}C]Acetate	0.70±0.13 (4) 0.79	1.41±0.17 (4) 1.39	0.17±0.08 (3) 0.10	0.02±0.48 (3) 0.52	9.59±1.49 (9) 8.48	15.97±2.26 (9) 14.34	1.70±1.28 (4) 1.69	1.77±0.39 (4) 1.46
[2-^{14}C]Acetate	1.88±0.76 (5) 1.95	4.89±0.98 (4) 4.20	0.28±0.14 (3) 0.24	0.86±0.53 (3) 1.57	1.50±0.36 (8) 1.40	4.63±1.13 (7) 4.87	4.47±2.69 (4) 4.25	4.78±2.25 (4) 4.42

Table 2. *Experimental and computed incorporation of ^{14}C-labelled substrates into alanine, glutamate, lipid glycerol and fatty acid in control hepatocytes*
For details see the legend to Table 1.

	Incorporation (nmol/20 min per mg of protein)							
	Alanine		Glutamate		Lipid glycerol		Fatty acid	
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.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	0.90	0.62	0.21	0.09	0.05	0.16	0.00	0.01
[6-^{14}C]Glucose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	2.25	1.25	0.53	0.18	0.10	0.08	0.00	0.02
[U-^{14}C]Glucose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	1.60	0.91	0.26	0.09	0.08	0.13	0.00	0.01
[U-^{14}C]Fructose	21.74 ± 4.23 (3)	10.65 ± 2.11 (3)	4.09 ± 0.30 (3)	2.34 ± 0.80 (3)	1.25 ± 0.12 (2)	1.29 ± 0.04 (2)	0.17 ± 0.06 (2)	0.90 ± 0.15* (2)
	24.28	8.92	3.94	0.89	1.26	1.31	0.03	0.08
[1-^{14}C]Ribose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	0.76	0.76	0.03	0.07	0.05	0.21	0.00	0.01
[1-^{14}C]Alanine	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	66.54	23.87	0.12	0.05	0.04	0.06	0.00	0.00
[U-^{14}C]Alanine	N.M.	N.M.	N.M.	N.M.	0.36 ± 0.11 (2)	1.78 ± 0.57 (2)	0.09 ± 0.01 (2)	0.18 ± 0.01 (2)
	70.11	25.92	10.81	1.96	0.06	0.12	0.09	0.18
[1-^{14}C]Acetate	0.98 ± 0.03 (2)	0.75 ± 0.43 (2)	5.76 ± 0.73 (2)	−0.46 ± 1.13 (2)	0.01 ± 0.03 (5)	0.01 ± 0.05 (5)	0.76 ± 0.58 (4)	2.20 ± 0.96 (3)
	1.12	0.57	6.21	0.94	0.01	0.02	0.76	2.45
[2-^{14}C]Acetate	3.60 ± 1.34 (2)	1.23 ± 0.35 (2)	7.53 ± 1.14 (2)	0.36 ± 1.04 (2)	0.03 ± 0.03 (5)	0.03 ± 0.09 (3)	0.74 ± 0.38 (4)	3.23 ± 1.97 (4)
	2.81	1.73	7.57	1.39	0.02	0.05	0.77	2.47

Table 3. *Experimental and computed incorporation of ^{14}C-labelled substrates into glucose, glycogen, CO_2 and lactate in glucagon-treated hepatocytes*
For details see the legend to Table 1. Best-fit values are in Fig. 3.

	Incorporation (nmol/20min per mg of protein)							
	Glucose		Glycogen		CO_2		Lactate	
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.	2.54±0.44 (3)	−1.09±0.09 (3)	6.37±1.02 (4)	5.08±1.24 (4)	<5.71 (2)	<5.71 (2)
	48.00	74.08	2.70	−1.03	5.73	4.77	0.21	0.04
[6-^{14}C]Glucose	N.M.	N.M.	2.83±0.28 (4)	−0.87±0.26 (4)	0.69±0.19 (4)	0.88±0.13 (4)	<5.71 (2)	<5.71 (2)
	50.91	77.55	2.87	−1.11	0.82	0.91	0.90	0.16
[U-^{14}C]Glucose	N.M.	N.M.	3.11±1.01 (4)	−0.83±0.52 (4)	N.M.	N.M.	N.M.	N.M.
	51.25	77.39	2.88	−1.13	1.42	1.38	0.57	0.10
[U-^{14}C]Fructose	190.19±37.45 (9)	152.35±8.15 (9)	10.49±0.77 (4)	−6.10±0.87 (4)	8.73±0.99 (9)	8.31±1.29 (9)	15.31±3.88 (4)	3.25±2.27 (4)
	181.74	156.94	10.23	−6.09	9.63	8.85	12.77	1.34
[1-^{14}C]Ribose	22.58±6.01 (3)	26.43±5.57 (4)	N.M.	N.M.	0.32±0.08 (4)	0.43±0.09 (4)	N.M.	N.M.
	20.78	23.07	1.17	−0.64	0.33	0.44	0.32	0.04
[1-^{14}C]Alanine	6.05±2.03 (7)	10.16±3.21 (7)	0.37±0.06 (2)	0.01±0.03* (2)	65.81±9.94 (9)	67.22±10.95 (10)	N.M.	N.M.
	6.59	11.03	0.37	−0.13	57.88	69.76	59.46	6.05
[U-^{14}C]Alanine	11.27±2.81 (4)	23.72±4.00 (4)	N.M.	N.M.	33.36±7.63 (10)	41.74±4.67 (11)	57.32±6.62 (4)	7.15±2.10 (4)
	12.24	23.22	0.69	−0.19	28.92	43.23	61.81	8.07
[1-^{14}C]Acetate	1.09±0.16 (4)	2.06±1.05 (4)	0.10±0.05 (3)	0.09±0.06 (3)	7.45±2.08 (9)	12.92±4.32 (9)	0.35±0.26 (4)	0.50±0.31 (3)
	1.17	2.81	0.07	−0.01	6.10	14.32	0.47	0.46
[2-^{14}C]Acetate	2.54±1.53 (5)	7.51±3.82 (5)	0.26±0.07 (3)	0.21±0.21 (3)	1.91±0.70 (9)	4.29±1.22 (8)	1.14±0.58 (3)	1.37±1.00 (3)
	2.96	8.32	0.17	0.00	1.25	4.61	1.21	1.38

Table 4. *Experimental and computed incorporation of ^{14}C-labelled substrates into alanine, glutamate, lipid glycerol and fatty acid in glucagon-treated hepatocytes*
For details see the legend to Table 1. Best-fit values are in Fig. 3.

	Incorporation (nmol/20min per mg of protein)							
	Alanine		Glutamate		Lipid glycerol		Fatty acid	
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.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	0.23	0.00	0.06	0.01	0.02	0.04	0.00	0.00
[6-^{14}C]Glucose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	1.00	0.01	0.24	0.03	0.08	0.07	0.00	0.00
[U-^{14}C]Glucose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	0.63	0.01	0.10	0.01	0.05	0.06	0.00	0.00
[U-^{14}C]Fructose	12.76±2.93 (3)	−0.04±0.12 (3)	2.37±0.25 (3)	−0.14±0.30 (3)	1.19±0.14 (2)	0.94±0.15 (2)	0.05±0.02 (2)	0.21±0.10 (2)
	14.22	0.11	2.29	0.18	1.14	0.98	0.01	0.03
[1-^{14}C]Ribose	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	0.35	0.00	0.01	0.00	0.03	0.05	0.00	0.00
[1-^{14}C]Alanine	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
	73.09	1.24	0.18	0.00	0.04	0.06	0.00	0.00
[U-^{14}C]Alanine	N.M.	N.M.	N.M.	N.M.	0.16±0.06 (2)	0.99±0.54 (2)	0.07±0.01 (2)	0.18±0.03 (2)
	75.70	1.41	11.01	1.05	0.08	0.12	0.07	0.18
[1-^{14}C]Acetate	0.53±0.06 (2)	0.07±0.11 (2)	4.06±0.60 (2)	−0.12±0.54 (2)	0.01±0.01 (5)	0.02±0.02 (5)	0.22±0.11 (4)	0.46±0.28 (3)
	0.52	0.04	3.78	0.38	0.01	0.01	0.26	0.50
[2-^{14}C]Acetate	1.86±0.68 (2)	0.51±0.20 (2)	4.55±0.36 (2)	1.52±0.64 (2)	0.02±0.02 (5)	0.07±0.05 (5)	0.24±0.13 (4)	0.60±0.23 (4)
	1.35	0.11	4.71	0.62	0.02	0.04	0.26	0.54

Table 5. *Predicted and observed metabolite flux values for control and glucagon-treated hepatocytes*
Predicted flux values were taken from Figs. 2 and 3. Observed flux values are from measurements of metabolite concentrations in neutralized $HClO_4$ extracts of hepatocytes isolated from fed rats and incubated in standard substrate mixture as described in the Methods section. Both the predicted and observed fructose fluxes neglect accumulation of fructose phosphate esters. Values are means ± S.D. for the number of experiments shown in parentheses.

	Flux parameter(s) and metabolites	Control cells in interval		Glucagon-treated cells in interval	
		I	II	I	II
Predicted	$V_{12}-V'_{12}$	−6	54	−9	−212
Observed	Glycogen*	28±64 (10)	44±47 (10)	−18±109 (10)	−255±74 (10)
Predicted	V'_G-V_G	198	60	245	404
Observed	Glucose	167±74 (14)	128±121 (13)	294±65 (14)	289±94 (14)
Predicted	$-V_F$	−248	−145	−234	−165
Observed	Fructose	−281±40 (6)	−167±21 (6)	−303±52 (6)	−160±11 (6)
Predicted	$V'_{AL}-V_{AL}$	−71	−82	−75	−91
Observed	Alanine	−87±30 (5)	−38±25 (5)	−96±34 (5)	−90±34 (5)
Predicted	V_{LDH}	216	112	106	15
Observed	Lactate	143±30 (6)	100±38 (7)	53±12 (6)	25±10 (6)
Predicted	V_{GDH}	67	40	80	63
Observed	Urea	33±4 (5)	33±6 (5)	44±4 (5)	52±11 (5)

* Glycogen at $t=0$ was 1054±255 nmol of glucose equivalents/mg of protein ($n=10$).

tion. The high rates of gluconeogenesis and ureogenesis (Table 5) indicate that the concentration of fructose employed allowed good maintenance of cellular metabolism and ATP levels, in agreement with the findings of Wood *et al.* (1981). Fructose decreased to about 1 mM at the end of the 40 min incubation, still above its K_m ($\simeq 0.5$ mM) for fructokinase (Van den Berghe, 1978). Alanine remained above 1.5 mM throughout the incubation. The low flow of label from ribose and acetate makes it very unlikely that these concentrations dropped below 0.3 of their initial values.

Gluconeogenesis during the first interval decreases the specific activity of glucose when it is the labelled substrate; computations for the second interval were corrected accordingly. Calculations indicated that negligible error would result from the recycling of label in input substrates that are also products (e.g. glucose, alanine, acetate) or from label acquisition by initially unlabelled substrates.

Statistical analysis of data

The number of measurements, N, used in the fitting process was generally 42. Because the measured incorporation of [1-^{14}C]alanine into glycogen (20–40 min, glucagon-treated and control cells) and of [U-^{14}C]fructose into fatty acids (20–40 min, control cells) were small numbers with improbably low error and were incompatible with other data, these were not used in the fitting process.

Goodness of fit was assessed with a weighted least squares parameter, E, defined elsewhere (Blum & Stein, 1982). The flux values that yielded the minimum value for E are presented in Figs. 2 and 3. Although Blum & Stein (1982) used a value of E comparable to N as indicative of a good fit, a preferable criterion may be to compare E to $N-I$, where I is the number of independent parameters. This allows E to be treated as a χ^2 deviate with $N-I$ degrees of freedom. The values of E obtained in the present studies were: 0–20 and 20–40 min control cells, $E=22$ and 27, respectively; 0–20 and 20–40 min glucagon-treated cells, $E=15$ and 24, respectively. A χ^2 test (subject to the qualifications detailed in question 3 of Appendix I) shows that $P<0.01$ for each interval and condition, indicating that the lack of a perfect fit is not highly statistically significant.

Given that the best-fit flux values shown in Figs. 2 and 3 yield a satisfactory fit to the data for each interval and condition, several questions arise concerning the interaction between the data and the model which require statistical analysis. The statistical instruments used and their limitations are presented in detail in Appendix I. The first question that may be asked is whether the between-substrate variation for each metabolic product measured could result from errors arising in the measurement of label flow into that product. This was evaluated by using the F-statistic described in Appendix I. In each interval and condition the P value for label incorporation into lipid fatty acids was large, ranging from 0.01–0.025 for control cells in interval I to 0.1–0.25 for the other three conditions. This is consistent with the small amounts (and hence relatively large uncer-

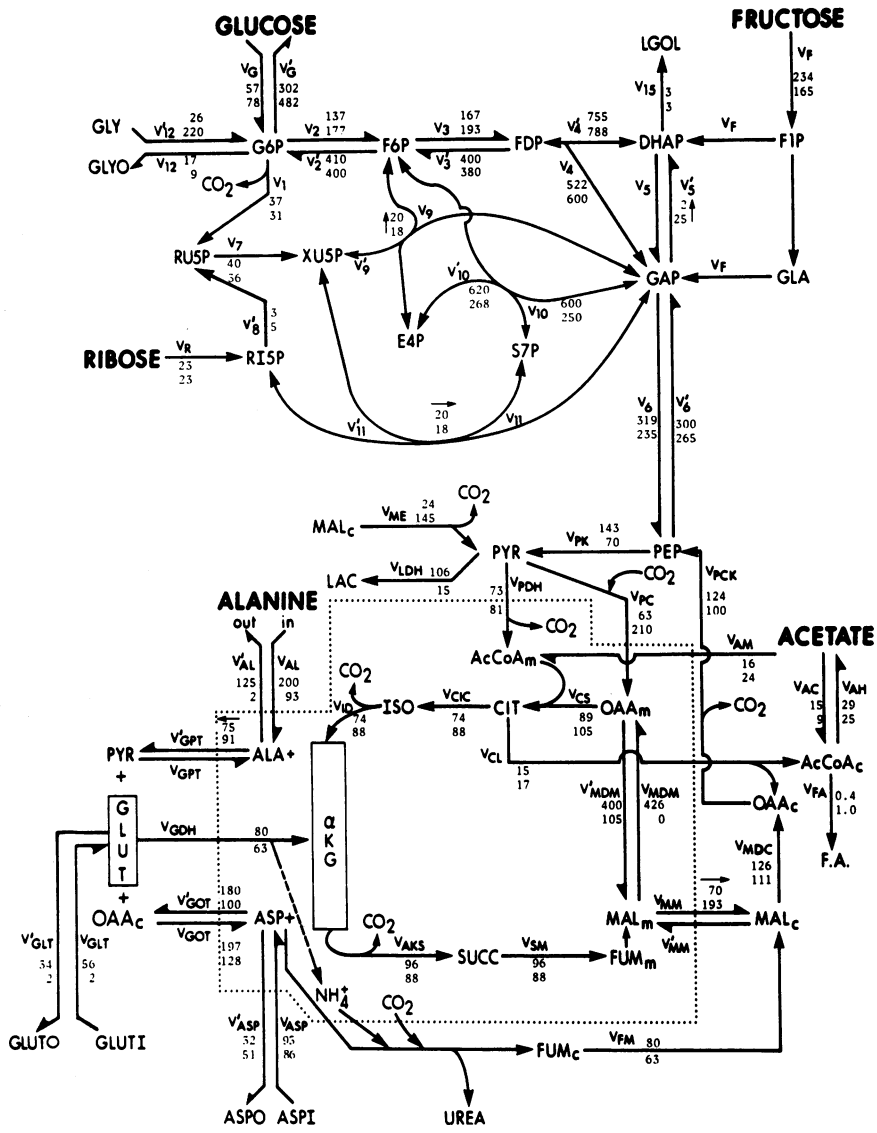

Fig. 3. *Best-fit model for data obtained from glucagon-treated hepatocytes*
Data presented in Tables 3 and 4 and oxygen consumptions given in the text were used to obtain the best-fit flux estimates. For further details, see the legend to Fig. 2.

tainties) of label flowing into fatty acids. Uncertainty in the small values of V_{FA}, however, has essentially no effect on the interpretation of the metabolic pattern. The measurements of label flow into glutamate from the three labelled substrates used might also have differed merely as a result of measurement error, since the P values were <0.005 for control cells in interval I and $0.1<P<0.25$ for glucagon-treated cells in interval II. Similarly, the P value for alanine in interval II for glucagon-treated cells was $0.1<P<0.25$. The P values for all other measurements were <0.005, with most of them being $<10^{-4}$.

A second question that may be asked is whether that portion of the between-substrate variation that is explained by the model can be attributed to measurement error. Use of a χ^2 test indicates that $P<10^{-5}$ for each condition and interval. A related question is whether the unexplained variation can be attributed to measurement error. Use of the χ^2 test outlined in Appendix I indicates that $P<10^{-5}$ for each condition and interval, i.e., that the (small

amount of) unexplained variation is highly significant and is most unlikely to be the result of errors of measurement. This implies that the model does not completely explain all the information contained in the data. A measure of the explanatory power of the model is the ratio of explained variation to total variation. This value was greater than 0.95 for each condition and interval, indicating that the model explained most of the variation in the data.

To evaluate properly the significance of changes in the best fit flux values between intervals or as a result of glucagon addition, it is necessary to estimate confidence limits for each flux value. Since the fitted flux values are highly interdependent, this is a complex statistical problem. In the absence of a statistically valid criterion, we present in Appendix II the range of variation obtained when each V_i was varied, one at a time, until the fit parameter, E, changed by more than 5% (see also Crawford & Blum, 1983). The allowable deviation of the V_i from their best fit values to within this arbitrary criterion is not to be taken as a confidence limit but instead provides a measure of the effect of varying each V_i on the goodness of fit, i.e., of the relative precision within which the individual V_i have been determined. It should be emphasized that while unidirectional fluxes for certain bidirectional flux pairs (e.g., V_{11} and V'_{11}) may be loosely determined or even indeterminate, the *net* flux through each such pair is tightly determined. In the absence of a statistically valid criterion for assessing the confidence limits on each V_i, the range of flux values given in Appendix II assists one in assessing the meaningfulness of differences in the best fit flux values between different intervals and conditions.

Handling of glycogen metabolism in the model

During the first interval, radioactivity accumulates in glycogen (Tables 1 and 3). If phosphorylase is active during the second interval, then label will flow from glycogen into the glucose 6-phosphate pool, even if net glycogen deposition is occurring. Two models of glycogen breakdown were examined: random degradation, observed in the liver of newborn rats (Devos & Hers, 1980), and ordered (last in, first out) degradation, thought to be the major mechanism in adult rat liver (Devos & Hers, 1979).

To perform the calculations with the random degradation model, it was assumed that all ^{14}C entering glycogen during interval I was homogeneously distributed into the 1054nmol of glucose equivalents/mg of protein initially present as glycogen. This resulted in predicted values for glycogenolysis and glucose release during the second interval that exceeded the observed values by several-fold.

Analysis according to a last in, first out model of glycogen degradation was performed as follows. Control hepatocytes did not exhibit net glycogenolysis in interval II (Table 1). The specific radioactivity of glycogen during the second interval was therefore set equal to that of newly synthesized glycogen in interval I; i.e., it was assumed that there was no dilution by the pre-existing glycogen. Glucagon-treated cells, however, had a glycogenolysis of 255nmol of glucose equivalents during interval II (Table 5). To calculate glycogen specific radioactivity, it was assumed that all the ^{14}C entering glycogen during interval I was distributed in this pool. With this approach, predictions of glycogen breakdown and glucose release were in good agreement with measured values for both control and glucagon-treated cells. These findings constitute an independent confirmation of the last in, first out model of Devos & Hers (1979).

Discussion

Validity of steady-state assumption

The equations used to analyse the data assume that the hepatocytes attain metabolic and isotopic steady state shortly after their addition to the substrate mixture. However, if changes in pool size occur slowly relative to the total flux of carbon through that pool, the system is in a quasi-steady-state wherein flux calculations remain valid (Borowitz *et al.*, 1977). The glucose 6-phosphate and fructose 1,6-bisphosphate pools are likely to be among the most rapidly changing metabolite pools upon addition of cells to the substrate mix. The concentration of glucose 6-phosphate did not change significantly in control hepatocytes throughout the 40min incubation, but rose to 3–4 times its initial value of 0.72nmol/mg of protein in glucagon-treated cells (Table 6). The concentration of fructose 1,6-bisphosphate dropped markedly at the start of the incubation but was essentially stable from 5 to 40min, with control cells maintaining approximately twice the fructose 1,6-bisphosphate concentration of glucagon-treated hepatocytes. Similar concentrations of glucose 6-phosphate and fructose 1,6-bisphosphate and the same pattern of alteration upon glucagon treatment have been observed by Van Schaftingen *et al.* (1980) in freeze-clamped livers from fed rats. Since the flow of carbon through the glucose 6-phosphate and fructose 1,6-bisphosphate pools far exceeds the changes in concentration of either metabolite in each condition and interval studied (Tables 1–4), the quasi-steady-state hypothesis for these nodes is valid.

Measurements of O_2 consumption and fluorescence of NADH in hepatocytes incubated under

Table 6. *Metabolite concentrations during a 40min incubation of control and glucagon-treated hepatocytes with standard substrate mixture*

Values are means ± S.D. for five experiments, except that $n = 4$ when indicated by an asterisk.

Time (min)	Concentration (nmol/mg of protein) of: Glucose 6-phosphate		Fructose 1,6-bisphosphate	
	Control	Glucagon	Control	Glucagon
0	0.72 ± 0.26		0.46 ± 0.22	
5	0.74 ± 0.34	1.11 ± 0.07	0.062 ± 0.013	0.032 ± 0.007
10	0.99 ± 0.39*	1.56 ± 0.31	0.055 ± 0.012	0.035 ± 0.006
20	0.98 ± 0.26	2.68 ± 0.94	0.079 ± 0.015	0.035 ± 0.016*
40	0.84 ± 0.17	2.20 ± 0.81	0.077 ± 0.019	0.024 ± 0.009*

similar conditions showed stabilization within less than 4min after addition of cells to the substrate mix (Balaban & Blum, 1982). Measurements performed with the present substrate mixture (results not shown) indicate that O_2 consumption remained essentially constant from the earliest time examined (6min) through 60min of incubation.

Further evidence concerning the validity of the quasi-steady-state assumption during most of the first interval was obtained from studies on the rate of passage of label from a number of labelled substrates into products and from measurements of net production of glucose, lactate, and urea and consumption of alanine and fructose. These studies may be summarized as follows (results not shown): evolution of glucose and lactate and consumption of fructose were linear from 0 to 20min; urea evolution was linear after a lag of less than 5min; the fluxes of label from [U-^{14}C]fructose to glucose and lactate and from [1-^{14}C]alanine to lactate were linear from 0 to 20min; the flux from [U-^{14}C]-fructose to CO_2 was linear after a lag period of less than 5min; the fluxes from [1-^{14}C]alanine to CO_2 and glucose increased slowly throughout the 0–20min interval. These data are consistent with a system that approaches steady state within a few minutes after addition of the cells to the substrate mixture. The flux values obtained therefore represent the average flow of carbon in these cells during each interval; values during the first interval may be subject to some error because steady-state conditions were not attained at some nodes for several minutes.

The data used to obtain the best fit flux values were measurements of oxygen consumption and of label incorporation into various metabolites. The flux values so computed predict the net amounts of glucose, fructose, and alanine that should have been consumed and the amounts of lactate and urea that should have been produced during the incubation for both control and glucagon-treated cells. Table 5 shows that the agreement between predicted and observed values is generally satisfactory except for urea production. This was considerably larger during interval I than the value predicted by the model, although the predicted and observed values agreed closely in interval II. To the extent that NH_4^+ may accumulate during the 5min lag period before urea production begins and urea cycle activity reaches its steady-state level, this will lead to an overestimation of urea cycle activity. The good agreement between other observed and predicted values in Table 5 reinforces the view that the flux values computed from our data provide a valid quantitative description of metabolic fluxes in control and glucagon-treated hepatocytes under these conditions.

Metabolic flow pattern

Net glucose release ($V'_G - V_G$) in control cells fell from 198 to 60nmol/20min per mg of protein in the second interval (Fig. 2). Glycogen synthesis and breakdown were almost balanced during the first 20min, but net glycogen synthesis occurred during interval II. Gluconeogenesis (i.e. $V'_2 - V_2$) decreased from 217 to 123nmol/20min per mg of protein in interval II, of which 34 and 26nmol/20min per mg of protein, respectively, were contributed by the pentose phosphate pathway ($V_9 - V'_9 + V'_{10} - V_{10}$). Fructose utilization (V_F) fell from 248 to 145nmol/20 min per mg of protein, while alanine uptake ($V_{AL} - V'_{AL}$) rose slightly from 71 to 82 nmol/20min per mg of protein. There was, however, no net contribution of alanine to gluconeogenesis under these conditions, since net flow in the lower Embden–Meyerhof pathway was glycolytic ($V_6 > V'_6$) throughout the incubation. Flux through pyruvate kinase (V_{PK}), phosphoenolpyruvate carboxykinase (V_{PCK}), pyruvate dehydrogenase (V_{PDH}), and pyruvate carboxylase (V_{PC}) did not change markedly, but lactate production (V_{LDH}), which was considerable, dropped by about 50% in interval II. Acetate

activation ($V_{AM}+V_{AC}$) was about equal in the mitochondrial and cytosolic compartments and far exceeded the amount of acetyl-CoA used for lipogenesis (V_{FA}). Flux from citrate to 2-oxoglutarate and from 2-oxoglutarate to fumarate changed little with time. There was net flow in the mitochondria from oxaloacetate to malate, and a large flow of malate occurred from mitochondria to cytosol. Some net utilization of glutamate ($V_{GLT}-V'_{GLT}$) and of aspartate ($V_{ASP}-V'_{ASP}$) occurred in the first interval, but by the second interval there was very little uptake or release of either amino acid.

Although glucose release was similar in control and glucagon-treated cells during interval I [200–250nmol/20min per mg of protein, (Figs. 2 and 3)], in the second interval it fell to 60 in control cells but rose to about 400 in glucagon-treated cells. This difference came predominantly from the large glycogenolysis of glucagon-treated cells in interval II, although net gluconeogenic flow through the fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle (V'_3-V_3) and net fructose 6-phosphate production by the pentose phosphate pathway ($V_9-V'_9+V'_{10}-V_{10}$) was also larger for glucagon-treated than for control cells. Fructose utilization (V_F) and alanine uptake ($V_{AL}-V'_{AL}$) were scarcely altered by the addition of glucagon, but the fate of alanine was changed significantly. This is shown by the alteration in flow along the lower Embden–Meyerhof pathway ($V_6-V'_6$) from net glycolysis during interval I to net gluconeogenesis during interval II in glucagon-treated cells and by the much smaller lactate output (V_{LDH}) in these cells relative to controls, especially in interval II.

Krebs cycle activity was virtually identical in control and glucagon-treated cells, as was ribose utilization (V_R) and lipid glycerol formation (V_{15}). While there was little difference in utilization of glutamate, glucagon appears to have increased the net utilization of (endogenous) aspartate, especially in the second interval.

Futile cycles

Reversal of flux along steps containing reactions displaced far from equilibrium requires that such steps be bypassed by other irreversible reactions. If paired opposing reactions operate simultaneously there is an apparently wasteful hydrolysis of ATP, often referred to as futile cycling (for recent reviews, see Katz & Rognstad, 1976; Hue, 1981, 1982). Stein & Blum (1978) have developed a general formalism for the analysis of such cycles. They define the futility, F, of a substrate cycle as the reverse flux per unit net flux. Another useful index of the effects of a given substrate cycle is the amount of ATP consumed by the 'wasteful' flux, expressed as a percentage of total cellular ATP production. These measures of futility are presented in Table 7 for the five futile cycles occurring in the metabolic pathways under consideration.

(*a*) *Glucose/glucose 6-phosphate cycle.* Transport of glucose across the plasma membrane of hepatocytes is very rapid (Craik & Elliott, 1979) relative to the maximal rate of cellular glucose phosphorylation (Bontemps *et al.*, 1978). The fluxes through glucokinase and glucose 6-phosphatase appear to be regulated entirely by substrate concentration in both subcellular preparations and intact hepatocytes (Hue, 1981; Crawford & Blum, 1983). Evidence of cycling between glucose and glucose 6-phosphate dates back to the work of Renold *et al.* (1954), who found that liver slices incorporated radioactivity from fructose into glucose at the same time as paired slices incorporated label from glucose into CO_2 and glycogen. The same findings may be seen in the present data for both control (Tables 1 and 2) and glucagon-treated cells (Tables 3 and 4). Carefully controlled experiments by Katz *et al.* (1978) provided strong support for the concept that changes in glucose uptake or release can be explained by purely substrate level effects, except for their finding that the addition of 10mM-glycerol reduced the flux through glucokinase. A reduction in flux through glucokinase was also observed upon addition of glyceraldehyde to isolated hepatocytes (Katz *et al.*, 1975). In a previous study (Crawford & Blum, 1983), glucose phosphorylation (V_G) was approx. 40–46nmol/20min per mg of protein, somewhat lower than the values of V_G found in the present studies, although the glucose concentration in the medium was the same. The lower glucokinase flux seen by Crawford & Blum (1983) may be attributable to the presence of glycerol in their substrate mixture. In the present studies V_G was approximately constant (82 and 86nmol/20min per mg of protein) in control cells, but was lower in glucagon-treated cells (57nmol/20min per mg of protein) during the first interval. If the transient accumulation of glyceraldehyde that occurs in pig liver perfused with fructose (Sestoft *et al.*, 1972) also occurred in glucagon-treated rat hepatocytes during the first interval, it might account for this low V_G.

Glucose release (V'_G) was higher from glucagon-treated than from control cells in both intervals. In control cells V'_G declined in the second interval, concomitant with a decline in gluconeogenesis and a switch from glycogenolysis to glycogen synthesis. While gluconeogenesis in glucagon-treated cells also declined in the second interval, these hepatocytes developed a profound glycogenolysis which led to an increase in glucose output. Changes in glucose 6-phosphate concentration (Table 6) were consistent with this pattern of glucose release,

Table 7. *Contribution of 'futile' substrate cycles to ATP consumption in control and glucagon-treated hepatocytes*
Futility values (F) are computed according to Stein & Blum (1978). ATP production is 'total production' from Table X of Rabkin (1984). ATP production and consumption values are in nmol/20min per mg of protein.

Substrate cycle	Parameter	Control cells in interval I	Control cells in interval II	Glucagon-treated cells in interval I	Glucagon-treated cells in interval II
Glucose/glucose 6-phosphate	Futility	0.41[a]	1.4	0.23	0.19
	ATP consumption	82	86	57	78
	ATP consumption/ATP production (%)	4.1	4.6	3.0	4.4
Glycogen/glucose 6-phosphate	Futility	5.8[b]	0.02[c]	1.9[b]	0.04[b]
	ATP consumption	35	1	17	9
	ATP consumption/ATP production	1.8	0.1	0.9	0.5
Fructose 6-phosphate/fructose 1,6-bisphosphate	Futility	1.2[d]	2.7	0.72	1.0
	ATP consumption	217	263	167	193
	ATP consumption/ATP production	10.9	14.2	8.8	11.0
Phosphoenolpyruvate/pyruvate/ oxaloacetate	Futility	0.75[e]	0.95[f]	0.79[f]	2.3[g]
	ATP consumption	108	122	63	70
	ATP consumption/ATP production	5.4	6.6	3.3	4.0
Acetate/acetyl-CoA	Futility	30[h]	9	29	25
	ATP consumption	60	54	58	50
	ATP consumption/ATP production	3.0	2.9	3.1	2.8

[a] $F = V_G/(V'_G - V_G)$; 'futile' ATP consumption = V_G.
[b] $V'_{12} > V_{12} \rightarrow F = V_{12}/(V'_{12} - V_{12})$; 'futile' ATP consumption = V_{12}.
[c] $V_{12} > V'_{12} \rightarrow F = V'_{12}/(V_{12} - V'_{12})$; 'futile' ATP consumption = V'_{12}.
[d] $F = V_3/(V'_3 - V_3)$; 'futile' ATP consumption = V_3.
[e] Glycolytic, mode I (net oxaloacetate and pyruvate production) $\rightarrow F = V_{PCK}/|V_6 - V'_6|$; 'futile' ATP consumption = V_{PCK}.
[f] Glycolytic, mode II (net pyruvate production only) $\rightarrow F = V_{PC}/|V_{GPT} + V_{LDH} + V_{PDH} - V_{ME} - V_{GPT}|$; 'futile' ATP consumption = V_{PC}.
[g] Gluconeogenic, mode VI (net phosphoenolpyruvate and oxaloacetate production) $\rightarrow F = V_{PK}/|V_6 - V'_6|$; 'futile' ATP consumption = V_{PK}.
[h] $F = V_{AH}/(V_{AC} + V_{CL} - V_{AH})$; 'futile' ATP consumption = 2 V_{AH} (two ATP equivalents are required to activate one molecule of acetate to acetyl-CoA).

except that we did not observe a drop in glucose 6-phosphate concentration during interval II in control hepatocytes, when V'_G decreased. It is possible that a local change in glucose 6-phosphate concentration occurred that was not seen in our measurements, or that our measurements were not sufficiently precise in this interval. Glucose-6-phosphate isomerase is also an enzyme whose activity *in vitro* is directly related to glucose 6-phosphate concentration (Zalitis & Oliver, 1967), and changes in forward flux through this enzyme (V_2) paralleled changes in V'_G in both the present studies and those of Crawford & Blum (1983). With the exceptions noted, our findings are consistent with substrate-mediated control of glucose uptake and release.

Katz *et al.* (1978) found that the futility of the glucose/glucose 6-phosphate cycle varied markedly with experimental conditions, ranging from approx. 14 with 10mM-glucose as sole substrate to approx. 0.7 during maximal gluconeogenesis from 10mM-fructose in the presence of 10mM-glucose. The futility of control hepatocytes incubated in the present substrate mixture was 0.41 in interval I and increased to 1.4 in the second interval as glucose output declined. Glucagon markedly reduced futility in both intervals (Table 7). However, there was also an increase in glucose output, so that the 'wastage' of ATP hardly changed. In both the present studies and those of Crawford & Blum (1983), ATP waste at this cycle was about 4% of total cellular ATP synthesis. Thus, despite the lack of regulation, ATP waste at this cycle was relatively constant.

(*b*) *Glycogen/glucose 6-phosphate cycle.* Although treatment of perfused liver with glucagon can cause a rapid and massive increase in autophagy (Aronson, 1980) and liver lysosomes contain an α-glucosidase (Jeffrey *et al.*, 1970), most studies, including those which employ glucagon, show a strong correlation between phosphorylase *a* levels and glycogenolytic rate (Stalmans, 1976; Hers, 1976). In the present studies there was very little change in glycogen content of control or glucagon-

treated cells in interval I, whether determined by direct measurement (Table 5) or by computation from tracer data (Figs. 2 and 3). It was only in interval II that the control and glucagon-treated cells diverged, with net glycogen synthesis by the former and glycogenolysis by the latter. While hepatocytes from fed rats have been shown to exhibit less glycogenolysis when incubated with 10mM-glucose than with no substrate, there was still net degradation over a 20min incubation (Bontemps, 1979*a*). Furthermore, while a 20min delay in the activation of glycogen synthase by high concentrations of K^+ has been noted (Bontemps, 1979*b*), full activation of phosphorylase generally occurs within 2min of glucagon addition (Birnbaum & Fain, 1977; Vandenheede *et al.*, 1976; Van de Werve *et al.*, 1977). Struder *et al.* (1984), however, have shown that while addition of glucagon to rat hepatocytes caused a rapid rise in phosphorylase *a* activity, glucose release lagged behind and did not reach its maximum until 15min after hormone addition, when phosphorylase *a* activity had already returned to basal levels. A possible explanation for our findings may lie in the presence of fructose and alanine in the substrate mixture. The addition of low fructose concentrations to a system that is otherwise balanced or even glycogenolytic can convert it to one where net glycogen synthesis occurs, and this effect may take 20min or more to develop (Wood *et al.*, 1981). Alanine has a specific, though not well understood, effect in increasing glycogen deposition from fructose and other gluconeogenic precursors (Katz *et al.*, 1976). The presence of fructose and alanine in the substrate mixture may, therefore, have acted to block glycogenolysis by covalently activated phosphorylase (cf. Van den Berghe *et al.*, 1973) and to promote glycogen deposition in the control cells.

Futility at the glycogen/glucose 6-phosphate cycle was high in control cells during interval I (Table 7), but ATP expenditure was less than 2% of cellular production. During interval II glycogen synthesis increased and glycogenolysis virtually ceased, leading to a very low futility and practically no ATP wastage. In the presence of glucagon, futility was lower in interval I than for control cells, and, again, virtually nil during interval II. To the extent that glycogen synthesis and breakdown may have occurred sequentially rather than simultaneously during interval I, futility may have been even lower than calculated. Thus, even in the absence of glucagon, less than 2% of ATP produced is wasted at this substrate cycle.

(*c*) *Fructose 6-phosphate/fructose 1,6-bisphosphate cycle.* The methodology employed in most previous studies of futile cycling at the fructose 6-phosphate/fructose 1,6-bisphosphate cycle depends on assumptions and/or estimates of the activities of the Cori cycle and of transaldolase, the degree of isotopic equilibration of the reactions catalysed by aldolase and triosephosphate isomerase, and, often, the efficiency of detritiation at one or more metabolic steps (for details, see Katz & Rognstad, 1976; Hue, 1981; Crawford & Blum, 1982). The present studies assume only that the 'upper' portion of the metabolic scheme is an adequate representation of these metabolic pathways and that quasi-steady-state conditions prevail, and are therefore not subject to the above mentioned ambiguities of interpretation.

Both phosphofructokinase and fructose bisphosphatase are subject to substrate, product, and allosteric regulation by a large number of metabolites (Uyeda, 1978; Pilkis *et al.*, 1978; Hue, 1981). The cytosolic concentrations of several potential modulators can be affected by glucagon (Siess *et al.*, 1977) but, as noted by Hue (1981), the apparent net effect of these changes would be to activate phosphofructokinase and inhibit fructose bisphosphate, opposite to the known effects of glucagon on glycolysis and gluconeogenesis. A possible resolution of this problem comes from the recent discovery of fructose 2,6-bisphosphate, a potent regulator of both phosphofructokinase and fructose bisphosphatase, whose concentration in hepatocytes can be changed by a number of effectors of metabolism, including glucagon (Hers & Van Schaftingen, 1982). A glucagon-induced fall in fructose 2,6-bisphosphate levels (Richards & Uyeda, 1980) would be expected to reduce phosphofructokinase activity and increase fructose bisphosphatase activity to an extent dependent on the phosphorylation states of these enzymes, which may be changed by glucagon (Furuya & Uyeda, 1980; Brand & Söling, 1982; Morikofer-Zwez *et al.*, 1981). The situation is further complicated by the fact that many of the effects of phosphorylation and of fructose 2,6-bisphosphate are changes in substrate affinity and/or affinity for allosteric activators and inhibitors, whose cellular levels can also vary. The ability to measure the fluxes through phosphofructokinase and fructose bisphosphatase is therefore of particular interest.

In the study of Crawford & Blum (1983), flux through phosphofructokinase (V_3) was low (about 9nmol/20min per mg of protein in both intervals) and cycling at fructose 6-phosphate/fructose 1,6-bisphosphate consumed less than 1% of cellular ATP production. In the present study, V_3 ranged between 167 and 263nmol/20min per mg of protein in control and glucagon-treated cells. Thus the substitution of alanine and fructose for mannose, glycerol, and ethanol in the substrate mixture led to a 20–30-fold increase in flux through phosphofructokinase.

Flux through fructose bisphosphatase, V'_3, was 400 nmol/20 min per mg of protein for control and glucagon-treated cells, about 8–10-fold higher than that seen by Crawford & Blum (1983). Since time-dependent increases in V_3 were in each case larger than decreases in V'_3, futility increased in interval II. In control cells, about 11% and 14% of the cellular ATP production was wasted at this cycle in intervals I and II, respectively. Glucagon decreased wastage to about 9% and 11%, respectively, i.e. it caused only a modest decline in futile energy expenditure. The failure of glucagon to raise the already high flux through fructose bisphosphatase may result from an increased effectiveness of fructose 2,6-bisphosphate as an inhibitor of fructose bisphosphatase at the decreased fructose 1,6-bisphosphate concentrations of glucagon-treated cells (Van Schaftingen & Hers, 1981), but this is speculative. In any case, considerable ATP is wasted at the phosphofructokinase/fructose bisphosphatase substrate cycle under the present conditions, even in the presence of glucagon.

(*d*) *Phosphoenolpyruvate/pyruvate/oxaloacetate cycle.* The metabolic crossroads of phosphoenolpyruvate, pyruvate and oxaloacetate is complex and has proven difficult to study because of compartmentation and the large number of possible regulatory sites (Hue, 1981). Although it has been suggested that a pathway with so many potentially regulated steps might not exhibit any futile cycling (Atkinson, 1966; Scrutton & Utter, 1968), studies by Clark *et al.* (1975), Friedmann *et al.* (1971), and Rognstad (1975) suggest that some futile cycling occurs at this crossroads and that glucagon reduces such cycling. Thomas & Halestrap (1981) have suggested that the activities of all the enzymes involved in the conversion of pyruvate to phosphoenolpyruvate are so closely matched that a change in any one of them can alter the rate of the whole pathway. Engstrom (1978) has reviewed the evidence that glucagon treatment *in vivo* leads to a cyclic AMP-dependent phosphorylation of pyruvate kinase that inhibits its activity and increases its affinity for inhibitors such as Mg^{2+}–ATP and alanine. In the present studies, flux through pyruvate kinase (V_{PK}) was substantial in control hepatocytes and did not change with time. The addition of glucagon caused a drop in V_{PK} of about 40% in the first interval, and a further halving of flux in interval II.

It is now accepted that glucagon stimulates pyruvate carboxylation both by increasing mitochondrial pyruvate transport and by activating pyruvate carboxylase (Jensen *et al.*, 1983; Claus *et al.*, 1983; Allan *et al.*, 1983). Glucagon-treated hepatocytes, surprisingly, had a lower V_{PC} than control cells during interval I, but exhibited the expected increase relative to control cells in the second interval. Since stable changes in mitochondrial pyruvate metabolism have been observed within a few minutes after glucagon addition (Siess & Wieland, 1980; Chan *et al.*, 1979), two questions are raised by our results: why is V_{PC} initially lower in glucagon-treated than in control hepatocytes, and why does it take so long for the expected increase in V_{PC} to occur? One possibility is that there is an initial reduction in mitochondrial pyruvate availability because of the rapid inactivation of pyruvate kinase. Later on, pyruvate for V_{PC} may be supplied via mitochondrial alanine transamination and increased flux through malic enzyme.

A third enzyme that could play a role in the regulation of the phosphoenolpyruvate/pyruvate/oxaloacetate substrate cycle is phosphoenolpyruvate carboxykinase. Lardy and co-workers have identified a cytosolic protein which can bind to and activate phosphoenolpyruvate carboxykinase in the presence of Fe^{2+}, and it has been suggested that this mechanism is activated by glucagon (Lardy & Merryfield, 1981). Flux through phosphoenolpyruvate carboxykinase (V_{PCK}) in control hepatocytes appeared to rise over 30% in the second interval. The increased supply of oxaloacetate to support this increase in flux stems mainly from a reversal of flow through malic enzyme (V_{ME}), which consumes cytosolic malate during the first interval, but produces it during the second interval. Very little data is available on the short-term regulation of malic enzyme; to our knowledge, the present study provides the first quantification of flux through this enzyme in intact cells. The flux through phosphoenolpyruvate carboxykinase in glucagon-treated cells was comparable with that in control cells in interval I, but decreased somewhat in interval II, in contrast to the marked increase in flux through pyruvate carboxylase. While it should be noted that V_{PC} and V_{ME} were not very tightly determined in interval II for glucagon-treated cells (Appendix II), it is nonetheless clear that the fluxes V_{PC} and V_{PCK} did not vary co-ordinately in response to glucagon under these conditions.

Stein & Blum (1978) have identified six basic modes of the phosphoenolpyruvate/pyruvate/oxaloacetate cycle; which reaction is considered wasteful depends on the particular configuration of the cycle under each condition, and the definition of futility changes accordingly (see Table 7). Because changes in configuration occurred in the present experiments, it is useful to look instead at 'futile' ATP consumption. Control cells wasted approx. 6% of ATP production in each interval at this crossroads; the presence of glucagon reduced this to 3–4%.

(e) *Acetate metabolism and the acetate/acetyl-CoA substrate cycle.* The rate of fatty acid synthesis (V_{FA}) was quite low, especially in the presence of glucagon. For both control and glucagon-treated cells, V_{FA} increased in the second interval. This may be explained by the findings of McGarry *et al.* (1978), who correlated the time-dependent increase in fatty acid synthesis with increased intracellular levels of malonyl-CoA; hepatocytes were depleted of this substance when first isolated, and glucagon retarded its reaccumulation.

A surprising finding was the cycling of acetyl-CoA via the cytosolic acetyl-CoA hydrolase, a futile cycle which has not, to our knowledge, been previously recognized. The futility of this cycle is high and variable (Table 7), because the reverse flux (V_{AH}) is large while net flux is small. Since ATP consumption depends on V_{AH} alone, ATP consumption was appreciable, accounting for about 3% of cellular ATP production under each condition studied.

It is of interest that both cytosolic acetate activation (V_{AC}) and citrate lyase activity (V_{CL}) proceeded at brisk rates despite the low rate of lipogenesis. While this may be attributable to the presence of 1.25mM-acetate [as compared to blood acetate levels of about 0.2mM (Knowles *et al.*, 1974)] in the case of acetyl-CoA synthetase, citrate lyase alone was producing cytosolic acetyl-CoA much faster than its utilization. Thus, under present conditions, hepatocytes had at most a limited ability to stem 'wasteful' production of cytosolic acetyl-CoA.

The finding that flux through citrate lyase was somewhat higher in glucagon-treated cells than in control cells argues against direct or indirect inhibition of this enzyme by glucagon. Janski *et al.* (1979) demonstrated glucagon-mediated phosphorylation of the enzyme in rat hepatocytes, but were unable to detect any changes in the enzyme's activity in hepatocyte extracts under a variety of conditions. Inhibition of citrate lyase could also arise indirectly from a glucagon-mediated decrease in cytosolic citrate concentration. Glucagon did not, however, cause changes in cellular citrate levels in hepatocytes from fed rats in the presence of 10mM-lactate plus 1mM-pyruvate (McGarry *et al.*, 1978), nor did it change cytosolic citrate concentrations in hepatocytes from fasted rats incubated with 10mM-lactate (Siess *et al.*, 1977). It appears, therefore, that glucagon does not lower citrate levels if a source of pyruvate is supplied which bypasses glucagon-inhibited steps of glycolysis, and the presence of alanine in the current substrate mixture provides such a source.

Isotopic equilibration

Many workers investigating futile cycling have assumed that isotopic equilibration occurs at one or more steps when interpreting their experimental data. Most commonly included are the reactions catalysed by hexose phosphate isomerase, aldolase, and triosephosphate isomerase. The computer program used in fitting the current model predicts the specific radioactivity of each carbon atom of every metabolite in the model. By comparing the specific activities of corresponding carbons across the enzyme step in question, one can determine for each labelled substrate and condition whether isotopic equilibration obtained. The results of such an analysis for each of the four conditions examined are presented in Table 8. If the present system had been studied with methods that assumed isotopic equilibration across these enzymes when the indicated substrate was labelled, erroneous conclusions might have been drawn whenever there is an 'N' in Table 8.

Pentose phosphate pathway

Flux through glucose 6-phosphate dehydrogenase (V_1) fell markedly in interval II. In glucagon-treated cells the initial value of V_1 was higher, and it dropped only slightly in the second interval. Except for control cells in interval II, the values of V_1 in the current studies were higher than in Crawford & Blum (1983). The regulation of flux through glucose 6-phosphate dehydrogenase is not well understood (Levy & Christoff, 1983), but it is generally agreed that a high [NADPH]/[NADP$^+$] ratio inhibits activity. If this ratio increased during interval II in control cells, for reasons which are not readily apparent, this might account for the decrease in V_1 and the reversal of flux through malic enzyme (V_{ME}). Since treatment with glucagon increases the [NADPH]/[NADP$^+$] ratio in subsequently isolated liver mitochondria (Prpić & Bygrave, 1980) and in the cytoplasm of isolated hepatocytes carrying out gluconeogenesis from lactate (Siess *et al.*, 1977), one might have expected a lower V_1 in glucagon-treated cells than in controls, contrary to our observations. Whatever the factors responsible for regulation of V_1, the present results show that the oxidative steps of the pentose phosphate pathway were active and that net flow in the transaldolase and transketolase steps contributed appreciably (at least 15% under all conditions so far studied) to the overall gluconeogenic flux.

Fructose metabolism

Fructose utilization (V_F) was similar in glucagon-treated and control hepatocytes, falling approx. 40% during the incubation. Since little change in cellular ATP levels is expected at the concentrations of fructose employed (Topping & Mayes, 1977), the decrease in V_F probably reflects

Table 8. *Isotopic equilibration at three enzyme steps*

Isotopic equilibration of all corresponding carbons at a given enzyme step when hepatocytes are incubated with the designated labelled substrate is indicated by the symbol 'Y'; lack of such equilibration is indicated by the symbol 'N'. Equilibration is said to exist when the specific radioactivities of all corresponding carbons across a given step differ by less than 20%.

		Labelled substrate				
	Interval	Glucose	Ribose	Fructose	Alanine	Acetate
(*a*) Control cells						
Glucose phosphate isomerase	I	N	N	N	N	Y
	II	N	N	N	N	N
Aldolase	I	N	N	Y	Y	Y
	II	N	N	Y	Y	Y
Triose phosphate isomerase	I	Y	Y	Y	Y	Y
	II	N	N	Y	N	N
(*b*) Glucagon-treated cells						
Glucose phosphate isomerase	I	N	Y	Y	Y	Y
	II	N	N	N	N	N
Aldolase	I	N	N	Y	Y	Y
	II	N	N	Y	Y	Y
Triose phosphate isomerase	I	Y	Y	Y	Y	Y
	II	N	N	Y	N	N

the gradual decrease in fructose concentration in the medium. As might be expected, net flux from the level of the triose phosphates was in the direction of glucose formation under all conditions studied. Net flux was from dihydroxyacetone phosphate to glyceraldehyde 3-phosphate in three of the four conditions studied, but from glyceraldehyde 3-phosphate to dihydroxyacetone phosphate in glucagon-treated cells during interval II, implying a change in relative pool sizes of these substances in the glucagon-treated versus control cells. It should also be noted that fructose was the primary gluconeogenic substrate used under all conditions and, along with ribose, was the only substrate used for net gluconeogenesis except for a relatively small contribution from alanine for glucagon-treated cells in interval II.

Lower Embden–Meyerhof pathway

Unidirectional glycolytic flux, V_6, was 10–20-fold higher in the present study than for hepatocytes incubated in the highly reducing substrate mixture of Crawford & Blum (1983), in accordance with the redox inhibition of glyceraldehyde-3-phosphate dehydrogenase by ethanol (Rawat & Lundquist, 1968). Furthermore, the presence of fructose in the current substrate mixture leads to a brisk influx of carbon at glyceraldehyde 3-phosphate which would also tend to increase V_6. The progressive inactivation of pyruvate kinase by glucagon appears to be the primary cause for the decrease in glycolysis and the development of net gluconeogenesis in this area of metabolism.

Amino acid metabolism

Alanine is the major hepatic source of amino nitrogen for urea synthesis (Remesy *et al.*, 1978) and of carbon for gluconeogenesis in fasting rats (Exton, 1972; Snell, 1980). Although transport into the cell appears to be rate limiting at concentrations below 0.5 mM (Sips *et al.*, 1980), this does not appear to be the case at higher concentrations such as were used here (McGivan *et al.*, 1981; Christiansen, 1984). Since the transamination reaction appears to be readily reversible under intracellular conditions (Sips *et al.*, 1980; Fafournoux *et al.*, 1983), changes in the metabolism of 2-oxoglutarate, glutamate, and pyruvate should play a major role in alanine metabolism. There was considerable flow of label both to and from alanine in three out of the four conditions examined, but net alanine utilization was approximately the same in all four conditions. Thus, the present results do not demonstrate a glucagon-mediated stimulation of net alanine consumption. This indicates that the rapid glucagon-induced increase in alanine transport observed by Edmondson & Lumeng (1980) does not play a major role in determining alanine consumption under these conditions. This is consistent with the view that, at alanine concentrations such as those employed here, alanine consumption is determined intracellularly.

Krebs *et al.* (1978) and Wanders *et al.* (1983) have show that most of the ammonia for urea synthesis is generated by mitochondrial glutamate dehydrogenase; in the present metabolic model it

is the sole source of ammonia, so that the rate of urea synthesis is V_{GDH}. V_{GDH} was somewhat higher in both intervals in glucagon-treated cells, as required by the larger total influx of aspartate. The source of endogenous amino acids is protein hydrolysis, and these should be more readily available in the presence of glucagon because of the rapid increase in autophagy mediated by this hormone (Aronson, 1980; Hopgood *et al.*, 1980). A glucagon-mediated increase in ureagenesis has been reported under a number of experimental conditions (Mallette *et al.*, 1969; Triebwasser & Freedland, 1977).

Pyruvate dehydrogenase

Control of flux through pyruvate dehydrogenase is thought to be mediated largely by phosphorylation, with inactivation being favoured by high intramitochondrial [acetyl-CoA]/[CoA], [NADH]/[NAD^+], and [ATP]/[ADP] ratios (Pettit *et al.*, 1975; Siess & Wieland, 1976). There are, however, no known effects of glucagon treatment on the activity of this enzyme *in vitro* (Denton *et al.*, 1981). In the study of Crawford & Blum (1983), flux through pyruvate dehydrogenase was almost nil, possibly because of the highly reduced state of the mitochondria in the presence of ethanol, inhibition by acetaldehyde, and absence of a substrate such as alanine which directly yields mitochondrial pyruvate. In the current studies, V_{PDH} was 62 and 74nmol/20min per mg of protein in control hepatocytes during the first and second intervals, respectively, and 10–15nmol/20min per mg of protein higher in the glucagon-treated cells. Since estimates of V_{PDH} appear to be narrowly determined (Appendix II), it is likely that V_{PDH} did rise with time of incubation and with glucagon treatment, but the reasons for this are not clear. It should be noted that the majority of acetyl-CoA generated in the mitochondria (and subsequently oxidized) was derived from pyruvate decarboxylation despite the presence of supraphysiological (1.25mM) acetate.

Tricarboxylic acid cycle

Although glucagon-treated cells have a somewhat higher O_2 consumption than control hepatocytes, their tricarboxylic acid cycle activities are almost identical. This accords with the suggestion (Kimmig *et al.*, 1983) that the major portion of glucagon-mediated stimulation of respiration may be secondary to inhibition of glycolysis. In the present conditions the loss of glycolytically derived ATP can easily be made up by oxidative phosphorylation of the NADH produced by the increased V_{GDH} and V_{PDH} fluxes, and thus no increase in tricarboxylic acid cycle activity was necessary.

Energetic considerations

The estimated flux values can be used in conjunction with the known stoichiometry of the corresponding reactions to compute the rates of ATP formation and consumption in each interval (Rabkin, 1984). The excess of production over consumption (presumably used for ion transport, protein and nucleic acid synthesis, etc.) ranged from 476nmol/20min per mg of protein in glucagon-treated cells during the second interval to 608nmol/20min per mg of protein in control cells in the second interval. Because a substantial amount of the carbon flow to lactate came from alanine rather than glucose or fructose, the consumption of reducing equivalents for lactate production exceeded the rate of glycolytic NADH formation. The vast majority of ATP production by both glucagon-treated and control cells, therefore, stemmed from reducing equivalents generated in the mitochondria.

Of the reactions shown, the largest expenditure of ATP was via fructokinase and triose kinase, which together consumed from 290 to 496nmol ATP/20min per mg of protein. Since some fructose 1-phosphate was probably also formed but is not included in V_F, the tendency of fructose to deplete cellular ATP levels (Van den Berghe, 1978) is readily apparent. Urea production was also an important ATP-consuming process. The expenditure of ATP in 'futile' cycles was substantial, ranging from about 19% of computed ATP production in glucagon-treated cells during interval I up to 28% of production in control cells during interval II. The largest part of this ATP expenditure occurred at the fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle. Whether this much waste would occur in the intact liver after a rich meal (and therefore in the presence of all precursors for protein, nucleic acid, and lipoprotein synthesis) is not certain.

This work was supported by NIH grant 5 RO1 HDO1269. M. R. was supported by the Medical Scientist Research Training Program (NIH grant 5T32-GM07171). We are grateful to Ms. Carolyn Edwards Hinton and Mr. A. Hayes for excellent technical support, to Rachel Hougom for typing this manuscript, to Dr. Michael Hines for extensive and invaluable assistance in setting up the computer programs, to Professor J. W. Moore for unrestricted access to his computer facility, to Professor D. Burdick for devising the statistical analysis of the results, and to Dr. J. Crawford for helpful discussions.

References

Allan, E. H., Chisholm, A. B. & Titheradge, M. A. (1983) *Biochem. J.* **212**, 417–426

Aronson, N. W. (1980) *Life Sci.* **27**, 95–104

Atkinson, D. A. (1966) *Annu. Rev. Biochem.* **35**, 85–124

Balaban, R. S. & Blum, J. J. (1982) *Am. J. Physiol.* **242**, C172–C177

Berry, N. M. & Friend, D. S. (1969) *J. Cell Biol.* **73**, 506–520

Birnbaum, M. J. & Fain, J. N. (1977) *J. Biol. Chem.* **252**, 528–535

Blum, J. J. & Rabkin, M. S. (1984) *Proc. Int. Congr. Myocardial Cell. Bioenerg. Compartmentation 2nd*, in the press

Blum, J. J. & Stein, R. B. (1982) in *Biological Regulation and Development* (Goldberger, R., ed.), vol. 3A, pp. 99–125, Plenum Press, New York

Bontemps, F. (1979*a*) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), p. 96, Elsevier, New York

Bontemps, F. (1979*b*) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), p. 107, Elsevier, New York

Bontemps, F., Hue, L. & Hers, H. G. (1978) *Biochem. J.* **174**, 603–611

Borowitz, M. J., Stein, R. B. & Blum, J. J. (1977) *J. Biol. Chem.* **252**, 1589–1605

Brand, I. A. & Söling, H. D. (1982) *Eur. J. Biochem.* **122**, 175–181

Chan, T. M., Bacon, C. B. & Hill, S. A. (1979) *J. Biol. Chem.* **254**, 8730–8732

Chen, R. F. (1967) *J. Biol. Chem.* **242**, 173–181

Christiansen, H. N. (1984) *Biosci. Rep.* **3**, 905–913

Clark, D. G., Lee, D., Rognstad, R. & Katz, J. (1975) *Biochem. Biophys. Res. Commun.* **67**, 212–219

Claus, T. H., Park, C. R. & Pilkis, S. J. (1983) *Handb. Exp. Pharmacol.* **66/I**, 315–360

Craik, J. D. & Elliott, K. R. F. (1979) *Biochem. J.* **182**, 503–508

Crawford, J. M. & Blum, J. J. (1982) *Arch. Biochem. Biophys.* **216**, 42–50

Crawford, J. M. & Blum, J. J. (1983) *Biochem. J.* **212**, 585–598

Denton, R. M., McCormack, J. G. & Oviasu, O. A. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 159–174, Elsevier, New York

Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120

DeRosa, G. & Swick, R. W. (1975) *J. Biol. Chem.* **250**, 7961–7967

Devos, P. & Hers, H. G. (1979) *Eur. J. Biochem.* **99**, 161–167

Devos, P. & Hers, H. G. (1980) *Biochem. J.* **192**, 177–181

Dieterle, P., Brawand, F., Moser, U. K. & Walter, P. (1978) *Eur. J. Biochem.* **88**, 467–473

Edmondson, J. W. & Lumeng, L. (1980) *Biochem. Biophys. Res. Commun.* **96**, 61–68

Engstrom, L. (1978) *Curr. Top. Cell. Regul.* **13**, 29–51

Exton, J. H. (1972) *Metabolism* **21**, 945–990

Fafournoux, P., Remesy, C. & Demigne, C. (1983) *Biochem. J.* **210**, 645–652

Friedmann, B., Goodman, E. H., Saunders, H. L., Kostos, V. & Weinhouse, S. (1971) *Arch. Biochem. Biophys.* **143**, 566–578

Furuya, E. & Uyeda, K. (1980) *J. Biol. Chem.* **255**, 11656–11659

Goldberg, R. P. & Brunengraber, H. (1979) *Adv. Exp. Med. Biol.* **132**, 413–418

Golden, S. & Katz, J. (1980) *Biochem. J.* **188**, 799–805

Groen, A. K., Sips, H. J., Vervoorn, R. C. & Tager, J. M. (1982) *Eur. J. Biochem.* **122**, 87–93

Groot, P. H. E., Scholte, H. R. & Hulsmann, W. C. (1976). *Adv. Lipid Res.* **14**, 75–126

Gutmann, I. & Wahlefeld, A. W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1464–1468, Academic Press, New York

Hems, R. & Saez, G. T. (1983) *FEBS Lett.* **153**, 438–440

Hers, H. G. (1976) *Annu. Rev. Biochem.* **45**, 167–189

Hers, H. G. & Van Schaftingen, E. (1982) *Biochem. J.* **206**, 1–12

Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1980) *Biochem. J.* **186**, 71–79

Hue, L. (1981) *Adv. Enzymol.* **52**, 247–331

Hue, L. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 71–97, Academic Press, New York

Janski, A. M., Srere, P. A., Cornell, N. W. & Veech, R. L. (1979) *J. Biol. Chem.* **254**, 9365–9368

Jeffrey, P. L., Brown, D. H. & Brown, B. I. (1970) *Biochemistry* **9**, 1403–1415

Jensen, C. B., Sistare, F. O., Hamman, F. M. & Haynes, R. C. (1983) *Biochem. J.* **210**, 819–827

Katz, J. & Rognstad, R. (1976) *Curr. Top. Cell. Regul.* **10**, 237–289

Katz, J., Wals, P. A., Golden, S. & Rognstad, R. (1975) *Eur. J. Biochem.* **60**, 91–101

Katz, J., Golden, S. & Wals, P. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3433–3437

Katz, J., Wals, P. A. & Rognstad, R. (1978) *J. Biol. Chem.* **253**, 4530–4536

Kimmig, R., Mauch, T. J. & Scholz, R. (1983) *Eur. J. Biochem.* **136**, 617–620

Knowles, S. E., Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) *Biochem. J.* **142**, 401–411

Kornacker, M. S. & Lowenstein, J. M. (1965) *Biochem. J.* **94**, 209–215

Krebs, H. A., Hems, R., Lund, P., Halliday, D. & Read, W. W. C. (1978) *Biochem. J.* **176**, 733–737

Lardy, H. A. & Merryfield, M. L. (1981) *Curr. Top. Cell. Regul.* **18**, 243–253

Levy, H. R. & Christoff, M. (1983) *Biochem. J.* **214**, 959–965

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lund, H. & Bremer, J. (1983) *Biochim. Biophys. Acta* **750**, 164–170

Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 5724–5728

McGarry, J. D., Takabayashi, Y. & Foster, D. W. (1978) *J. Biol. Chem.* **253**, 8294–8300

McGivan, J. D., Ramsell, J. C. & Lacey, J. H. (1981) *Biochim. Biophys. Acta* **644**, 295–304

Meijer, A. J. & Hensgens, H. E. S. J. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 259–286, Academic Press, New York

Mendes-Mourao, J., Halestrap, A. P., Crisp, D. M. & Pogson, C. I. (1975) *FEBS Lett.* **53**, 29–32
Morikofer-Zwez, S., Stoecklin, F. B. & Walter, P. (1981) *Biochem. Biophys. Res. Commun.* **101**, 104–111
Noll, F. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1475–1479, Academic Press, New York
Parry, M. J. & Walker, D. G. (1966) *Biochem. J.* **99**, 266–274
Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582
Pilkis, S. J., Park, C. R. & Claus, T. H. (1978) *Vitam. Horm.* **36**, 383–460
Prass, R. L., Isohashi, F. & Utter, M. F. (1980) *J. Biol. Chem.* **255**, 5215–5223
Prpić, V. & Bygrave, F. L. (1980) *J. Biol. Chem.* **255**, 6193–6199
Rabkin, M. S. (1984) Ph.D. Thesis, Duke University
Rawat, A. K. & Lundquist, F. (1968) *Eur. J. Biochem.* **5**, 13–17
Remesy, C., Demigne, C. & Aufrere, J. (1978) *Biochem. J.* **170**, 321–329
Renold, A. E., Hastings, A. B. & Nesbett, F. B. (1954) *J. Biol. Chem.* **209**, 687–697
Richards, C. S. & Uyeda, K. (1980) *Biochem. Biophys. Res. Commun.* **97**, 1535–1540
Roe, J. H. & Papadopoulos, N. M. (1954) *J. Biol. Chem.* **210**, 703–707
Rognstad, R. (1975) *Biochem. Biophys. Res. Commun.* **63**, 900–905
Scholte, H. R. & Groot, P. H. E. (1975) *Biochim. Biophys. Acta* **409**, 283–296
Scrutton, M. C. & Utter, M. F. (1968) *Annu. Rev. Biochem.* **37**, 249–302
Sestoft, L., Tonnesen, K., Hansen, F. V. & Damgaard, S. E. (1972) *Eur. J. Biochem.* **30**, 542–552
Siess, E. A. & Wieland, O. H. (1976) *Biochem. J.* **156**, 91–102
Siess, E. A. & Wieland, O. H. (1980) *Eur. J. Biochem.* **110**, 203–210
Siess, E. A., Brocks, D. G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235
Siess, E. A., Brocks, D. G. & Wieland, O. H. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 235–257, Academic Press, New York
Sillero, M. A. G., Sillero, A. & Sols, A. (1969) *Eur. J. Biochem.* **10**, 345–350
Sips, H. J., Groen, A. K. & Tager, J. M. (1980) *FEBS Lett.* **119**, 271–274
Snell, K. (1980) *Biochem. Soc. Trans.* **8**, 205–213
Stalmans, W. (1976) *Curr. Top. Cell. Regul.* **11**, 51–97
Stein, R. B. & Blum, J. J. (1978) *J. Theoret. Biol.* **72**, 487–522
Struder, R. S., Snowdowne, K. W. & Borle, A. B. (1984) *J. Biol. Chem.* **259**, 3596–3604
Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **198**, 551–564
Tomera, J. F., Goetz, P. G., Rand, W. M. & Brunengraber, H. (1982) *Biochem. J.* **208**, 231–234
Topping, D. L. & Mayes, P. A. (1977) *Biochem. Soc. Trans.* **5**, 1001–1002
Triebwasser, K. C. & Freedland, R. A. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1159–1165
Uyeda, K. (1978) *Adv. Enzymol.* **48**, 193–244
Van den Berghe, G. (1978) *Curr. Top. Cell. Regul.* **13**, 97–135
Van den Berghe, G., Hue, L. & Hers, H. G. (1973) *Biochem. J.* **134**, 637–645
Vandenheede, J. R., Keppens, S. & DeWulf, H. (1976) *FEBS Lett.* **61**, 213–217
Van de Werve, G., Hue, L. & Hers, H. G. (1977) *Biochem. J.* **162**, 135–142
Van Schaftingen, E. & Hers, H. G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2861–2863
Van Schaftingen, E., Hue, L. & Hers, H. G. (1980) *Biochem. J.* **192**, 263–271
Wanders, R. J. A., Meijer, A. J., Groen, A. K. & Tager, J. M. (1983) *Eur. J. Biochem.* **133**, 245–254
Williamson, D. H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.), 2nd edn., pp. 1679–1682, Academic Press, New York
Wood, C. L., Babcock, C. J. & Blum, J. J. (1981) *Arch. Biochem. Biophys.* **212**, 43–53
Wood, C. L. & Blum, J. J. (1982) *Am. J. Physiol.* **242**, E262–E272
Zalitis, J. & Oliver, I. T. (1967) *Biochem. J.* **102**, 753–759

APPENDIX I

Statistical considerations pertaining to analysis of metabolic models

Donald S. BURDICK
Department of Mathematics, Duke University, Durham, NC 27706, U.S.A.

This Appendix contains the details of the statistical procedures referred to in Crawford & Blum (1983) and in the present study. We first present the formulas that were used and then discuss the rationale behind them.

Notation

Y_{ijk} = the datum from the jth measurement of the kth metabolic product variate for the ith labelled substrate. In the present study,