Compartmentation between Glycolysis and Gluconeogenesis in Rat Liver

By C. J. THRELFALL AND D. F. HEATH

*Experimental Pathology of Trauma Section, Toxicology Research Unit,
 Medical Research Council Laboratories, Carshalton, Surrey*

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1. The specific radioactivity-time relationships of glucose, glucose 6-phosphate, glycerol 1-phosphate and UDP-glucose were determined in rat liver after the intravenous injection of [U-¹⁴C]fructose, and a kinetic analysis was carried out. The glucose 6-phosphate pool was found to be compartmented into gluconeogenic and glycolytic components, and evidence was obtained that the triose phosphates were similarly compartmented. The glycolytic pathway was fed by glycogenolysis and glucose phosphorylation. There was no direct evidence that glycogenolysis fed only the glycolytic pathway, but this interpretation would make the liver resemble other organs in this respect. 2. UDP-glucose was not formed solely from gluconeogenic glucose 6-phosphate, as there was some dilution of label in the intervening glucose 1-phosphate pool, probably from glycogenolysis, though other pathways cannot be excluded. 3. The data cannot be explained by isotopic exchange.

The metabolic interrelationships between glucose, fructose and glycogen are shown in Scheme 1. Isotopic studies have suggested that the role of glucose 6-phosphate in this scheme is more complex than is generally thought, and Muntz & Vanko (1962), Threlfall (1966) and Landau & Sims (1967) have adduced evidence for two pools of glucose 6-phosphate. Niemeyer and his colleagues (Figuerola

& Pfeifer, 1964, and references cited therein) and Smith, Taylor & Whelan (1967) have questioned the involvement of glucose 6-phosphate in the synthesis of UDP-glucose and glycogen in rat liver.

We have injected [U-¹⁴C]fructose into rats and have determined the specific radioactivities of glycerol 1-phosphate, glucose 6-phosphate, glucose and UDP-glucose in the liver. A kinetic analysis

Scheme 1. Glucose, fructose and glycogen interrelationships.

of the results established the existence of two pools of glucose 6-phosphate, and showed that the data were consistent with the formation of UDP-glucose via glucose 6-phosphate.

MATERIALS AND METHODS

Chemicals and enzymes. [^{14}C]Fructose (3.5 mc/m-mole) and [^{14}C]glucose 6-phosphate (3.5 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49), glycerol 1-phosphate dehydrogenase (L-glycerol 3-phosphate-NAD oxidoreductase, EC 1.1.1.8), glucose phosphate isomerase (D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.9), NAD⁺ and NADP⁺ were obtained from the Boehringer Corp. (London) Ltd., London, W. 5. Peroxidase (donor-hydrogen peroxide oxidoreductase, EC 1.11.1.7) and glucose oxidase (β -D-glucose-oxygen oxidoreductase, EC 1.1.3.4) were from the Sigma (London) Chemical Co., London, S.W. 6.

Ion-exchange resins. Dowex AG 1 (X8; 200-400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was cycled through Cl⁻ and OH⁻ forms twice each with 2N-HCl and 2N-NH₃ respectively before conversion into the formate form with 2N-formic acid. The columns were regenerated with 6N-formic acid-m-sodium formate (400 ml. per column) and washed to neutrality with ion-free water. Amberlite IR-120 (H⁺ form) (British Drug Houses Ltd., Poole, Dorset) was allowed to stand for several days with occasional stirring in 4N-HCl and washed by decantation with ion-free water to pH 7.0. De-Acidite J (The Permutit Co., London, W. 4) was converted into the CO₃²⁻ form by standing in 4 vol. of 5% (w/v) Na₂CO₃ overnight and washed with ion-free water to pH 7.0. Zeo-Karb 225 (The Permutit Co.) was converted into the H⁺ form with 2N-HCl and washed as above.

Animal techniques. Male albino Wistar rats of the Porton strain (200-220 g.) fed on M.R.C. diet 41B (Bruce & Parkes, 1956) were injected via a tail vein with [^{14}C]fructose (50 μC /ml., 0.2 ml./200 g. rat body wt.) at approx. 14.30 hr. These rats were post-absorptive and ceased feeding at 7.00 hr. They were stunned at set times by a blow on the head and about 4 g. of liver was rapidly removed into liquid N₂. This process took 5-7 sec.

Liver samples were obtained for the determination of glucose 6-phosphate and glycerol 1-phosphate by the following methods: (1) stunning and a lobe rapidly removed into liquid N₂; (2) light ether anaesthesia and liver frozen *in situ* by using tongs cooled in liquid N₂ as described by Hohorst, Kreutz & Bücher (1959); (3) sodium pentobarbitone (Veterinary Nembutal; Abbott Laboratories Ltd., Queenborough, Kent) anaesthesia (6 mg./100 g. body wt. intraperitoneally) and liver removed into liquid N₂ or frozen *in situ* as in (2).

Anion-exchange chromatography. The following operations were performed at 2-3°. The frozen liver was homogenized in 40 ml. of ice-cold 5% (w/v) HClO₄ in a Nelco 10 blender (Measuring and Scientific Equipment Ltd., London, S.W. 1) and centrifuged. The supernatant was neutralized with 10N-KOH (Universal Indicator paper) and left for 30 min. The KClO₄ was removed by centrifugation, and the supernatant was applied to a column (30 cm. $\times$ 2 cm.) of Dowex I (X8; formate form; 200-400 mesh) and the

column washed with a small volume of ion-free water. Fractionation was achieved by the formic acid-ammonium formate method of Brumm, Potter & Siekevitz (1956) as modified by Gilbert & Yemm (1958) by using a mixing flask containing twice the volume of water used by the latter and eluting two columns in parallel. A D.C.L. micro pump (series II; F. A. Hughes and Co. Ltd., Great Burgh, Epsom, Surrey) was used and the flow rate adjusted to 60 ml./hr. per column. The effluent was monitored by u.v.-absorbing material with a Uvicord I flow analyser (LKB Instruments Ltd., South Croydon, Surrey) and 10 ml. fractions were collected in a fraction collector (Central Ignition Co., London, N. 1).

Identification and treatment of fractions. (1) Determinations of specific radioactivities. (a) Glucose. Glucose and fructose were hardly retained by the column and were eluted as a highly radioactive peak with the glycogen, recognizable by its opalescence. The radioactive fractions were pooled and the glycogen was precipitated with an equal volume of ethanol. After standing overnight at 2-3° the glycogen was removed by centrifugation and the supernatant evaporated to dryness at room temperature in a stream of air. The residue was dissolved in 5 ml. of water and passed down a column (8 cm. $\times$ 1 cm.) of Amberlite IR-120 (H⁺ form). The column was washed with water to neutrality and the effluent dried in an air stream. The residue was taken up in water (2-3 ml.) and passed down a mixed-bed resin column (6 cm. $\times$ 1 cm.) of equal parts De-Acidite J (CO₃²⁻ form) and Zeo-Karb 225 (H⁺ form), and the column was washed with water to give 10 ml. of effluent. Of this 8.5 ml. was evaporated to dryness in an air stream and 1.5 ml. was used to obtain an approximate glucose content. The dried portion was dissolved in 100 μl . of water and chromatographed on Whatman no. 1 paper for 40 hr. in butan-2-one-2-methylpropan-2-ol-98% formic acid-water (10:10:3:3, by vol.) (Fink, Cline & Fink, 1963) to separate glucose and fructose. The glucose band was located by reference markers and eluted from a Perspex trough with water (5 ml.) into a deposit vessel. The dried eluate was dissolved in water (0.1-1.0 ml., depending on the glucose content as determined on the 1.5 ml. sample above), and applied in multiples of 2 μl . to fenestrated Whatman no. 540 paper (Gey, 1956) and run for 40 hr. in the same solvent system as the band separation. The glucose was located by a radiochromatogram scanner (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.), except in experiments soon after the fructose injection when glucose labelling was low and glucose markers were used. The glucose was eluted from a Perspex trough with water (0.2-0.3 ml.) and passed down Dowex 1 (X8; Cl⁻ form; 200-400 mesh) columns (5 cm. $\times$ 0.5 cm.) to remove an inhibitor of the glucose oxidase reaction present in the chromatography paper (Gaitonde, 1965). (The use of Whatman no. 540 paper, the collection of a small volume of eluate and the elution of the minimal area of paper decreased this inhibition to a minimum, but did not remove it.) The columns were washed with water (2 $\times$ 0.5 ml.) and the volume was adjusted to 2.5 ml. Glucose was determined on a 1 ml. sample by the glucose oxidase method as modified by Stoner, Heath & Collins (1960), and radioactivity was measured on a separate 1 ml. sample.

(b) UDP-glucose. The UDP-glucose was identified on the LKB monitor as a u.v.-absorbing peak eluted after a yellow flavine derivative. Radioactivity determinations

showed a well-defined peak, and the fractions containing most of the radioactivity were pooled and heated in a boiling-water bath for 15 min. to liberate the glucose. To remove Na^+ the cooled solution was passed down a column of Amberlite IR-120 (H^+ form) (1 cm. diam. $\times$ 2.5 cm. resin height for each 10 ml. of effluent). The column was washed to neutrality with water and the combined effluent evaporated to dryness. The residue was dissolved in water and passed down a mixed-bed resin as described for glucose. The effluent and washings were evaporated to dryness, dissolved in water (150 μl .) and divided equally between three spots on fenestrated Whatman no. 540 paper, and thereafter the procedure was as for glucose.

(c) Glucose 6-phosphate. The approximate position of glucose 6-phosphate relative to the u.v.-absorption profile was determined by the addition of $[\text{U-}^{14}\text{C}]\text{glucose 6-phosphate}$ to an extract from an uninjected animal. The glucose 6-phosphate was accurately located by assay of dried 0.5 ml. samples of the fractions with glucose 6-phosphate dehydrogenase (Hohorst, 1963a), or at times up to 6 min. after the injection by the highly radioactive peak associated with fructose 1-phosphate that coincided with glucose 6-phosphate and fructose 6-phosphate. The required fractions were combined and dried down in an air stream, dissolved in 0.1 N-HCl (1.0 ml.) and heated for 1 hr. in a boiling-water bath to hydrolyse the fructose phosphates. The mixture was dried down, and the residue was dissolved in water (200 μl .) and chromatographed on Whatman no. 1 paper for 36 hr. as a band in propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.). The glucose 6-phosphate was located by its radioactivity with a radiochromatogram scanner (Gauging Systems Ltd., Baldock, Herts.). The appropriate zone was eluted with water, the eluate was dried down, and the residue was dissolved in water and chromatographed as above in 2-methylpropan-2-ol-0.1 N-HCl-methanol (3:1:1, by vol.) and located as described above. The eluted and dried residue was dissolved in water (3.5 ml.), and glucose 6-phosphate was determined on a sample by the method of Hohorst (1963a) by using a Unicam SP.800 recording spectrophotometer. The remainder was used for radioactivity determination. The absence of fructose 6-phosphate was checked by the addition of glucose phosphate isomerase.

(d) Glycerol 1-phosphate. Glycerol 1-phosphate was eluted from the column about 30 fractions later than the glucose 6-phosphate, and was located by the radioactive peak and by enzymic assay on dried 0.5 ml. samples of the fractions by using the enzymic method of Hohorst (1963b). The combined fractions were treated as described for the glucose 6-phosphate fractions, except that the hydrolysis was omitted and glycerol 1-phosphate dehydrogenase was used (Hohorst, 1963b).

(2) Chemical determinations in rat liver. (a) Glucose 6-phosphate and glycerol 1-phosphate. Frozen liver (approx. 1.5 g.) was rapidly weighed and homogenized in ice-cold 5% (w/v) HClO_4 (5 vol.) in a Nelco 10 homogenizer with a 10 ml. glass vessel. After centrifugation at 2° a sample (6 ml.) was neutralized with 5 M- K_2CO_3 , and glucose 6-phosphate and glycerol 1-phosphate were determined (Hohorst, 1963a,b) by using a Unicam SP.800 automatic recording spectrophotometer.

(b) UDP-glucose. UDP-glucose was determined from the glucose released by the acid hydrolysis from the pooled UDP-glucose fractions obtained from the Dowex 1 (formate form) fractionation of the liver after the $[\text{U-}^{14}\text{C}]\text{fructose}$ injection.

Radioactivity measurements. Samples were counted in a Packard Tri-Carb series 4000 liquid-scintillation spectrometer by using the scintillation medium of Bruno & Christian (1961) but with 1,4-bis-(5-phenyloxazol-2-yl)-benzene omitted. Counts were corrected to disintegrations/min. by addition of a ^{14}C toluene standard.

RESULTS

Concentrations of metabolic intermediates in liver. The glucose 6-phosphate, glycerol 1-phosphate and UDP-glucose contents of rat liver found by the different procedures are given in Table 1. The rapid freezing of the liver of a rat anaesthetized with ether *in situ* with cooled tongs gave tissue contents of glucose 6-phosphate and glycerol 1-phosphate not significantly different from those found by rapidly removing liver from a stunned animal and freezing in liquid nitrogen.

Table 1. *Effect of sampling method on the concentrations of metabolic intermediates in rat liver*

Experimental details are given in the text. Results are given as means $\pm$ s.e.m. with the numbers of animals in parentheses.

Sampling method	Concn. ($\mu\text{moles/g. wet wt.}$)		
	Glucose 6-phosphate	Glycerol 1-phosphate	UDP-glucose
1. Stunning and lobe removed	0.36 $\pm$ 0.02 (8)	0.42 $\pm$ 0.03 (8)	0.23 $\pm$ 0.02 (3)
2. Ether and tongs	0.39 $\pm$ 0.05 (8)	0.46 $\pm$ 0.06 (8)	—
3. Nembutal (8 min.) and tongs	0.16 $\pm$ 0.01 (4)	0.57 $\pm$ 0.13 (4)	—
4. Nembutal (8 min.) and lobe removed	0.20 $\pm$ 0.02 (4)	0.64 $\pm$ 0.17 (4)	—
5. Nembutal (30 min.) and tongs	0.080 $\pm$ 0.005 (3)	0.43 $\pm$ 0.04 (3)	—
<i>P values for glucose 6-phosphate</i>			
1 versus 2	Not significant		
1 versus 3	< 0.001		
1 versus 4	< 0.001		
3 versus 4	Not significant		
3 versus 5	< 0.01

Fig. 1. Specific radioactivities of intermediates after $[U-^{14}C]$ fructose. The standard deviations are about 20%. There was a tendency for values to be correlated, i.e. in a given rat for all specific radioactivities to be higher or lower than the mean, so that specific-radioactivity ratios were more constant at a given time than individual values. The values are means of two to four determinations except at 12 min., when only one liver was analysed. All specific radioactivities in it were unexpectedly low. They were normalized so that the glucose specific radioactivity fitted the mean glucose specific-radioactivity-time curve drawn through points at other times. $\circ$, Glucose; $\bullet$, UDP-glucose; $\square$, glycerol 1-phosphate; $\triangle$, glucose 6-phosphate.

Nembutal anaesthesia for 8 min. gave a glucose 6-phosphate value significantly lower than that found by stunning or ether anaesthesia, and this value was decreased significantly by prolonging anaesthesia to 30 min. Freezing *in situ* or in liquid nitrogen after excision gave similar values.

The glycerol 1-phosphate content of the liver was not significantly affected by any of the procedures.

Specific radioactivities of $[U-^{14}C]$ fructose metabolites. The specific radioactivities of liver glucose, glucose 6-phosphate, glycerol 1-phosphate and UDP-glucose at time-intervals up to 35 min. after the injection of $[U-^{14}C]$ fructose ($10 \mu\text{C}/200 \text{ g. rat body wt.}$) are shown in Fig. 1. The specific radioactivity of the glycerol 1-phosphate rose very rapidly above that of any other compound to a maximum at about 1–1.5 min. Glucose 6-phosphate specific radioactivity also rose rapidly to a maximum at approx. 3 min. that was several-fold lower than the value for glycerol 1-phosphate. The specific-radioactivity curves for these compounds crossed at approx. 8 min. after the injection of the fructose, and at 20 and 35 min. the (glycerol 1-phosphate)/(glucose 6-phosphate) specific-radioactivity ratios were almost identical (0.64 and 0.61), indicating that isotopic equilibrium had almost been reached and that most of the injected fructose had been utilized. The specific radioactivities of glucose and UDP-glucose rose more slowly than those of the glucose

and glycerol phosphates. That of glucose was somewhat higher than that of UDP-glucose initially, but from 2 min. it was lower. The specific radioactivity of glucose reached a maximum at approx. 5 min. and declined slowly for the remainder of the time studied, as expected from its large pool size (cf. Ashby, Heath & Stoner, 1965). The UDP-glucose specific radioactivity reached a peak at 7 min. that was 1.5 times the value for glucose and then fell; at 20–35 min. it approached that of glucose.

The specific radioactivity of glucose 6-phosphate was less than that of glucose at 20–35 min. The (glucose 6-phosphate)/glucose specific-radioactivity ratios at 20 and 35 min. (0.47 and 0.57) were similar to that (0.52) found by Muntz & Vanko (1962) in the liver of the fed rat after $[1-^{14}C]$ fructose infusion into the portal vein for 1–2 min. At this time in the present experiments the ratio exceeded unity.

DISCUSSION

Metabolic intermediates in rat liver

The concentrations of glucose 6-phosphate and glycerol 1-phosphate found in livers of rats that had been stunned or anaesthetized with ether were not significantly different, even though the latter were frozen *in situ* with tongs. The glucose 6-phosphate content was similar to that found by Hohorst *et al.* (1959), who used ether anaesthesia and tongs, and by Burch (1965), who froze the liver in Freon-12 (dichlorodifluoromethane) after stunning the rat, but was four times that found by Young (1966) after Nembutal anaesthesia, stunning, cervical dislocation or guillotining and rapid freezing of the liver by cooled tongs. The values found in the present experiments after Nembutal anaesthesia were similar to those obtained by Young (1966), who concluded that the higher values found by other authors were due to glycogenolysis. In the present experiments the liver from the stunned rat was frozen in liquid nitrogen in 5–7 sec., less than the time shown by Young (1966) to be required for appreciable glucose 6-phosphate formation by glycogenolysis. On the available evidence the fourfold difference in the glucose 6-phosphate values found in the present experiments and those of Young (1966) can only be attributed to factors other than experimental technique.

The glucose 6-phosphate content after freezing the liver *in situ* with cooled tongs was not significantly different from that found by cooling rapidly excised liver in liquid nitrogen (Table 1, sampling methods 1 and 2), and the latter method was used as a routine to obtain larger liver samples.

In interpretation of the data considerable weight is placed on the finding that for a long time after injection of $[U-^{14}C]$ fructose the specific radioactivity

of glucose 6-phosphate was consistently lower than the specific radioactivity of glucose. This could be an artifact due to the release of glucose 6-phosphate from glycogen immediately after the rat was killed. But, as two different methods of sampling the liver gave the same concentrations of glucose 6-phosphate, we have assumed that the finding was genuine.

The glycerol 1-phosphate concentration did not change significantly with the different techniques and was similar to those found by Burch (1965) in the livers of rats anaesthetized with ether.

The UDP-glucose content of the liver from stunned rats was lower than that found by Burch (1965) in rats anaesthetized with ether, but was similar to that found by Young (1966) in rats anaesthetized with Nembutal.

Kinetic treatment of data

Symbols. The following symbols are used:

S , specific radioactivity (disintegrations/min./ μ mole);

C , concentration and pool size (μ moles/g. wet wt. of liver);

Q , pool size (μ moles/g. wet wt. of liver), where distinguishable from C ;

k , velocity constant (per min.);

t , time (min.);

V or R , rates (μ moles/min./g. wet wt. of liver);

q , total label in a pool.

The following subscripts are used: T, triose phosphates; 1, glycerol 1-phosphate; 6, glucose 6-phosphate; G, glucose; U, UDP-glucose.

Subscripts to rates, V , were chosen to conform with Heath (1968).

No allowance has to be made for exchange of label between fructose and glucose via sorbitol, as sorbitol was found to be unlabelled in similar experiments (Ashby *et al.* 1965).

The metabolism of fructose is nearly confined to the liver and kidneys *in vivo* and is similar in both organs (Klein, Hurwitz & Olsen, 1946; Wick, Sherrill & Drury, 1953; Hers, 1955; Reinecke, 1956; Salomon, Lanza & Smith, 1961; Opie, Shipp,

Evans & Leboeuf, 1962; Opie, Evans & Renold, 1962), and about 90% of the label injected as $[U-^{14}C]$ fructose can be accounted for in $[^{14}C]$ glucose and $^{14}CO_2$ (Ashby *et al.* 1965). The quantities of label that can be supposed to be transported in metabolites to the liver from other organs must therefore be very small, and can be neglected. By 20 min. after injection about 90% of the injected dose has been utilized (Ashby *et al.* 1965). Of the $[U-^{14}C]$ fructose utilized Ashby *et al.* (1965) showed that most is probably utilized by the liver. For the present purposes all that is needed is a minimum estimate of the total fructose label turned over in the first 20 min. This will be taken as 60% of the injected dose, i.e. $0.6 \times 5 \mu c/100$ g. rat or, taking the liver weight as 4 g./100 g., 1.7×10^6 disintegrations/min./g. of liver. The best estimate from the results obtained by Ashby *et al.* (1965) is 2.1×10^6 disintegrations/min./g. of liver.

Compartmentation of glucose 6-phosphate. The specific radioactivities of glucose 6-phosphate, glycerol 1-phosphate and glucose (Table 2) were related by:

$$S_6 = 0.2S_1 + 0.45S_G \quad (1)$$

This relationship holds from 0.25 min. As S_G was low until 1 min. (Fig. 1) it follows that glucose 6-phosphate was in substantial isotopic equilibrium with glycerol 1-phosphate by 0.25 min., and hence that the turnover of glucose 6-phosphate was very rapid. (A possible proviso is discussed below.)

How far should the triose phosphates be regarded as in isotopic equilibrium with each other?

Hers (1957), Rose, Kellermeyer, Stjernholm & Wood (1962) and Rauschenbach & Lamprecht (1964) showed that isotopic equilibration between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate was only about 70% complete. In our experiments, however, both glyceraldehyde 3-phosphate and dihydroxyacetone phosphate received label independently from $[U-^{14}C]$ fructose (see also Lamprecht, Diamanstein, Heinz & Balde, 1959). Consequently inequality of labelling could only occur insofar as the labelling of these two trioses followed different time-courses. Dihydroxyacetone

Table 2. Relationship between the specific radioactivities of glycerol 1-phosphate, S_1 , glucose, S_G , and glucose 6-phosphate, S_6

The calculated values of S_6 were obtained by eqn. (1).

		$10^{-3} \times$ Specific radioactivity (disintegrations/min./ μ mole)									
Time (min.)	...	...	0.25	0.5	1	1.5	3	6	12	20	35
Experimental values	$\left\{ \begin{array}{l} S_1 \\ S_G \\ S_6 \end{array} \right.$	S_1	13.8	40	72	61	52	15.6	6.7	3.3	3.2
		S_G	0	1.9	7.9	9.8	13.7	12.6	12.0	11.6	8.9
		S_6	2.8	11.2	15.7	16.9	17.7	13.3	6.3	5.5	5.1
Calculated S_6			2.8	8.9	17.9	16.6	16.6	8.8	6.7	5.9	4.6

phosphate will tend to be labelled first, as glyceraldehyde 3-phosphate is labelled via a longer pathway (Scheme 1). In the extreme case at very short times label might be imagined to enter only dihydroxyacetone phosphate, so that after 70% equilibration with glyceraldehyde 3-phosphate the specific radioactivity of dihydroxyacetone phosphate would be 1.18 times that of the combined pool; whereas after longer times the converse would be the case, and the specific radioactivity of dihydroxyacetone phosphate would be about 0.85 times that of the combined pool. It follows that, if equilibration between the trioses and glucose 6-phosphate was very rapid, the experimental specific radioactivities of glucose 6-phosphate should show a systematic trend with respect to an equation such as (1); they should be low at short times and high at long times. There was, in fact, no marked trend (Table 1).

Such complete separation in the time-course of the labelling of the trioses from [$U\text{-}^{14}\text{C}$]fructose is highly unlikely, as at all times on any kinetic scheme some label must reach both trioses simultaneously. Any likely trend could easily be obscured by the experimental errors. It could also be compensated for at very short times after injection if glycerol 1-phosphate was not quite in instantaneous equilibrium with dihydroxyacetone phosphate. Then at short times the specific radioactivity of glycerol 1-phosphate would somewhat underestimate that of dihydroxyacetone phosphate, which in turn would somewhat overestimate that of the combined dihydroxyacetone phosphate and glyceraldehyde 3-phosphate pool. The overall conclusion is, however, that the specific radioactivity of glycerol 1-phosphate is likely to represent the specific radioactivity of the precursor pool within the experimental error, and this is taken to be the case, i.e. both C_3 precursors will have had the same specific radioactivity, S_T , as glycerol 1-phosphate, S_1 ; and the total specific radioactivity of both precursors, S_T' , will be given by:

$$S_T' = 2S_T = 2S_1 \quad (2)$$

Eqn. (1) could in principle be explained in any of three ways: by compartmentation of glucose 6-phosphate; or by isotopic exchange between glycerol 1-phosphate, glucose 6-phosphate and glucose, i.e. by a flux of label very much faster than any net flux of compound into and out of the system; or by isotopic exchange combined with a contribution to glucose 6-phosphate labelling via the conversion of fructose into fructose 6-phosphate by hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1) (DiPietro, 1964).

It is first shown that isotopic exchange cannot explain these results without causing them to conflict with others.

Suppose isotopic exchange is the correct explanation. The system can be represented by Scheme 2, in which the two C_3 precursors of glucose 6-phosphate are combined into one pool, with specific radioactivity S_T' (eqn. 2).

The specific radioactivity of glucose 6-phosphate eventually fell to only 0.45 times that of glucose. This can only be explained if label in glucose 6-phosphate was diluted by an input of unlabelled glucose 6-phosphate, at rate R_2 . Then, with the symbolism of Scheme 2, equating input and output of glucose 6-phosphate:

$$V_8 + V_{10} = V_7 + V_9 + R_2 \quad (3)$$

Equating input and output of label:

$$(V_8 + V_{10})S_6 = V_7S_T' + V_9S_G \quad (4)$$

Eqn. (1) can be rewritten:

$$S_6 = 0.1S_T' + 0.45S_G \quad (5)$$

Rearranging eqn. (4), substituting in it eqn. (3) and equating the coefficients of S_T' and S_G with those in eqn. (5) gives:

$$V_9 = R_2; \quad V_7 = R_2/4.5 \quad (6)$$

The only source of unlabelled glucose 6-phosphate is glycogen. There was no significant exchange of label with glycogen, as UDP-glucose remained

Scheme 2. Relationship between triose phosphates, glucose 6-phosphate and glucose in terms of isotopic exchange. ($V + V' = R_2$; the individual values of V and V' are irrelevant.)

only lightly labelled for the first 1 min. after injection. So R_2 can be equated with the net rate of glycogenolysis. In rats of the same colony and in the same nutritional state this was found to be $0.6 \mu\text{mole/min./g.}$ (Stoner, 1958; and later confirmed by him and communicated personally). Hence by eqn. (6): $V_9 = 0.6$; $V_7 = 0.1333$.

Each rate corresponds to a velocity constant:

$$(k_8 + k_{10}) = (V_8 + V_{10})/C_8 \quad (7)$$

With $C_8 = 0.38$ (Table 1), $k_8 + k_{10} = 3.51/\text{min.}$

Shortly after injection $S_G \ll S_8$, so glucose contributed negligible label to glucose 6-phosphate up to 0.5 min. after injection, and standard methods then give:

$$dS_8/dt = 0.351(S_T' - 10S_8) \quad (8)$$

Values of S_T' were obtained at short time-intervals (0.001–0.005 min.) by a combination of graphical and numerical extrapolation from the experimental specific radioactivities of glycerol 1-phosphate from 0 to 1.5 min. Eqn. (8) was then integrated numerically. Up to 0.5 min. the calculated values of S_8 were less than one-third of those found (Table 2). The calculated values of S_8 did not agree with those found within experimental error until 3 min. after injection. Exchange theory in this form was plainly unable to explain eqn. (1).

A different approach was next tried. Eqn. (8) was rewritten:

$$dS_8/dt = k(S_T' - 10S_8) \quad (9)$$

where:

$$10k = (k_8 + k_{10}) \quad (10)$$

and k was varied to see how fast the glucose 6-phosphate pool must have turned over to reach substantial isotopic equilibrium with glycerol 1-phosphate in 0.25 min. For $k = 1/\text{min.}$ S_8 was calculated to be 45% lower at 0.25 min. and 25% lower at 0.5 min. than calculated by eqn. (1), which fits the data adequately. A good fit required $k \geq 3/\text{min.}$ Even for $k = 1/\text{min.}$, eqns. (10), (7), (6) and (3) showed that $R_2 = 1.71$ and $V_9 = 1.71$, both impossibly high.

This value of R_2 is obviously too high. The value of V_9 , which is the rate of glucose 6-phosphate formation from glucose in the liver, can be shown to be too high by considering the results of Katz & Dunn (1967) and Ashby *et al.* (1965).

Katz & Dunn (1967) gave rats [$2\text{-}^3\text{H}$]glucose simultaneously with [$1\text{-}^{14}\text{C}$]glucose. They found that in all organs the glycogen laid down, as measured by ^{14}C deposition, was almost devoid of ^3H , because glucose 6-phosphate equilibrated very rapidly with fructose 6-phosphate by the action of glucose phosphate isomerase, during which most of the ^3H is lost. Thus ^3H disappearance from glucose measures the rate of glucose 6-phosphate formation;

and it was shown that this was 1.5 times the rate of glucose utilization as measured by [^{14}C]glucose disappearance. Ashby *et al.* (1965) showed that in the whole rat the disappearance of injected [^{14}C]glucose corresponded to a net rate of glucose utilization of $6.6 \mu\text{moles/min./100g.}$, and hence, from the result obtained by Katz & Dunn (1967), to a rate of glucose 6-phosphate formation from glucose of $1.5 \times 6.6 = 10 \mu\text{moles/min./100g.}$ rat body wt. Ashby *et al.* (1965) concluded that less than 20% of the glucose utilized was utilized in the liver. Taking the liver weight as 4g./100g. rat body wt., in the liver $V_9 < 0.5$. The minimum discrepancy is the factor $1.71/0.5$. The likely discrepancy is a factor greater than 10.

Exchange theory was also inconsistent with the total quantity of label passing through glycerol 1-phosphate. Denote by V_{11} the rate of utilization of the triose phosphates expressed as C_8 units/min. The rate of flow of label through them is:

$$dq_T/dt = V_{11}S_T' \quad (11)$$

whence:

$$q_T = V_{11} \int_0^\infty S_T' dt$$

i.e.:

$$V_{11} = q_T/A \quad (12)$$

where A is the area under the S_T' -time curve. To calculate V_{11} the integral must exclude the contribution made to the specific radioactivity due to recycling by pathways outside the system shown in Scheme 2. As by 10 min. the liver [$1\text{-}^{14}\text{C}$]fructose concentrations have fallen very low (Ashby *et al.* 1965), it is certain that by 20 min. all the label in the trioses came from recycling processes, so the area was taken as that from 0 to 20 min. This is an overestimate. The total label, q_T , is that in the fructose utilized, i.e. $> 1.7 \times 10^6$ disintegrations/min./g., whence $V_{11} > 2.4 \mu\text{moles of } C_8 \text{ units/min./g.}$ or $> 4.8 \mu\text{moles of trioses/min./g.}$ In the symbolism of Scheme 2:

$$V_{11} = V_7 + V \quad (13)$$

where V represents the rate of entry into the Embden-Meyerhof pathway. As of the label injected 70% can be accounted for in glucose (Ashby *et al.* 1965), only 30% can enter the Embden-Meyerhof pathway. Ashby *et al.* (1965) showed that this was probably an overestimate. Thus:

$$V \leq 30V_7/70 \quad (14)$$

The best estimate of V_7 has been shown to be 0.133, whence from eqn. (14) $V < 0.057 \mu\text{mole/min./g.}$, and from eqn. (13) $V_{11} = 0.19 \mu\text{mole/min./g.}$ The value of V_{11} is far too low. The value of V is also far too low to account for the rapid rise in the specific radioactivity of liver glutamate after the injection

of [$U\text{-}^{14}\text{C}$]glucose (Heath & Threlfall, 1968). With k about 4 (eqn. 9) these discrepancies disappear, but then the rates of glycogenolysis and of glucose $\rightleftharpoons$ glucose 6-phosphate equilibration are calculated to be, as before, over 10 times too big.

The exchange hypothesis therefore can account for either the rapid turnover of the triose phosphates and the rapid initial rise in the specific radioactivity of glucose 6-phosphate or for the eventual dilution of label in the glucose 6-phosphate pool and for the rate of glucose 6-phosphate $\rightleftharpoons$ glucose equilibration; but it cannot correlate the two pairs within the factor 10.

The hypothesis cannot be saved by assuming that label enters glucose 6-phosphate by the pathway:

This pathway, if it operates, would donate label faster to glucose 6-phosphate than do the triose phosphate pathways, which are longer, and in which the pool sizes are great enough to hold label up for a time, so that at short times the apparent equilibration of label between the trioses and glucose 6-phosphate could be an artifact: the slow donation of label from the trioses would be compensated for by a rapid donation via the hexokinase reaction. The overall effect of the hexokinase pathway, however, would always be the donation of additional label to glucose 6-phosphate, i.e. the triose phosphate pathways would contribute less than the total label observed, and the coefficient, 0.2, in eqn. (1) would be too high. Thus V_7 would be still lower, and the exchange hypothesis would be even less satisfactory. It seems, however, intrinsically likely that very little label entered glucose 6-phosphate from fructose via the hexokinase reaction. The concentration of injected fructose in the liver was about $10\mu\text{M}$ shortly after injection (calculated from the data given by Ashby *et al.* 1965). The K_m for fructose on hexokinase is about $7 \times 10^{-3}\text{M}$ (DiPietro, 1964), whereas for glucose it is about $4 \times 10^{-5}\text{M}$ (Lange & Kohn, 1961; Walker & Rao, 1963). The endogenous glucose concentration of about 8mM should have excluded fructose almost completely by competition.

Glucose 6-phosphate is therefore compartmented, in agreement with the conclusions of London (1966) and Threlfall (1966) about liver and of Landau & Sims (1967) about rat diaphragm. Henceforth it is assumed that isotopic exchange between glucose and glucose 6-phosphate was negligible, as in this context was probably the case. Compartmentation allows very rapid labelling of glucose 6-phosphate from the triose phosphates in the gluconeogenic chain, and isotopic exchange between these compounds may take place. Compartmentation implies that there was a glycolytic pool of glucose 6-phos-

phate which was labelled from glucose, but that label in this pool was diluted by unlabelled glucose 6-phosphate from glycogen. Hence eqn. (5) can be rewritten:

$$S_6 = \alpha S_T' + (1 - \alpha)\beta S_G \quad (15)$$

where $\alpha = 0.1$ and $\beta = 0.5$. The constant α represents the fraction of the total glucose 6-phosphate pool in the gluconeogenic chain, and β the dilution of label in the glycolytic chain. Hence, if R_1 is the rate of glucose 6-phosphate formation in the glycolytic chain, and R_2 is the rate of glycogenolysis:

$$\beta = R_1/(R_1 + R_2) \quad (16)$$

In experiments with [$U\text{-}^{14}\text{C}$]glucose $\beta = 0.38$ (Heath & Threlfall, 1968), in fair agreement with the present estimate, and, with $R_2 = 0.6$ (see above), $R_1 = 0.4\text{--}0.6\mu\text{mole/min./g.}$

The assumption that glycogenolysis only feeds the glycolytic chain brings the liver in line with other organs and tissues, in which glycogenolysis is associated with the formation of lactate. For further discussion, see below under 'UDP-glucose synthesis'.

Compartmentation of the triose phosphates. The turnover of the triose phosphates was shown above to be $\geq 2.4\mu\text{moles of C}_6 \text{ units/min./g.}$ This is the rate V_{11} in eqn. (11). It is somewhat greater than is consistent with the results obtained by Ashby *et al.* (1965).

The interaction of the glycolytic and gluconeogenic chains can be represented either as in Scheme 3(a), with separation to phosphoenolpyruvate, or as in Scheme 3(b), i.e. with the two chains joining at the triose phosphate level and isotopic exchange (chemical equilibration) between the triose phosphates and phosphoenolpyruvate. In Scheme 3 the symbolism is that used by Heath (1968) and Heath & Threlfall (1968), in which V_1 , V_5 and V_6 refer to the rate of movement of C_3 moieties, and V_{11} is the total rate of turnover of pairs of triose phosphates, i.e. of C_6 moieties, so in Scheme 3(a): $V_{11} = 0.5V_6$; and in Scheme 3(b): $V_{11} = 0.5(V_1 + V_6)$. The maximum acceptable value of V_{11} on both schemes can be calculated independently.

On both schemes net utilization of glucose by the tricarboxylic acid cycle is given by: $0.5(V_5 - V_6)$.

There is a small net utilization in post-absorptive rats (Heath & Threlfall, 1968), but, as the maximal estimate of V_{11} is obtained with maximal V_6 , it will be assumed that:

$$V_5 = V_6 \quad (17)$$

Now:

$$V_5 = 2(R_1 + R_2) \approx 2\mu\text{moles/min./g.} \quad (18)$$

(end of preceding section); so, on Scheme 3(a), $V_{11} = 0.5V_6 \approx 1$.

Scheme 3. Interaction of glycolysis and gluconeogenesis. The symbols V_1 etc. are as defined by Heath (1968).

Scheme 3(b) can be analysed by using the results of Ashby *et al.* (1965), who found that of label injected as $[U-^{14}C]$ fructose 70% appeared in glucose; and of label injected as $[U-^{14}C]$ pyruvate 30% can be shown (Heath & Threlfall, 1968) to have appeared in glucose. V_1 represents the rate of formation of pyruvate. Then:

$$(V_6 + 0.3V_1)/(V_6 + V_1) = 0.7 \quad (19)$$

whence, with $V_6 = V_5 \approx 2 \mu\text{moles/min./g.}$ (above), $0.5(V_1 + V_6) = V_{11} \approx 1.8 \mu\text{moles/min./g.}$

The present data do not allow us to make any choice between Schemes 3(a) and 3(b). On Scheme 3(a) there is a big discrepancy between the minimum rate of turnover of the triose phosphates calculated from their specific radioactivities, i.e. $2.4 \mu\text{moles/min./g.}$, and the rate of turnover required by the results with $[U-^{14}C]$ fructose obtained by Ashby *et al.* (1965), i.e. $1 \mu\text{mole/min./g.}$ If Scheme 3(a) is correct then the two rates can only be reconciled by compartmentation of the triose phosphates, only about 0.4 of which are in the gluconeogenic chain. On Scheme 3(b) the discrepancy is smaller, > 2.4 against $1.8 \mu\text{moles/min./g.}$ This probably implies some compartmentation, but the evidence is obviously less convincing. Heath & Threlfall (1968), however, show that some other data cannot be correlated on Scheme 3(b), but can be on Scheme 3(a), and for this reason we think that Scheme 3(a) is more nearly correct, i.e. that the gluconeogenic and glycolytic chains are to a considerable extent separated from glucose to phosphoenolpyruvate. It is not necessary, in view of the short time-scale of the experiments, to assume complete separation, only that mixing between pools is slow compared with their turnover times. On Scheme 3(a) S_T'' , the specific radioactivity of the gluconeogenic triose phosphates, was about $2.5S_T'$, whence, in eqn. (15), $\alpha = 0.04$, $\beta = 0.47$.

UDP-glucose synthesis. UDP-glucose is usually accepted as arising from glucose by the following sequence of reactions:

Glycogen is also converted into glucose 1-phosphate by glycogen phosphorylase (α -1,4-glucan-orthophosphate glucosyltransferase, EC 2.4.1.1).

As the specific radioactivity of UDP-glucose rose only slowly (Fig. 1) UDP-glucose turnover must have been slow; and as its specific radioactivity rose above that of glucose it must have been labelled from gluconeogenic glucose 6-phosphate. This argument also establishes that the glucose 1-phosphate in the gluconeogenic chain must have been compartmented from that involved in glycogenolysis. Assuming compartmentation was complete:

$$dS_U/dt = k_U(S_6' - S_U) \quad (20)$$

where S_6' is the specific radioactivity of gluconeogenic glucose 6-phosphate, and hence of gluconeogenic

genic glucose 1-phosphate. S_6' is twice the specific radioactivity of the gluconeogenic trioses, i.e.:

$$S_6' = S_T'' = 2.5S_T' = 5S_1 \quad (21)$$

The factor 2.5 is introduced to allow for compartmentation of the trioses (end of preceding Section).

After substituting eqn. (21) in eqn. (20), eqn. (20) was integrated numerically. The shape of the S_U -time curve (Fig. 1) was well reproduced up to 12 min. with $k_U = 0.05/\text{min.}$, but all the calculated values were twice the experimental ones. (After 12 min. the calculated curve did not fall quite as rapidly as the experimental one; this was to be expected, as by 20 min. much of the label in the triose phosphates was probably in the glycolytic chain.) The discrepancy is removed if there was a leakage of unlabelled glucose 1-phosphate from glycogenolysis into the gluconeogenic glucose 1-phosphate pool, sufficient to halve the specific radioactivity of the latter. The turnover of UDP-glucose, $k_U Q_U$, was $0.012 \mu\text{mole}/\text{min.}/\text{g.}$ ($Q_U = 0.23 \mu\text{mole}/\text{g.}$; Table 1). An exact assessment of the rate of leakage of glucose 1-phosphate from the glycogenolytic pool cannot be made, as it is not known how far this pool may be labelled by exchange with glycolytic glucose 6-phosphate, and the calculation also depends on the compartmentation of the triose phosphate pools, for which there is no precise estimate. The rate must, however, be very small.

Other interpretations of the UDP-glucose specific-radioactivity-time curve are possible, including that suggested by Smith *et al.* (1967), in which glucose 1-phosphate is supposed to be formed from glucose without glucose 6-phosphate as an intermediate. On this hypothesis UDP-glucose would be formed both from gluconeogenic glucose 6-phosphate and from glucose. Our interpretation is, however, more simple and more in accordance with accepted pathways.

Dilution of label in gluconeogenic glucose 6-phosphate by glucose 6-phosphate from glycogen is not a possible explanation, as can be seen by repeating the whole calculation assuming it is, but keeping R_2 constant (see above under 'Compartmentation of glucose 6-phosphate').

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