Factors Affecting Hepatic Glycolysis and some Changes that Occur during Development

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1. Glycolysis by the supernatant fraction of homogenates of liver from guinea pigs and rats at various stages of development (foetal, newborn and adult) has been examined in a suitably fortified medium by measurement of inorganic phosphate uptake and production of lactate and glycerol 1-phosphate. 2. Starting with glucose as substrate, two rate-determining steps in glycolysis occur at the stages of glucose phosphorylation and the phosphofructokinase reaction in liver tissue from animals of all ages. Effects of the post-natal development of glucokinase are recorded. 3. The appearance of microsomal glucose 6-phosphatase activity around birth has an effect on glycolysis owing to competition for glucose 6-phosphate. 4. A stimulating effect of the nuclear fraction, especially from foetal liver, on glycolysis by the supernatant fraction is interpreted as being due to stimulation by adenosine-triphosphatase activity at the 3-phosphoglycerate-kinase stage.

Although the glycolytic sequence of reactions is not quantitatively the most important pathway of glucose utilization in liver tissue (Weber, 1963), it is nevertheless an essential metabolic pathway in this organ. With liver slices and manometric assay methods, higher rates of anaerobic glycolysis have been recorded in foetal liver than in the adult tissue (Rosenthal & Lasnitzki, 1928; Burk, 1942; Vilee & Hagerman, 1958; Van Rossum, 1963). Glycolysis may also be measured in tissue homogenates fortified with appropriate cofactors (Utter, Wood & Reiner, 1945), and the enzymes of the glycolytic pathway are localized mainly in the soluble fraction of liver homogenates (LePage & Schneider, 1948; Kennedy & Lehninger, 1949). In experiments with fortified homogenates, Dawkins (1959) reported similar glycolytic rates in foetal and adult rat liver, and Oliver, Ballard, Shield & Bentley (1962) found no change during liver growth in the early post-partum rat.

In an attempt to explain the apparent discrepancy between the results obtained with slices and homogenates, and also to examine the effect, if any, of the development of the enzymes glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) (Lea & Walker, 1964a) and glucokinase (a phosphotransferase of the type EC 2.7.1.2) (Walker, 1963b; D. G. Walker & G. Holland, unpublished work) around parturition and during the neonatal period, the present investigation was designed to define possible rate-limiting enzymes of glycolysis in the supernatant fraction of homo-

genates of rat and guinea-pig liver. The influence of other subcellular fractions on the supernatant fraction have been examined and some changes in such effects recorded during development.

A preliminary account of this work has been given (Lea & Walker, 1964b).

MATERIALS AND METHODS

Animals. The type of guinea pigs and rats used, their diet and the methods for estimating the gestational age were as described by Walker (1963a).

Chemicals and enzymes. Glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), ATP, ADP, NAD⁺, NADH and sodium pyruvate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Yeast hexokinase (EC 2.7.1.1) (crystalline; activity 140 units/mg.), lactate dehydrogenase (EC 1.1.1.27) (360 units/mg.), glycerol 1-phosphate dehydrogenase (EC 1.1.1.8) (36 units/mg.), aldolase (EC 4.1.2.7) (9 units/mg.) and 3-phosphoglyceric acid (barium salt) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The barium salt of 3-phosphoglyceric acid was converted into the sodium salt by dissolving it in the minimum volume of 2N-HCl and precipitating the barium with the calculated quantity of Na₂SO₄. Heavy meromyosin prepared by chymotrypsin digestion of myosin was made by Mrs J. Frost in this Department according to the method of Mueller & Perry (1962). The adenosine-triphosphatase activity of the preparation under optimum conditions was 0.65 μ mole of inorganic phosphate released/min./ml. of solution having $E_{280}^{1\text{cm}}$ 1. All other chemicals were the best quality obtained from British Drug Houses Ltd., Poole, Dorset.

Tissue extracts. Unless stated otherwise, the procedures for preparing tissue homogenates were as described by

Lea & Walker (1964a). More concentrated homogenates (20%, w/v) were sometimes prepared to provide sufficient activity.

In the experiments on the effects of the whole particulate fraction on glycolysis by the supernatant fraction, the particulate fraction was prepared by centrifuging the whole homogenate at 100000g for 45 min. in an MSE Super-Speed 40 centrifuge (8 × 10 ml. head). Complete fractionation of liver-cell homogenates was performed by a procedure essentially that of Schneider & Hogeboom (1950). The nuclear fraction was obtained at 700g for 10 min., the mitochondrial fraction at 5000g for 20 min. and the microsomal fraction at 100000g. for 45 min. Each fraction was washed once in 0.25M-sucrose and finally suspended in a volume equal to that of the original whole homogenate. The supernatants from the washing steps were rejected to avoid dilution of the supernatant fraction. Thus when equal volumes of two or more fractions were included in a reaction mixture (see below) they were present in proportions equivalent to those in the original homogenate.

Assay of glycolysis. All the estimations of glycolytic (and enzymic) activities were performed at 30° under optimum conditions giving zero-order kinetics. This was frequently checked by performing assays with two enzyme concentrations and two incubation periods.

Glycolysis was assayed in a reaction mixture that included 0.1 ml. samples of whole homogenates or of one or more fractions of homogenates in a total volume of 1 ml. at pH 7.6 in the presence of: MgCl₂, 7 mM; ATP, 1 mM; NAD⁺, 1 mM; nicotinamide, 33 mM; potassium phosphate, 2.5 mM; KHCO₃, 25 mM. The acidic solutions were previously neutralized with 1N-NaOH and sufficient of a mixture of all these reactants was prepared for a whole experiment. This compound solution was then adjusted to pH 7.6 with a few drops of 0.1N-HCl and samples were pipetted into the series of incubation tubes. Except where otherwise noted, one of the following substrates was also included in the incubation mixture at the following final concentrations (these concentrations having been found in preliminary tests to be the optimum for preparations of livers from animals of all ages): glucose, 100 mM; glucose 6-phosphate, 5 mM; fructose 6-phosphate, 5 mM; fructose 1,6-diphosphate, 5 mM.

The first experiments (see the Results section) were performed in standard Warburg vessels with either O₂ + CO₂ (95:5) or N₂ + CO₂ (95:5) as the gas phase. In these cases the reaction was stopped by tipping in 0.2 ml. of 18% (w/v) perchloric acid from a side arm. The remaining experiments (i.e. the majority) were performed in 12 ml. conical centrifuge tubes with air as the gas phase. The reaction was then stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid. After centrifuging, samples of the protein-free supernatant were used for the determination of one or more of the following: (a) inorganic phosphate by the method of Fiske & Subbarow (1925); (b) lactate by the enzymic method of Hohorst, Kreutz & Bücher (1959); (c) glycerol 1-phosphate by the enzymic method of Hohorst *et al.* (1959). Preliminary experiments showed that neither of these enzymic assays was affected by either the trichloroacetic acid or the perchloric acid in the supernatant.

Modifications of and additions to the glycolysis reaction mixture are described in the Results section.

Duplicate incubations of all assays of glycolytic activity were performed and average values of the inorganic phos-

phate, lactate and glycerol 1-phosphate determinations are recorded. The duplicate values for estimations of these metabolites differed by less than 5%.

Enzyme-assay procedures. In order that direct comparisons might be made, all the following enzyme assays were performed at the same pH as used for the estimation of glycolysis, i.e. pH 7.6.

Adenosine-triphosphatase activity was assayed by the method of Bonting, Simon & Hawkins (1961), except that inorganic phosphate was determined as described above.

Phosphofructokinase (EC 2.7.1.11) activity was assayed in an incubation mixture, total volume 1 ml., containing (final concentrations): MgCl₂, 7 mM; ATP, 2 mM; tris buffer, pH 7.6, 50 mM; hydrazine (freshly prepared by neutralization of a solution of the sulphate with 2N-NaOH), 55 mM; aldolase, 1 µg. (0.009 unit); substrate, 5 mM. Glucose 6-phosphate and fructose 6-phosphate gave identical results when used as substrate [this being attributable to the high phosphoglucose-isomerase (EC 5.3.1.9) activity found throughout hepatic development (Burch *et al.* 1963; Lea & Walker, 1964a)], and the former was used in routine assays. The incubation was terminated by the addition of 1 ml. of 10% (w/v) trichloroacetic acid. The triose phosphate formed by the action of aldolase on the product of the phosphofructokinase reaction was assayed by a procedure based on the aldolase-assay procedure of Sibley & Lehninger (1949). Samples of the protein-free supernatant were added to 1.2 vol. of 2N-NaOH, and the alkali-labile phosphate liberated after 20 min. at room temperature was measured after neutralizing with 1 vol. of 2N-HCl by the method of Fiske & Subbarow (1925). Supernatant fractions prepared from guinea-pig liver were examined, and it was found that ATP concentrations up to 6 mM did not result in any inhibition of phosphofructokinase activity and that the presence of 2 mM-ADP had no effect (cf. Lardy & Parks, 1956; Passonneau & Lowry, 1962).

All enzyme activities are expressed as µmoles of substrate utilized or product formed/min./g. wet wt. of tissue or on a nitrogen basis (nitrogen being determined by a micro-Kjeldahl procedure).

RESULTS

Influence of the gas phase on lactate production. Experiments with either whole homogenates or the supernatant fractions from both foetal and adult livers of guinea pigs and rats showed that the rate of lactate production with a saturating concentration of either glucose or glucose 6-phosphate as substrate was the same when the gas phase was either oxygen + carbon dioxide (95:5) or nitrogen + carbon dioxide (95:5). As high partial pressures of oxygen or nitrogen made no difference to the observed rates of lactate formation, in subsequent studies air was used as gas phase.

Influence of fluoride and pyruvate on glycolysis by the supernatant fraction of homogenates of guinea-pig liver. Glycolysis was estimated as a routine by two procedures, lactate production and the esterification of inorganic phosphate (at the 3-phosphoglyceraldehyde-dehydrogenase stage). For the purpose of measuring the latter, sodium fluoride and

sodium pyruvate (final concentrations 10mm and 5mm respectively) were added to the incubation medium. The presence of fluoride minimizes the adenosine-triphosphatase activity and results in the accumulation of 3-phosphoglycerate owing to inhibition of enolase (EC 4.2.1.11), whereas the pyruvate facilitates the reoxidation of NADH with the formation of lactate (LePage, 1948). In the absence of fluoride and pyruvate a net increase in the inorganic phosphate concentration was recorded. The importance of glycerol 1-phosphate formation due to the presence of glycerol 1-phosphate dehydrogenase (EC 1.1.1.8) (Boxer & Shonk, 1960) was examined because changes in this could greatly influence the formation of lactate. Table 1 shows the effect of the addition of both fluoride and pyruvate on lactate and glycerol 1-phosphate production starting with three different substrates (at saturating concentrations: see below). Reasonably good agreement between inorganic phosphate utilization and lactate formation were recorded, as is to be expected from the fact that these two reactions occur during the further metabolism of glyceraldehyde 3-phosphate. The ratio of lactate formation to glycerol 1-phosphate formation was lower in adult liver than foetal liver.

A decrease in glycerol 1-phosphate formation and a subsequent rise in the proportion of lactate formed was recorded when pyruvate but no fluoride was added. In the absence of fluoride enolase is not inhibited. This glycolytic enzyme has a comparatively low activity in mammalian liver (Fellenberg, Eppenberger, Richterich & Aebi, 1962). The stimulation of lactate production by the addition of pyruvate alone might therefore be due to the by-passing of the rate-limiting enolase stage. The possible presence of such a controlling reaction was examined in supernatant preparations of livers from both foetal and adult guinea pigs by measuring lactate formation in the standard incubation medium to which was added (final concentrations) 3-phosphoglycerate (5mm) (adjusted to pH 7.6 with 2N-sodium hydroxide), ADP (2mm) and NADH (2mm). In both cases examined (one adult liver and one foetal liver preparation) the result was the same as that obtained in parallel determinations of the lactate formed from 5mm-fructose 1,6-diphosphate as substrate in the presence of pyruvate but not fluoride. Further, again in the absence of fluoride, the presence of 5mm-3-phosphoglycerate had essentially the same effect as pyruvate in increasing the ratio of lactate formation to glycerol 1-phosphate formation. The implication of these results is that enolase is not a rate-limiting step in these studies *in vitro* at any stage of liver development and maturation. Table 1 also shows that the preparations from livers of several different ages all showed the greatest ratio of lactate

Table 1. *Inorganic phosphate uptake and formation of lactate and glycerol 1-phosphate by supernatant fractions of guinea-pig liver, and the effects of the addition of pyruvate and fluoride to the incubation mixture*

The results for foetal liver are the means of determinations on pooled liver samples of two sets of litter mates of gestational age 39 and 48 days. The results for adult liver are each the means of determinations on two adult animals (at least 4 months old). Experimental details are given in the text. Final concentrations of substrates were: glucose, 100 mM; glucose 6-phosphate, 5 mM; fructose 1,6-diphosphate, 5 mM. Final concentrations of sodium fluoride and sodium pyruvate, where added, were 10 mM and 5 mM respectively. The incubation mixture contained ADP (2 mM) in addition to the standard constituents.

Tissue	Substrate	Additions to incubation mixture	Lactate production (μ moles/min./g. wet wt.)	Glycerol 1-phosphate production (μ moles/min./g. wet wt.)	Inorganic phosphate uptake (μ moles/min./g. wet wt.)
Foetal liver	Glucose	None	0.50	0.10	—
		Sodium fluoride + sodium pyruvate	0.63	0.02	0.37
	Glucose 6-phosphate	None	1.60	1.13	—
		Sodium fluoride + sodium pyruvate	2.13	0.81	2.13
Adult liver	Fructose 1,6-diphosphate	None	1.48	1.47	—
		Sodium fluoride + sodium pyruvate	2.29	1.03	2.36
	Glucose	None	0.79	0.40	—
		Sodium fluoride + sodium pyruvate	1.31	0.11	0.94
	Glucose 6-phosphate	None	1.66	1.58	—
		Sodium fluoride + sodium pyruvate	2.47	0.71	2.14
	Fructose 1,6-diphosphate	None	1.36	2.59	—
		Sodium fluoride + sodium pyruvate	2.76	1.94	3.35

Table 2. *Glycolytic rates (measured as inorganic phosphate esterified) in supernatant fractions prepared from guinea-pig liver at various ages with saturating concentrations of glucose or glucose 6-phosphate as substrate*

The activities are expressed as μ moles of inorganic phosphate esterified/min. either per g. wet wt. of tissue or per 100 mg. of nitrogen, as indicated. Results are given as means $\pm$ S.D., with the numbers of determinations in parentheses. Final concentrations of substrates were: glucose, 100 mM; glucose 6-phosphate, 5 mM. Experimental details are given in the text. Adult animals were at least 4 months old.

Age of animals	Substrate	Inorganic phosphate uptake	
		(μ moles/min./g. wet wt.)	(μ moles/min./100 mg. of N)
Foetuses (gestational age 40–52 days)	Glucose	0.45 $\pm$ 0.10 (6)	1.68 $\pm$ 0.39 (6)
	Glucose 6-phosphate	2.70 $\pm$ 0.49 (6)	10.0 $\pm$ 1.6 (6)
Foetuses (gestational age 53–68 days)	Glucose	0.42 $\pm$ 0.11 (6)	1.56 $\pm$ 0.54 (6)
	Glucose 6-phosphate	3.71 $\pm$ 0.63 (5)	13.5 $\pm$ 3.6 (5)
Newborn (1–3 days)	Glucose	0.24 $\pm$ 0.02 (4)	0.94 $\pm$ 0.16 (4)
	Glucose 6-phosphate	3.11 $\pm$ 0.28 (3)	12.4 $\pm$ 0.2 (3)
Newborn (6 and 9 days) (9 days)	Glucose	0.91, 0.85 (2)	3.25, 2.74 (2)
	Glucose 6-phosphate	3.34 (1)	10.7 (1)
Adults	Glucose	1.01 $\pm$ 0.39 (8)	3.63 $\pm$ 1.15 (8)
	Glucose 6-phosphate	2.92 $\pm$ 0.74 (7)	10.5 $\pm$ 2.8 (7)

formation to glycerol 1-phosphate formation with glucose as substrate, and the lowest ratio with fructose 1,6-diphosphate as substrate; with glucose 6-phosphate as substrate an intermediate result was recorded.

Phosphorylation of glucose as a rate-limiting step in hepatic glycolysis. No significant changes in glycolysis during development were recorded when a saturating concentration (5 mM) of glucose 6-phosphate was used as substrate. This is shown in Table 2, which further indicates that, with 100 mM-glucose as substrate, the glycolytic rate was significantly higher in adult liver than in foetal liver, with the most abrupt increase occurring a few days after birth. The rates with glucose were always less than those with glucose 6-phosphate as substrate. The effects of glucose concentration on glycolysis are illustrated in Fig. 1. Measurement of both lactate formation and inorganic phosphate esterification indicated that a glucose concentration of 1 mM gave almost maximum rates with preparations of foetal liver tissue, whereas concentrations of about 100 mM-glucose were necessary to elicit maximum rates with preparations of adult liver. The transition from foetal to adult behaviour with respect to glucose concentration occurs within the first week after birth (Table 3).

Further evidence that the phosphorylation of glucose is a rate-limiting step in glycolysis was obtained by adding crystalline yeast hexokinase. The addition of 1 μ g. (equivalent to 0.14 unit) was found to be optimum, and resulted in the increase of the rates obtained with saturating concentrations of glucose as substrate to those with glucose 6-phosphate as substrate for preparations from foetal, neonatal and adult guinea-pig liver. (Higher concentrations of added hexokinase resulted in

Fig. 1. Effect of initial glucose concentration on lactate formation and inorganic phosphate esterification by supernatant fractions of guinea-pig liver. Each point represents the mean of duplicate determinations. Experimental details are given in the text. $\circ$, Lactate production by preparation from adult guinea-pig liver; $\bullet$, inorganic phosphate esterification by preparation from adult guinea-pig liver; Δ , lactate production by preparation from foetal guinea-pig liver (gestational age 50 days); $\blacktriangle$, inorganic phosphate esterification by preparation from foetal guinea-pig liver (gestational age 51 days).

lower glycolytic rates. This was probably due to excessive breakdown of ATP to ADP, which might make the ATP concentration limiting for phosphofructokinase.)

Table 3. *Influence of glucose concentration on glycolysis (measured as inorganic phosphate uptake and lactate production) in liver-supernatant preparations from neonatal guinea pigs*

The glucose concentrations recorded are those of added glucose. The activity in the incubations containing no added glucose is due to the endogenous glucose present in the supernatant preparation, and this small quantity is also present in all the other incubations. The incubation mixture contained sodium fluoride (10mm) and sodium pyruvate (5mm) in addition to the standard constituents. Other experimental details are given in the text.

Age of animal (post-natal)	Final concn. of glucose (mm)	Inorganic phosphate uptake (μ mole/min./g. wet wt.)	Lactate production (μ mole/min./g. wet wt.)
1 day	0	—	0.24
	1	—	0.24
	10	0.27	0.28
	50	—	—
	100	0.27	0.29
3 days	0	0.12	0.14
	1	0.12	0.14
	10	0.14	0.16
	50	0.20	0.22
	100	0.22	0.23
6 days	0	0.29	0.30
	1	0.28	0.33
	10	0.66	0.80
	50	0.88	0.93
	100	0.91	0.98
9 days	0	0.29	0.31
	1	0.29	0.36
	10	0.57	0.60
	50	0.82	0.98
	100	0.85	0.98

In contrast with these results with liver tissue and in agreement with the work of DiPietro, Sharma & Weinhouse (1962), who noted that glucose oxidation by rat-kidney homogenates does not possess the great dependence on substrate concentration, we found that supernatant preparations of kidney tissue from both foetal and adult guinea pigs gave as high a glycolytic rate (measured as inorganic phosphate esterification) with 10mm-glucose as with 100mm-glucose. The addition of an optimum amount of yeast hexokinase again increased the rate with glucose as substrate to that starting with glucose 6-phosphate.

Phosphofructokinase reaction as a second rate-determining step in hepatic glycolysis. Both inorganic phosphate uptake and lactate production of supernatant fractions of guinea-pig liver from animals of all ages were identical in the presence of saturating concentrations (5mm) of glucose 6-phosphate and fructose 6-phosphate, owing to the high phosphoglucose-isomerase activity (Lea & Walker, 1964a). For the measurement of the glycolytic rate with 5mm-fructose 1,6-diphosphate as substrate ADP had to be added to the incubation medium. Optimum activity with the substrate occurred in the presence of 2mm-ADP. (The addition of a lower concentration of ADP, up to

1mm, to incubation media in which glucose, glucose 6-phosphate or fructose 6-phosphate was substrate had only a minor enhancing effect because ADP is generated from ATP in one or two phosphotransferase reactions.) In all of 16 liver samples examined, the glycolytic rate was always a little higher with fructose 1,6-diphosphate as substrate than with glucose 6-phosphate, but again no trends with development were noted. These results suggested that the phosphorylation of fructose 6-phosphate was a rate-limiting step. This was confirmed by the direct assay of phosphofructokinase activities, which agreed remarkably well with the glycolytic rates with glucose 6-phosphate as substrate (Table 4). The results recorded for phosphofructokinase activities are twice the actual rates of fructose 6-phosphate phosphorylation (as μ moles of fructose 6-phosphate phosphorylated/min./g. wet wt.), owing to the method of enzyme assay; this permits direct comparison of the recorded results with the glycolytic rates, for in each case 2 moles of phosphate are involved/mole of hexose or hexose derivative metabolized. Expression of all these results as μ moles/min./100mg. of N gave essentially the same pattern.

Influence of cell-particulate fractions on glycolysis by the supernatant fraction of rat liver. This phase

Table 4. *Phosphofructokinase as a rate-limiting step in hepatic glycolysis at all stages of development*

Glycolysis was assayed in liver-supernatant preparations as inorganic phosphate uptake in the presence of ADP (2 mM), sodium fluoride (10 mM) and sodium pyruvate (5 mM). All other experimental details are given in the text. The six foetal guinea pigs were of gestational ages 42, 51, 52, 53, 54 and 58 days. The second group of six guinea pigs consisted of four adults and two newborn animals aged 1 and 5 days. Results are given as means $\pm$ S.D., with the numbers of determinations in parentheses. Final concentrations of substrates were 5 mM.

Substrate	Inorganic phosphate uptake (μ moles/min./g. wet wt.)	
	Foetuses	Newborn and adult animals
Glucose 6-phosphate	2.59 $\pm$ 0.69 (6)	2.86 $\pm$ 0.61 (6)
Fructose 1,6-diphosphate	3.27 $\pm$ 0.37 (6)	3.82 $\pm$ 0.82 (6)
Fructose 6-phosphate	Phosphofructokinase activity (μ moles of alkali-labile phosphate formed/min./g. wet wt.)	
	Foetuses	Newborn and adult animals
	2.66 $\pm$ 0.63 (6)	2.92 $\pm$ 0.70 (6)

Table 5. *Comparison of glycolytic rates (measured as lactate production) in whole homogenates and supernatant fractions prepared from rat liver at different ages*

The activities are all expressed/g. wet wt. Results are given as means $\pm$ S.D., with the numbers of determinations in parentheses. Experimental details are given in the text. Final concentrations of substrates were: glucose, 100 mM; glucose 6-phosphate, 5 mM. Adult animals weighed 200–300 g.

Age of animal	Tissue preparation	Lactate production (μ moles/min./g. wet wt.)		
		Substrate	Glucose	Glucose 6-phosphate
Foetuses (gestational age 18–19 days)	Whole homogenate		1.64 $\pm$ 0.17 (4)	2.53 $\pm$ 0.29 (3)
	Supernatant fraction		0.49 $\pm$ 0.20 (4)	0.97 $\pm$ 0.71 (3)
Foetuses (gestational age 20–21 days)	Whole homogenate		0.90 $\pm$ 0.19 (5)	3.07 $\pm$ 0.55 (5)
	Supernatant fraction		0.35 $\pm$ 0.19 (5)	0.89 $\pm$ 0.23 (5)
Adult rats	Whole homogenate		0.42 $\pm$ 0.18 (9)	1.52 $\pm$ 0.25 (8)
	Supernatant fraction		1.44 $\pm$ 0.34 (9)	1.50 $\pm$ 0.25 (8)

of the present study was performed with rat liver, and after first ascertaining that the above results obtained for the guinea pig were at least qualitatively applicable to the rat, especially with respect to the rate-limiting steps and the differences between foetal and adult tissue. With 100 mM-glucose as substrate, the supernatant fraction of adult rat liver gave a greater lactate production than did similar preparations of foetal rat liver, but the reverse was the case with whole homogenates (Table 5). With 5 mM-glucose 6-phosphate as substrate, homogenates of adult rat liver and supernatant fractions gave similar glycolytic activities but, under the same conditions, homogenates of foetal rat liver were more active than their supernatant fractions (Table 5).

These effects were clearly due to the action of the particulate material on the supernatant fraction, and they were further examined in experiments in which whole-particulate fractions, obtained by centrifuging whole homogenates at 100 000g for

45 min., were recombined with supernatant preparations in amounts equivalent to those present in the original whole homogenates. The addition of a particulate fraction to the supernatant from which it had been removed resulted in the restoration of the original activity of the whole homogenate within reasonable limits of accuracy (Table 6). The addition of particulate fractions from foetal rat liver to supernatant preparations of both adult and foetal rat liver resulted in the stimulation of lactate production, whereas particulate fractions of adult rat liver had inhibitory effects on supernatant fractions of both adult and foetal rat liver (Table 6). Rat-liver homogenates were fractionated by the method of Schneider & Hogeboom (1950) and further recombination experiments performed in an attempt to ascertain which particulate fractions were responsible for such effects. In view of the findings of Paigen & Wenner (1962), who reported that cell particles were able to adsorb lactate dehydrogenase from media of low ionic strength

Table 6. *Effect of particulate fractions of adult and foetal rat liver on glycolytic rates (measured as lactate production) of supernatant fractions of adult and foetal liver*

The substrate was glucose (100 mM). Results are given as means $\pm$ S.D., with the numbers of determinations in parentheses. Experimental details are given in the text. Adult animals weighed 200–300 g.

Source of whole homogenate or supernatant fraction	Lactate production (μ moles/min./g. wet wt.)			
	Whole homogenate	Supernatant fraction	Supernatant fraction + adult particulate fraction	Supernatant fraction + foetal particulate fraction
Foetuses (gestational age 18–22 days)	1.10 $\pm$ 0.27 (5)	0.29 $\pm$ 0.09 (5)	0.23 $\pm$ 0.13 (5)	1.06 $\pm$ 0.25 (5)
Adult rats	0.35 $\pm$ 0.09 (6)	1.20 $\pm$ 0.28 (6)	0.43 $\pm$ 0.10 (6)	1.80 $\pm$ 0.36 (6)

Table 7. *Lactate production by whole homogenates and supernatant fractions of rat livers and the influence of the particulate fractions on the activity of the supernatant fractions*

The homogenates were prepared in 0.25 M-sucrose in all experiments except Expt. 2, in which the homogenizing medium was 0.15 M-KCl containing 8 ml. of 0.02 M-KHCO₃/l. Experimental details are given in the text. H, Whole homogenate; S, supernatant fraction; N, nuclear fraction; M, mitochondrial fraction; Mic, microsomal fraction.

Expt. no.	Age of animals	Lactate formed (μ moles/min./g. wet wt.)								
		H	S	N	M	Mic	S+N	S+M	S+ Mic	S+N+ M+Mic
1	Foetuses (gestational age 20 days)	0.93	0.51	0.03	0.01	0.07	0.94	0.50	0.62	0.93
2	Foetuses (gestational age 21 days)	0.99	0.50	0.04	0.03	0.04	1.01	0.68	0.56	0.97
3	Foetuses (gestational age 22 days)	0.71	0.52	0.05	0.05	0.04	0.75	0.59	0.65	0.72
4	Newborn rats (7 days)	0.20	0.55	0.03	0.01	0.03	0.51	0.45	0.26	0.26
5	Adult	0.55	1.04	0.02	0.02	0.06	1.07	1.06	0.49	0.52
6	Adult	0.40	0.80	0.03	0.03	0.04	0.68	0.67	0.38	0.36

and release it again on the addition of salt, and because all our previous studies had been performed with homogenates made in the potassium chloride homogenizing medium, a few fractionations were also made on homogenates prepared in the 0.15M-potassium chloride (containing 8ml. of 0.02M-potassium hydrogen carbonate/l.) medium, and these gave similar results to fractionations made in the standard 0.25M-sucrose medium of Schneider & Hogeboom (1950). Table 7 presents the results of typical experiments, from which it appears that the stimulating effect of the particulate fraction of foetal rat liver was due mainly to the nuclear fraction, whereas the inhibitory effect of the particulate fraction of adult rat liver on glycolysis was due to the microsomal fraction. The effects were further examined as follows.

Effect of microsomal fraction on glycolysis. In a typical experiment, the microsomal fractions from homogenates of foetal liver (gestational age 19 days) and adult liver were combined with the corresponding supernatant fractions or vice versa. With 100mM-glucose as substrate the microsomal fraction of adult rat liver decreased the lactate production by the supernatant fractions from foetal and adult livers by 23 and 32% respectively. No such

inhibition was observed with 5mM-glucose 6-phosphate as substrate with the same recombinations. This suggests that the site of the inhibitory effect lies around the interconversion of glucose and glucose 6-phosphate. The microsomal fraction of foetal rat liver showed no inhibitory effect when recombined with supernatant preparations of either adult or foetal rat livers with glucose or glucose 6-phosphate as substrate. Other experiments on the effects of microsomal fractions from livers of animals of several ages showed that the appearance of the inhibitory effect of the microsomal fraction coincided with the rapid increase in glucose 6-phosphatase activity around parturition (Burch *et al.* 1963).

Effect of nuclear fraction. The nuclear fraction responsible for the stimulating effects noted above contained numerous erythrocytes. In attempts to prepare a less-contaminated nuclear fraction the method of Hogeboom, Schneider & Striebig (1952) was employed, but it was found that, though lowering of the centrifugal force to 300g enabled nuclei of adult rat liver to be prepared almost free from erythrocytes as revealed by phase-contrast microscopy, nuclear preparations of foetal rat liver still contained many nucleated foetal erythrocytes. The

following experiments were therefore performed with twice-washed nuclear preparations obtained with a centrifugal force of 600g for 10 min. and with 0.15M-potassium chloride (containing 8 ml. of 0.02M-potassium hydrogen carbonate/l.) as homogenizing and washing medium.

The addition of nuclear preparations of adult rat liver did, in a number of individual experiments, give rise to a small stimulation of glycolysis by the supernatant fraction with glucose as substrate. This is illustrated in Expt. 1 of Table 8, but such stimulation was not always observed. The stimulatory effect on lactate production was more marked and was always obtained with glucose 6-phosphate or fructose 1,6-diphosphate as substrate. At the same time there was an increase in the ratio of lactate formation to glycerol 1-phosphate formation, but without a decrease in the absolute amount of glycerol 1-phosphate produced (Table 8). Such effects were always observed with all three substrates when nuclear preparations of foetal rat liver were examined (e.g. Expt. 2 of Table 8).

Jones, Norris & Landon (1963), using rat-kidney preparations, and Wenner & Cereijo-Santalo (1963), using mouse liver, showed that the formation of glycolytic end products was affected markedly by

ADP or ADP-generating systems acting at the 3-phosphoglycerate-kinase (EC 2.7.2.3) stage. Thus they demonstrated that the addition of such factors as arsenate, adenosine-triphosphatase activity or an ADP-generating system (consisting of ATP, 2-deoxyglucose and hexokinase) accelerated lactate formation by supernatant preparations, especially with fructose 1,6-diphosphate as substrate, and that such stimulation occurred even in the presence of an optimum concentration of ADP. The following observations suggest that the supernatant fraction of rat liver is also susceptible to this type of effect.

In a typical experiment, the presence of 5 mM-2-deoxyglucose and an optimum concentration (0.10 unit) of crystalline yeast hexokinase to the supernatant fraction of foetal rat liver (gestational age 22 days) increased lactate production from 5 mM-glucose 6-phosphate as substrate from 1.25 to 1.70 μ moles/min./g. wet wt. of tissue. The addition of the nuclear fraction alone to the supernatant fraction increased the lactate production to 1.87 μ moles/min./g. wet wt., and the further addition of 2-deoxyglucose and yeast hexokinase as before increased this by only a small amount to 1.95 μ moles/min./g. wet wt. In control incubations

Table 8. *Effect of nuclear fractions on lactate and glycerol 1-phosphate production by supernatant fractions of adult and foetal rat liver*

In these experiments the supernatant and nuclear fractions of adult rat liver were prepared from an adult rat weighing 300g., and the supernatant and nuclear fractions of foetal rat liver from pooled livers from a litter of gestational age 20 days. Experimental details are given in the text. Final concentrations of substrates were: glucose, 100 mM; glucose 6-phosphate, 5 mM; fructose 1,6-diphosphate, 5 mM. The incubation mixtures contained ADP (2 mM) in addition to the standard constituents.

Expt. no.	Source of supernatant and nuclear fractions	System assayed	Substrate	Lactate formed (μ moles/min./g. wet wt.)	Glycerol 1-phosphate formed (μ moles/min./g. wet wt.)
1	Adult rats	Supernatant fraction	Glucose	0.75	0.26
			Glucose 6-phosphate	1.71	0.60
			Fructose 1,6-diphosphate	0.98	2.99
			Glucose	1.02	0.42
		Supernatant fraction + nuclear fraction	Glucose 6-phosphate	2.01	0.83
			Fructose 1,6-diphosphate	1.90	3.39
			Glucose	0.42	0.13
			Glucose 6-phosphate	1.35	0.61
2	Foetuses	Supernatant fraction	Fructose 1,6-diphosphate	0.91	1.84
			Glucose	0.88	0.15
			Glucose 6-phosphate	2.04	0.81
			Fructose 1,6-diphosphate	1.73	2.02
		Supernatant fraction + nuclear fraction	Glucose	0.88	0.15
			Glucose 6-phosphate	2.04	0.81
			Fructose 1,6-diphosphate	1.73	2.02
			Glucose	0.88	0.15

Table 9. *Influence of arsenate and pyruvate on the stimulating effect of nuclear fractions of foetal rat liver on lactate production by supernatant fractions of foetal rat liver*

Expt. no.		Gestational age of foetuses (litter mates) used for preparation of liver supernatant and nuclear fractions	Substrate	System assayed		Lactate production (μ moles/min./g. wet wt.)									
						Final concn. of sodium pyruvate (mM)	...	0	0.5	0	5	0	5	5	5
1	18	Glucose 6-phosphate	Supernatant fraction	Supernatant fraction + nuclear fraction	0.89	0.95	0.89	0.92	1.38	1.25	1.33	1.38	1.33	1.25	1.33
2	22	Glucose 6-phosphate	Supernatant fraction	Supernatant fraction + nuclear fraction	1.61	1.69	1.61	1.59	1.97	1.67	1.87	1.97	1.87	1.67	1.87
3	18	Fructose 1,6-diphosphate	Supernatant fraction	Supernatant fraction + nuclear fraction	1.50	2.09	1.50	1.77	2.64	1.94	2.20	2.64	2.20	1.94	2.20
4	21	Fructose 1,6-diphosphate	Supernatant fraction	Supernatant fraction + nuclear fraction	2.47	2.61	2.47	2.24	2.95	2.35	2.74	2.95	2.74	2.35	2.74
			Supernatant fraction	Supernatant fraction + nuclear fraction	1.31	1.83	1.31	2.10	4.03	4.15	4.03	4.03	4.03	4.15	4.15
			Supernatant fraction	Supernatant fraction + nuclear fraction	1.91	2.21	1.91	2.41	4.13	4.75	4.60	4.13	4.60	4.75	4.75
			Supernatant fraction	Supernatant fraction + nuclear fraction	1.67	2.79	1.67	3.20	5.38	5.78	5.78	5.38	5.78	5.78	5.78
			Supernatant fraction	Supernatant fraction + nuclear fraction	2.56	3.51	2.56	3.76	5.78	5.98	5.82	5.78	5.82	5.98	5.98

In each separate experiment, the effect of the nuclear fraction on the supernatant from the same pooled liver specimen was examined. Experimental details are given in the text. Final concentrations of substrate were 5 mM.

neither 2-deoxyglucose nor hexokinase alone had any effect on lactate production. Low concentrations (less than 0.5 mM) of sodium arsenate caused no stimulation of lactate production from glucose by the supernatant fraction of foetal rat liver, and concentrations of sodium arsenate above 1 mM caused inhibition. The addition of arsenate to the same supernatant fractions with glucose 6-phosphate or fructose 1,6-diphosphate as substrate stimulated lactate production (Table 9). This stimulation, and also the stimulating effect of the nuclear fraction, was much less in the presence of 5 mM-sodium pyruvate, the addition of which will permit reoxidation of NADH and thus by-pass a rate-controlling step in the region of 3-phosphoglycerate kinase in the glycolytic sequence of reactions.

Direct estimations of adenosine-triphosphatase activity (Table 10) showed that, as with homogenates of adult rat liver (Bonting, Caravaggio & Hawkins, 1962), about half of the total activity of the whole homogenate occurred in the nuclear fraction. These results point to nuclear adenosine triphosphatase as being the stimulating factor. However, it is not the formation of ADP that is important, because control experiments showed that ADP at concentrations of 2 mM or more gave optimum glycolytic rates with the cell soluble fraction starting with any of the substrates. Further, at any concentration of ADP in the range 2–5 mM, stimulation of supernatant glycolysis by the nuclear fraction with fructose 1,6-diphosphate as substrate was always observed. In other experiments, the addition of a meromyosin preparation (see the Materials and Methods section) resulted in a similar stimulation of glycolysis, again in the presence of a range (2–5 mM) of ADP concentrations.

DISCUSSION

The determination of the maximum activity *in vitro* of an enzyme in a tissue preparation may not give a true indication of the actual activity *in vivo* for a variety of reasons. Nevertheless, it is the first stage in assessing the metabolic role of an enzyme in a known reaction sequence, and probably has a greater significance when the activity is low compared with other enzymes with that sequence. Previous studies (Lea & Walker, 1964a; Dawkins, 1963; Kornfeld & Brown, 1963) together revealed that the activities of enzymes involved in the metabolism of glucose 6-phosphate and glycogen were all higher than the minimum required to permit the changes in glycogen concentrations known to occur around birth in many species (Shelley, 1961; Kornfeld & Brown, 1963). The present study reveals two stages in the transformation of glucose into lactate that are limiting on the

Table 10. *Adenosine-triphosphatase activity of whole homogenates and nuclear fractions of foetal and adult rat livers*

The activities are expressed as μ moles of substrate metabolized/min. either per g. wet wt. of tissue or 100 mg. of nitrogen, as indicated. Results are given as means $\pm$ S.D., with the numbers of determinations in parentheses. Experimental details are given in the text. Adult rats weighed 200–300 g.

Age	Tissue preparation	Adenosine-triphosphatase activity	
		(μ moles/min./g. wet wt.)	(μ moles/min./100 mg. of N)
Foetuses (gestational age 17–22 days)	Whole homogenate	3.22 ± 0.27 (2)	16.8 ± 1.1 (2)
	Nuclear fraction	1.77 ± 0.33 (5)	9.0 ± 1.3 (5)
Adult rats	Whole homogenate	5.80 ± 0.01 (2)	20.4 ± 2.4 (2)
	Nuclear fraction	2.69 ± 0.30 (5)	9.0 ± 1.1 (5)

whole process of glycolysis. The phosphorylation of glucose by the two enzymes available for this purpose in liver tissue (Walker, 1963b) represents the first rate-limiting stage of glucose utilization, for cell-wall permeability in liver tissue is not limiting (Cahill, Ashmore, Earle & Zottu, 1958). The dependence of the glycolytic rate on glucose concentration is consistent with the kinetic characteristics of the phosphorylating system available during development (Walker, 1963b). Foetal hepatic glycolysis will be at maximum rate at the glucose concentrations normally present in liver *in vivo*. Even when the maximum rate of glucose phosphorylation is operating in adult liver (and the glucose concentration never reaches 100 mM, so that glucokinase probably never operates at maximum rate), this process still represents a limiting stage in glycolysis, as in other aspects of glucose metabolism by the liver (Niemeyer, González & Figueroa, 1956; DiPietro *et al.* 1962; Fain & Wilhelmi, 1962).

Fructose 6-phosphate may arise via glucose phosphorylation or the breakdown of glycogen. The control of phosphofructokinase activity has been the subject of many recent studies, and the liver enzyme appears to be no exception to that from other sources in being affected by the concentrations of substrate, product, ATP, ADP and other metabolites (Passonneau & Lowry, 1964). The conditions for phosphofructokinase assay in the present study have been uniform throughout, so that changes or lack of changes in activity are of significance, and the results indicate that the phosphorylation of fructose 6-phosphate is another rate-limiting step in lactate formation at all stages of hepatic development.

The appearance of glucose 6-phosphatase around birth in both rat and guinea-pig liver is well documented (for references see Lea & Walker, 1964a), and it is this change in the microsomes that must account for the fact that the liver microsomal fraction from adult, but not foetal, animals inhibits glycolysis with glucose as substrate but not with

5 mM-glucose 6-phosphate as substrate. In the latter case there is sufficient substrate present to maintain glycolysis at maximal rate in spite of some of the glucose 6-phosphate being hydrolysed by the specific phosphatase. The results indicate how *in vivo*, at much lower glucose 6-phosphate concentrations, changes in glucose 6-phosphatase activity can affect other metabolic pathways leading from this key intermediate compound. They may also explain the lower rates of anaerobic glycolysis obtained with slices of liver from adult animals compared with those from foetal animals, and why other workers (Dawkins, 1959; Oliver *et al.* 1962), who used fructose 1,6-diphosphate as a substrate for measuring glycolysis by whole homogenates of rat livers, observed no change in lactate formation during development. Under such conditions the effect of glucose 6-phosphatase development would not be apparent.

The conditions used in our experiments permitted no appreciable effect of the mitochondrial fraction on the glycolytic rate of the supernatant fraction. This is in contrast with many other studies (Schneider, Graffi, Bielka & Venker, 1957; Sisakian & Pinus, 1958; Aisenberg, 1959; Potter & Niemeyer, 1959; Cremer, 1960; Schwartz & Lee, 1960; Pinus & Sisakian, 1961; Fain & Wilhelmi, 1962), and is probably due to the concentrations of cofactors used in our assay system. In particular, our concentrations of ADP and inorganic phosphate are not in any way limiting.

The interaction of the nuclear fraction with the glycolytic enzymes has received little attention previously. LePage & Schneider (1948) observed a stimulating effect with fractions prepared from homogenates of adult rabbit liver. The present results indicate that the nuclear fraction has an effect throughout the development of rat liver in the presence of an adequate concentration of ADP such that more ADP gave no further effect. Several systems that lower the ATP/ADP ratio caused a stimulation analogous to that by the nuclear fraction: (i) the addition of arsenate, which interacts

during the oxidation of 3-phosphoglyceraldehyde to prevent ATP formation; (ii) the addition of 2-deoxyglucose plus hexokinase; (iii) the addition of an adenosine triphosphatase (meromyosin). The nuclear fraction possesses adenosine-triphosphatase activity, and the addition of pyruvate lowered the stimulating effect of this fraction, as it did that of the other ADP-forming systems. These results are in many ways similar to those observed by Jones *et al.* (1963) for the effect of the endoplasmic reticulum of kidney tissue on glycolysis by kidney supernatant preparations. Jones *et al.* (1963) demonstrated a direct action of adenosine triphosphatase on the reaction catalysed by a purified preparation of 3-phosphoglycerate kinase. The present effect of the liver nuclear fraction may well occur at the same site; such a mechanism is consistent with all the findings, and explains why it is not the ADP concentration that affects the stimulation. An apparent difference in the distribution of adenosine-triphosphatase activity in the particulate fractions of homogenates of kidney and liver tissue is indicated on comparing the results of Jones *et al.* (1963) with those in the present paper. Most of the experiments of Jones *et al.* (1963), including the demonstration of the stimulatory effect of the microsomal fraction, were performed with fructose 1,6-diphosphate as substrate. Many of our experiments on the effect of the nuclear fraction (which, unlike the microsomal fraction, does not carry glucose 6-phosphatase activity) employed glucose as substrate. By acting at an earlier point in the glycolytic sequence of reactions, the inhibitory effect of glucose 6-phosphatase activity may mask a stimulatory effect of adenosine triphosphatase with glucose as substrate.

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