The Existence of a Glyconeogenic Pathway in Rat Skin

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The existence of a glyconeogenic pathway in rat skin has been demonstrated by measurement of three of the key glyconeogenic enzymes, fructose 1,6-bisphosphatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, and by studies on the incorporation *in vitro* of carbon from pyruvate and alanine into skin glycogen.

The ability of liver and kidney to convert C_3 compounds, such as lactate, pyruvate and certain amino acids into glycogen is well established (Exton, 1972; Scrutton & Utter, 1968). In skin, however, demonstration of the possible existence of this glyconeogenic pathway has been much neglected and is generally dismissed as unlikely (Johnson & Fusaro, 1973), even though as long ago as 1948 Wöhnlich reported that cattle and human skin could synthesize glycogen from lactate *in vitro* (Wöhnlich, 1948, 1949).

Materials and Methods

Tissues were dissected from rats (white Wistar, CFHB strain, 200–230 g; Anglia Laboratory Animals, Huntingdon, U.K.) after anaesthesia with sodium pentobarbitone (Nembutal; Abbott Laboratories, Kent, U.K.). After shaving of the ventral surface of the animals, skin and liver samples were removed together with the vastus lateralis muscle.

Tissues for enzyme assays were homogenized in the appropriate medium at 4°C by using a Polytron homogenizer (type PT10; Northern Media Supply, Hull, U.K.) at maximum speed for four periods of 30 s each, allowing the cutter to cool between the homogenization periods. A supernatant was prepared by centrifugation of the homogenate at 26000g for 15 min.

Enzyme assays

Fructose 1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) was measured after homogenization of the tissue in 0.15 M-KCl and subsequent dialysis of the 26000g supernatant against 0.5 M-KCl (Wimshurst & Manchester, 1970; Taketa & Pogell, 1965). The reduction of $NADP^+$ (0.2 mM) was followed spectrophotometrically at 37°C in the presence of fructose 1,6-bisphosphate (0.05 mM), phosphoglucose isomerase (EC 5.3.1.9) (7 units), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (3.5 units) (Boehringer Corp., London W.5, U.K.), mercaptoethanol (20 mM), $MgSO_4$ (5 mM)

and EDTA (0.05 mM) in a total volume of 3.11 ml at pH 7.5.

Pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP), E.C. 6.4.1.1.] was measured by using the method of Wimshurst & Manchester (1970).

Phosphoenolpyruvate carboxykinase [orthophosphate-oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] was measured by the method of Ballard & Hanson (1967).

In both these assays excess of labelled CO_2 was removed from the residual acidic solutions by drying them under vacuum over P_2O_5 and KOH.

Protein in the supernatant was measured by using the method of Lowry *et al.* (1951).

Incorporation studies *in vitro*

For the measurement of the incorporation of C_3 compounds into glycogen and its precursors, skin pieces were incubated in Krebs-Ringer bicarbonate buffer, pH 7.2 (Krebs & Henseleit, 1932), containing penicillin (50 units/ml) and streptomycin (50 µg/ml) in an atmosphere of $O_2 + CO_2$ (95:5) at 37°C in the presence of either [$U-^{14}C$]pyruvate (The Radiochemical Centre, Amersham, Bucks., U.K.) (5 mM) and malonate (9 mM) or [$U-^{14}C$]alanine (5 mM), malonate (9 mM) and 2-oxoglutarate (2 mM). Labelled glucose 6-phosphate and UDP-glucose were isolated from skin pieces, frozen in liquid N_2 after incubation, by methods based on the work of Das *et al.* (1971) and Hardingham & Phelps (1968). Powdered skin, prepared by pulverization in a liquid- N_2 mill (Spex Freezer Mill; Glen Greston, Middlesex, U.K.), was extracted with ice-cold $HClO_4$ (Hems & Brosnan, 1970) and, after neutralization, the solution was applied to a column (11 cm × 1.4 cm) of Dowex 1 (formate form) anion-exchange resin (Hurlbert *et al.*, 1954). The column was eluted with a formic acid/ammonium formate gradient. The stationary vessel contained 4.5 M-formic acid (350 ml) and the mixing vessel contained water (350 ml). The mixture was pumped on to the column at a rate of 46 ml/h for 7 h, after which the stationary vessel was filled with 0.5 M-

ammonium formate/4M-formic acid (450ml) and the mixing vessel with 4M-formic acid (450ml). This mixture was pumped on to the column at the same rate for a further 17h. Glucose 6-phosphate and UDP-glucose were isolated from the collected fractions after removal of NH_4^+ ions and formic acid; they were then hydrolysed to release free glucose. The hydrolysis of glucose 6-phosphate was carried out by using alkaline phosphatase (EC 3.1.3.1) (Sigma Chemical Co., London S.W.6, U.K.) at pH9, and of UDP-glucose by heating at 100°C in 0.1 M-HCl for 30min. Glucose was isolated by paper chromatography (Albon & Gross, 1950) and elution of the glucose-containing areas with water. The specific radioactivities of these extracts were calculated by counting the radioactivity in Bray's (1960) scintillator fluid and measuring the glucose by following spectrophotometrically the reduction of NADP^+ (0.4mm) at 25°C in the presence of glucose 6-phosphate dehydrogenase (3.5 units), hexokinase (EC 2.7.1.1) (2.8 units) (Boehringer), ATP (0.32mm), MgCl_2 (12mm) and Tris buffer, pH7.4 (60mm).

Radio-labelled glycogen was isolated from powdered skin as follows. The skin (1 g) was extracted with 30 % (w/v) KOH (5ml) at 100°C. The extract was centrifuged, and glycogen was precipitated from the supernatant (4.5ml) with ethanol (6ml), by using Na_2SO_4 (0.5ml of a satd. soln.) as a precipitation aid. The precipitate was redissolved in water (4.5ml) and precipitated with ethanol (6ml). This precipitate was dissolved in water, dialysed against deionized water, and the non-diffusible material was freeze-dried to obtain the glycogen. The specific radioactivities of glucose moieties of glycogen were determined after hydrolysis of the glycogen by using either 1 M-HCl at 100°C for 2.5h and isolation of the glucose by chromatography, as already described, or by using amyloglucosidase (EC 3.2.1.3) (1 mg/ml) (Sigma) at pH4.8 and 50°C for 1h, followed by radioactivity counting and glucose measurement in the supernatant after centrifugation.

Glucose *p*-nitroanilide derivatives were prepared by the method of Weygand *et al.* (1951).

Results and Discussion

The synthesis of glycogen from pyruvate and alanine by reversal of the glycolytic pathway (glyconeogenesis) requires the by-pass of two irreversible glycolytic steps, catalysed by phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40). The reverse reactions are controlled by fructose 1,6-bisphosphatase and pyruvate carboxylase plus phosphoenolpyruvate carboxykinase respectively. The activities of these enzymes have been measured in skin, and compared with those found in liver and in skeletal muscle, which is considered to be a non-glyconeogenic tissue owing to the absence of significant activities of pyruvate carboxylase (Scrutton & Utter, 1968; Opie & Newsholme, 1967). Enzyme activities have been expressed on the basis of wet weight of tissue and on the protein content of the supernatant used for assay (Table 1).

The results show that, although the activities of all three enzymes in skin are approx. 20 times lower than in liver on a wet-weight basis, when based on protein content they are only approx. four times lower. It can also be seen that the activity of phosphoenolpyruvate carboxykinase in skin is four times that in muscle based on wet weight, and seven times on a protein basis, whereas the activity of pyruvate carboxylase is 30 times that in muscle based on tissue weight, and 50 times when based on protein. Fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase activities have already been demonstrated in human epidermis by Halprin & Ohkawara (1966), although no experimental procedures were described.

The individual presence of these enzymes in skin does not necessarily indicate functional glyconeogenesis, as they can fulfil other roles unconnected with this pathway. In skeletal muscle, for instance, it is

Table 1. *Comparison of the activities of the key glyconeogenic enzymes in skin, liver and muscle*

Results are expressed as the mean of each group $\pm$ S.E.M. The number of animals in each group is shown in parentheses. Enzyme assay methods are described in the Materials and Methods section.

Enzyme	Tissue		
	Skin	Liver	Muscle
Phosphoenolpyruvate carboxykinase			
Units/g wet wt.	0.208 $\pm$ 0.007 (8)	4.24 $\pm$ 0.612 (4)	0.051 $\pm$ 0.004 (5)
Units/g of protein	8.97	38.5	1.3
Pyruvate carboxylase			
Units/g wet wt.	0.304 $\pm$ 0.041 (6)	5.10 $\pm$ 1.26 (4)	0.011 $\pm$ 0.0015 (4)
Units/g of protein	13.1	46.4	0.28
Fructose 1,6-bisphosphatase			
Units/g wet wt.	0.423 $\pm$ 0.030 (4)	7.57 $\pm$ 0.498 (4)	0.41 $\pm$ 0.018 (4)
Units/g of protein	18.2	68.8	10.56

Fig. 1. Incorporation *in vitro* of [U-¹⁴C]pyruvate (●) and [U-¹⁴C]alanine (○) into skin glycogen

The methods used are described in the Materials and Methods section.

thought that fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase decrease the concentrations of accumulated intermediates of the α -glycerophosphate-dihydroxyacetone phosphate and the malate-oxaloacetate cycles (Opie & News-holme, 1967). Similarly, in adipose tissue, pyruvate carboxylase and phosphoenolpyruvate carboxykinase are thought to be involved in lipogenesis and the formation of α -glycerophosphate by 'glyceroneogenesis' (Hanson *et al.*, 1971). However, in both these tissues, synthesis of glycogen from pyruvate is precluded, owing to a lack of one of the key enzymes: in muscle a lack of pyruvate carboxylase and in adipose tissue a lack of fructose 1,6-bisphosphatase. In skin therefore, where all three enzymes are present at similar activities, it is likely that different mechanisms are in operation and pyruvate carboxylase and phosphoenolpyruvate carboxykinase operate in harness to convert pyruvate, via oxaloacetate, into phosphoenolpyruvate, which is then available for glycogen synthesis via fructose 1,6-bisphosphate and fructose 1,6-bisphosphatase.

Confirmation of the glycogenic potential of skin was provided by work on the incorporation *in vitro* of [U-¹⁴C]pyruvate into glycogen, and the precursors of glycogen synthesis, glucose 6-phosphate and UDP-glucose. Incubation of skin pieces with [U-¹⁴C]pyruvate in the presence of malonate, to block the tricarboxylic acid cycle, showed that after 60 min of incubation 17.7 pmol of pyruvate was incorporated per nmol of glucose in glucose 6-phosphate, 15.8 pmol was incorporated per nmol of glucose in UDP-glucose and 0.47 pmol was incorporated per nmol of glucose in glycogen. The possibility of radioactive contamination of the recovered glucose by

other substances was ruled out by isotope dilution by using the glucose *p*-nitroanilide derivative. These results show that there is a positive incorporation of pyruvate into glycogen via glucose 6-phosphate and UDP-glucose, although the rate of incorporation appears to be very low.

Similar incubations were carried out to measure the rate of incorporation of both [U-¹⁴C]pyruvate and [U-¹⁴C]alanine into skin glycogen during the 60 min period (Fig. 1). Uptake of [U-¹⁴C]pyruvate was measured in the presence of malonate as before, and uptake of [U-¹⁴C]alanine in the presence of malonate and of 2-oxoglutarate. The addition of 2-oxoglutarate, which is required for the transamination of alanine to pyruvate, replaced the normal production of 2-oxoglutarate via the tricarboxylic acid cycle, which was blocked by malonate. In both experiments linearity of incorporation was reached approx. 30 min after the start of the incubation. The rate of incorporation of alanine into glycogen was about 30% of the rate of incorporation of pyruvate. However, as a large proportion of the alanine taken up by the tissue will be preferentially incorporated into protein, the availability of alanine for glycogenesis will be considerably lower than that of pyruvate. Incubation in the presence of cycloheximide (5 mM), to block the protein-synthetic route, slowed the incorporation rate even further. It would seem therefore that glycogen synthesis via this route is inhibited in some way by cycloheximide. Data have been presented on the interference of cycloheximide and other protein-synthesis inhibitors in glycogen synthesis (Vremsen, 1970), although information on this effect seems to be confined to the state *in vivo*. Inhibition of mucopolysaccharide synthesis from glucose by puromycin has also been demonstrated (de la Haba & Holtzer, 1965).

These results demonstrate therefore that a pathway exists in rat skin for the synthesis of glycogen from C₃ compounds. Although it appears to be somewhat limited when compared with recognized glycogenic tissues such as liver, comparison of the total mass of skin and liver in the rat (Crandall & Drabkin, 1946) suggests that in the whole body the relative contribution of skin to glycogenesis could be considerable.

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