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Glycogen Accumulation in Cultured Skin Fibroblasts

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The use of skin fibroblasts in the study of inborn errors of metabolism is now a well-established technique (Hsia, 1970); coupled with histological and chemical investigations, it has provided an additional insight into a number of lysosomal storage disorders (Matalon & Dorfman, 1969). Hitherto, however, application of such methods to the glycogen-storage diseases has been limited to enzyme assays (Dancis *et al.*, 1969). Hug *et al.* (1971) were able to demonstrate a Best's Carmine-positive substance in fibroblasts from a case with α -glucosidase deficiency (type II glycogenosis). Other workers (Nitowsky & Grunfeld, 1967) have been unable to demonstrate glycogen accumulation in fibroblasts from cases of glycogen-storage disease. Recently it has proved possible to promote glycogen accumulation in skin fibroblasts by blocking the lysosomal α -glucosidase with the disaccharide trehalose (Brown & Brown, 1972).

Using a histochemical technique developed in this laboratory (Lake, 1970), we have been able to demonstrate glycogen accumulation in skin fibroblasts and to show the dependence of such accumulation on the glucose concentration of the culture medium of the cells.

Skin fibroblasts were obtained by standard procedures (Paul, 1970) and maintained on medium 199 supplemented with foetal calf serum (20%, v/v). Glycogen accumulation was noted in normal skin fibroblasts within 24 h of renewal of the culture medium. As the glucose concentration of the culture medium became exhausted (2–3 days in our experiments), so the glycogen content of the fibroblasts diminished. Virtually complete glycogen depletion of normal skin fibroblasts was noted within 24 h of cells, initially replete glycogen, being transferred to medium 199 without glucose (with or without supplementation with foetal calf serum). Glycogen reaccumulated when such cells were returned to the normal medium containing glucose,

Skin fibroblasts were grown from a case of glycogen-storage disease of unknown type (liver glycogen content 8.5%, w/w). The depletion of glycogen from these fibroblasts maintained in glucose-free medium was much slower than in control cultures maintained similarly. Some glycogen was still evident after 48 h under these conditions.

From our experiments we conclude that glycogen accumulation is a normal function of skin fibroblasts; such accumulation may to some extent cushion these cells against the effects of glucose deprivation. Such histochemical observations may serve to complement other techniques used in the study of glycogen-storage diseases, and the fact that, in cell cultures, glycogen metabolism bears an apparently simple relationship to glucose availability may be of value in studying the control of such metabolism and pathological situations.

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Activation and Phosphorylation of the Subunits of Phosphorylase Kinase

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Phosphorylase kinase is a key enzyme in the nervous and hormonal regulation of glycogenolysis in skeletal muscle. Its activity is totally dependent on Ca^{2+} (Heilmeyer *et al.*, 1970; Brostrom *et al.*, 1971) released from the sarcoplasmic reticulum after electrical excitation, and it is also linked to adrenaline via adenylate cyclase and cyclic-AMP (adenosine 3':5'-cyclic monophosphate)-dependent protein kinase, the latter enhancing the activity a further 20–30-fold at physiological pH (Walsh *et al.*, 1971).

Purified rabbit muscle phosphorylase kinase was homogeneous by sedimentation velocity (22.4S) but showed four bands staining for protein on gel electrophoresis in sodium dodecyl sulphate. Proteolytic enzyme inhibitors added during the preparation, or denaturation of the final product in various ways designed to eliminate proteolysis and ensure complete reduction and denaturation, did not alter the gel pattern. The subunit molecular weights were: α , 132000;

α' , 130000; β , 118000; γ , 41000. The α' component, although always present, stained faintly and is tentatively assumed to be derived from the α component. Molar proportions obtained from densitometric tracings of the gels gave: ($\alpha + \alpha'$), 0.83; β , 1.00; γ , 1.06; these suggest a minimal binding weight ($\alpha\beta\gamma$) of 290000 daltons. The absorbance index, $E_{280}^{1\%}$, determined refractometrically was 12.1 ± 0.2 .

The pH 6.8/8.2 activity ratio of phosphorylase kinase rose from 0.015 to 0.38 on incubation with cyclic AMP (0.01 mM), purified cyclic-AMP-dependent protein kinase, Mg^{2+} (2 mM), [γ - ^{32}P]ATP (0.2 mM), ethanedioxybis(ethylamine)tetra-acetate (0.2 mM), EDTA (0.4 mM) and glycerophosphate (10 mM), pH 6.8. Activity and ^{32}P incorporation both reached maximum values within 20 min, but did not exactly parallel one another. It was found that 1.82 ± 0.1 mol of phosphate was incorporated/290000 daltons. Both the lack of correspondence between phosphorylation and activation and the phosphate stoichiometry disagreed with the results reported by Walsh *et al.* (1971) (0.42 mol of phosphate incorporated/100000 daltons under essentially identical experimental conditions).

Examination of the phosphorylation of each sub-unit showed that the first phosphate entered the β component, paralleling the activity increase, whereas the second phosphate entered the ($\alpha + \alpha'$) component ten times more slowly. At 1 min activation was 55%, the β component was 58% phosphorylated but the ($\alpha + \alpha'$) component was only 6% phosphorylated. After 20 min the ($\alpha + \alpha'$) and β components contained equal amounts of phosphate. No radioactivity appeared in the γ component. Phosphorylation of the ($\alpha + \alpha'$) component depended on either cyclic-AMP-dependent protein kinase or phosphorylation of the β component. Experiments are in progress to determine whether (a) cyclic-AMP-dependent protein kinase phosphorylates the α and β components at different rates or (b) phosphorylation of the β component activates phosphorylase kinase, which then autophosphorylates the α component.

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Temporal Aspects of Glucose Metabolism in the Rat at Birth

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Previous work on glucose metabolism in neonatal rats included the use of naturally delivered newborn animals, whose postnatal ages therefore varied by up to 60 min within a litter (Vernon & Walker, 1972). In the present work rats were delivered within minutes of their littermates by caesarian section in order to examine more accurately the temporal changes occurring after birth. In the first 2 h immediately after delivery there is a dramatic fall in the plasma concentrations of glucose and lactate. Specific radioactivities of plasma glucose or lactate measured at intervals after a single intraperitoneal injection of tracer amounts of [6 - ^{14}C]glucose or [U - ^{14}C]lactate were used to calculate the rates of formation and utilization of these metabolites (Wrenshall, 1955). Postnatal hypoglycaemia was due to a high rate of glucose utilization at birth that exceeded the rate of glucose formation. Hepatic glycogen concentrations, which were high at birth, remained constant for up to 2 h before any measurable decrease occurred, and during this period injection of glucagon or dibutyryl cyclic AMP (*N*-6, *O*-2'-dibutyryl adenosine 3':5'-cyclic monophosphate) were without effect, although at later ages these agents had a pronounced glycogenolytic effect. The rate of lactate utilization at birth was very high, and, in view of the delay in glycogen mobilization, it seems likely that a proportion of this lactate is being converted into glucose.

Injection of actinomycin D at birth completely blocked glucose formation, virtually abolished incorporation of [U - ^{14}C]lactate into glucose and accelerated the postnatal hypoglycaemia, but had no effect on the time-course of glycogen mobilization. The inhibitor has no effect on the postnatal development of glycogen phosphorylase (Cake & Oliver, 1969), but blocks phosphopyruvate carboxylase development (Yeung & Oliver, 1968). It thus appears that glucose formation immediately after birth is entirely dependent on gluconeogenesis.

The block in hepatic glycogen mobilization at birth may be due to a relative deficiency in the phosphorylase-activating system, as has been observed by Dawkins (1963). Schaeffer *et al.* (1969) have found that glucagon activation of phosphorylase in adult rats is dependent on the presence of glucocorticoids. We found that injection of dibutyryl cyclic AMP and cortisol together at birth had no effect on glycogen concentrations measured 1 h later; if they were injected together 1 h after birth glycogen concentrations fell markedly within 1 h, but did not if either