ampholytes. The separated 'isoenzymes' show no differences in kinetic properties, and have identical electrophoretic mobilities. Attempts to re-focus the sub-forms give rise to additional bands.

The molecular weight of the enzyme estimated by gel filtration (375000) does not alter during purification. Ultracentrifugation experiments (Barnes *et al.*, 1971) indicate a molecular weight nearer 300000 for enzyme purified by another method.

The pure enzyme contains two types of subunit in equal numbers. Both types have a molecular weight of 40000, estimated by gel electrophoresis in sodium dodecyl sulphate, so the enzyme is probably an octamer. The subunits can be separated by electrophoresis on polyacrylamide gels in 8M-urea, and differ in amino acid composition and tryptic-peptide 'fingerprints'. Cysteine residues are found only in one type of subunit.

When excited at 290 nm the enzyme fluoresces at 340 nm. After the binding of NADH the emission

maximum shifts to 440 nm, and the 340 nm peak is suppressed. This allows a direct estimate of the NAD^{+} - and NADH-binding constants. Isocitrate binds to the enzyme-NADH complex and modifies the emission spectrum. Unfortunately the low binding affinities prevent an estimate of the number of active sites.

Results obtained from fluorescence titrations can be combined with new kinetic measurements to build up a model for the reaction mechanism. The minimal hypothesis needed to explain the experimental findings is an extended form of the allosteric theory of Monod *et al.* (1965) in which there are three rather than two interconvertible enzyme conformations.

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COMMUNICATIONS

Regulation of Granulose Synthesis in *Clostridium pasteurianum*

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Species of butyric clostridia synthesize granulose, an intracellular glucan whose structure resembles that of amylopectin (Gavard & Milhaud, 1952). In one strain of *Clostridium pasteurianum* maximal synthesis of granulose coincided with sporulation (Mackey & Morris, 1971), so that knowledge of the means of regulation of granulose biosynthesis could contribute to our understanding of the condition of the organism when sporulation is initiated.

Like glycogen synthesis in other bacteria (Preiss, 1969), granulose was synthesized in *Cl. pasteurianum* by the sequential action of an ADP-glucose pyrophosphorylase (EC 2.7.7.-) and an ADP-glucose-granulose transglycosylase (i.e. granulose synthetase). Throughout its exponential anaerobic growth in a glucose - ammonium - vitamins - salts medium (Mackey & Morris, 1971) wild-type *Cl. pasteurianum* N.C.I.B. 9486 synthesized considerable quantities of granulose (15% of the dry weight of the organism); under these conditions both enzymes were present at a specific activity of approx. 6nmol/min per mg of protein. Several granulose-negative mutant strains were isolated that grew in minimal medium at a simi-

lar rate to the wild-type. These lacked either ADP-glucose pyrophosphorylase or granulose synthetase.

The soluble ADP-glucose pyrophosphorylase was partially purified (33-fold) and was found to be specific in its utilization of ATP and glucose 1-phosphate. Its ATP-saturation curve was not sigmoidal (K_m 2mM for ATP) and its activity was not enhanced by phosphorylated glycolytic intermediates, pyruvate, NADPH or pyridoxal 5'-phosphate. It was inhibited only by relatively high concentrations of ADP (K_i 20mM); both AMP and inorganic orthophosphate were without effect.

Partial purification (22-fold) of the granulose synthetase was facilitated by its being bound to the particles of granulose isolated by low-speed centrifugation of crude extracts of *Cl. pasteurianum*. The enzyme proved to be specific for ADP-glucose and its activity was not enhanced by hexose phosphates; ADP and to a smaller extent AMP were inhibitory. Pyridoxal 5'-phosphate (1mM) was also inhibitory, suggesting the presence of an essential lysine residue at the catalytic site (Schnackerz & Noltmann, 1971).

These findings suggest that, although the rate of granulose biosynthesis will probably reflect the adenylate-energy-charge status of the organism (Shen & Atkinson, 1970), it is not subject to the fine control effected by allosteric activation of ADP-glucose pyrophosphorylase that is a feature of glycogen production in many bacteria (Preiss, 1969).

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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;