concentrations may inhibit phosphofructokinase, but the increase of citrate is small relative to that of ADP. It appears probable that control at these sites is rather insensitive at physiological substrate concentrations, and may serve primarily to shut off gluconeogenesis and enhance glycolysis under conditions of severe ATP deficiency, such as anoxia. The extent *in vivo* of the energetically wasteful cycle of fructose diphosphate formation by phosphofructokinase and destruction by fructose diphosphatase, and also possible uncoupling of oxidative phosphorylation by fatty acids, is not known. However, calculations by Rognstad & Katz (1966) of energy balances in adipose tissue, and the excess of oxygen consumption of kidney-cortex slices producing glucose in the presence of acetoacetate (Krebs *et al.* 1965), suggest the possibility that the energy cost of gluconeogenesis may be considerably higher than the theoretical 6 molecules of ATP/molecule of glucose. The changes of enzyme patterns observed in the livers of diabetic, starved and cortisone-treated rats (Weber *et al.* 1965) presumably serve to increase the efficiency as well as the capacity of gluconeogenesis, but they are too slow to account for its initiation.

Adrenaline probably acts on liver in a similar manner to glucagon (Exton, Jefferson, Butcher & Park, 1966; Exton & Park, 1966). The present studies suggest that the effect of these hormones on gluconeogenesis is mediated by the release of fatty acids. It appears that gluconeogenesis is 'switched on' primarily by the rise in acetyl-CoA and NADH concentrations, which facilitate, respectively, the pyruvate-carboxylase and triose phosphate-dehydrogenase steps. Whether glycolysis is effectively 'switched off' by analogous control mechanisms requires further clarification.

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Mechanism of Action of Bovine Pancreatic Ribonuclease

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The binding of nucleotides to ribonuclease may be investigated by spectrophotometric methods. The spectra of purine and pyrimidine nucleotides are all perturbed on interaction with the active site of ribonuclease, with a lowering of the extinction of the

base, and, sometimes, a small bathochromic shift. These changes are attributed to the nucleotide rather than the protein because their nature varies with the character of the base. For uridine nucleotides (2', 3'- and 5'-) the position of maximum decrease in the

Fig. 1. Enzyme-substrate complex of ribonuclease and an ester of cytidine 3'-phosphate. In the model, imidazole II lies between the purine ring of R and the cytosine ring.

spectrum on interaction with ribonuclease is at 255–257 $m\mu$ with a smaller increase at 283–285 $m\mu$ (pH 5.2 and 10.02; nucleotide and protein concentration 50 μM). The spectral shifts of adenine nucleotides differ from those of the uridine nucleotides in the position of the minimum, but they are similar in that the wavelengths of maximum increase and decrease are independent of the position of the phosphate group. For cytidine nucleotides, however, the position of the phosphate group has a marked effect. The 5'-mono, 5'-di- and 5'-tri-nucleotides give minima at 275 $m\mu$ whereas the 2'- and 3'-phosphates have minima in the region 263–265 $m\mu$. All these nucleotides inhibit the hydrolysis of cytidine 2',3'-(cyclic)-phosphate competitively under conditions identical with those used for the measurement of the difference spectra, except cytidine 5'-phosphate, which behaves more like a non-competitive inhibitor. Inhibition constants have been determined for these compounds and a range of other substances related to the substrate. The results indicate that, for a cyclic phosphate substrate, interaction occurs at the phosphate group, the ribofuranose ring and the pyrimidine. For a pyrimidine nucleotide ester, for example cytidyl-(3'-5')-adenosine, additional interactions can occur at the ester group, in this case adenosine.

The results summarized above can be related to our proposed mechanism of action of this enzyme (Deavin, Mathias & Rabin, 1966). The enzyme-substrate complex for an ester substrate is shown in Fig. 1. The formation of the linked system, ester oxygen-histidine I-water-pyrimidine oxygen, produces a configuration of the substrate in which the 2'-hydroxyl group (the attacking nucleophile) is juxtaposed to the phosphorus atom. This system cannot form with a purine replacing the pyrimidine, accounting for this aspect of the specificity of the enzyme. If lyxose replaces ribose, or the glycosidic

link is α instead of β , it is also impossible for the linked system to form, explaining why compounds of this type are not substrates (for review see Witzel, 1963). The juxtaposition of the 2'-oxygen to the phosphorus atom will favour an addition reaction, giving rise to an intermediate with five oxygen atoms attached to phosphorus. Removal of the alkoxide group, assisted by histidine I, completes the formation of the cyclic phosphate. The attack of the 2'-hydroxyl group is promoted by hydrogen-bonding to imidazole II. Model building reveals that this imidazole lies above, parallel to and in partial contact with the pyrimidine ring of the substrate. If the ester group is a purine nucleoside, the purine ring can lie above and in contact with imidazole II. The orbital overlap if the purine is replaced by a pyrimidine is greatly diminished. This would account for the variation of activity with structure for substrates of this type (Witzel, 1963) and in particular for the effect of the structure of the leaving group on the velocity of decomposition of the enzyme-substrate complex.

Our view of the formation of the enzyme-substrate complex is that the first step involves electrostatic and hydrogen-bond interactions of the phosphate oxygen atoms with a lysine amino group and histidine residues I and II. This is followed by the formation of the pyrimidine-water-imidazole I-phosphate oxygen system. A conformational change in the protein, accompanied by acid ionization of imidazole II, leads to the interposition of imidazole II between the two nucleotide bases and allows hydrogen-bonding with the 2'-hydroxyl group. The isomerization of enzyme-nucleotide complexes found for cytidine 3'-phosphate but not for the other ligands investigated (Cathou & Hammes, 1965) could well derive from an analogous movement. Our investigations by optical rotatory dispersion reveal conformational

changes in the protein only when the pyrimidine nucleotide bound to it has an oxygen atom in the 2'-position of the sugar suitable for hydrogen-bonding of the type suggested. It is probable that there are additional interactions between the protein and the pyrimidine and purine rings. The spectral changes of purine nucleotides resemble the effects of protonation of the adenine and guanine rings. This indicates that a cationic acid group (probably another lysine) interacts at N-1 on the purine ring. The changes in the spectrum of uridine nucleotides are similar to the transfer of this group to an alkaline environment, suggesting that a carboxylate ion interacts with the 6-hydroxyl group of the enol form. This carboxylate group might be paired with the amino group involved in interaction with the purine ring. Interaction with the sub-

strate does not necessitate the disturbance of the geometry of this pair. The water molecule bridging imidazole I and the pyrimidine ring could be positioned by hydrogen-bonding to an acceptor group on the protein.

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α -1,4-Glucosidase Activity in Leucocytes from the Family of Two Brothers Who Lack this Enzyme in Muscle

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Since the discovery by Hers that tissues obtained from patients with generalized glycogen-storage disease (Pompe's disease) fail to hydrolyse maltose and the outer chains of glycogen at an acid pH whereas normal tissues have this activity, a deficiency of an α -1,4-glucosidase with an acid pH optimum (lysosomal α -1,4-glucosidase) has been implicated as an important factor in the accumulation of glycogen in tissues of patients with this type of glycogenosis (type II) (Hers, 1961, 1963; Baudhuin, Hers & Loeb, 1964). Recently two papers have appeared in which deficiencies were shown of such an α -1,4-glucosidase in skeletal muscles of individuals who did not have type II glycogen-storage disease. In one, studies were described on a 14-year-old boy who had been diagnosed as having progressive muscular dystrophy (Courtécuisse, Royer, Habib, Monnier & Demos, 1965). In the second paper clinical, histological and biochemical findings were reported on two brothers, age 15 years (A.W.) and 4½ years (T.W.), with mild manifestations limited to their skeletal muscles

(Zellweger, Brown, McCormick & Tu, 1965). These reports focus attention on the need for further investigation of the role of lysosomal α -1,4-glucosidase in glycogen metabolism. In the present communication, results are presented from recent studies of the leucocytes of A.W. and T.W. obtained 2 years after the initial work on these brothers. Although previously enzymic activity in hydrolysing maltose at pH 4.5 could not be demonstrated in homogenates of muscle samples obtained from the patients, leucocyte homogenates are now shown to have undiminished ability to hydrolyse maltose at an acid pH.

Leucocytes were isolated from 10ml. of heparin-treated blood essentially by the method of Huijing (1964). The well-washed leucocyte films were homogenized in 1ml. of 0.05M-NaF. Reaction mixtures containing 0.02M-maltose in 0.02M-acetate buffer, pH 4.5, and 50–100 μ g. of protein in a final volume of 0.3ml. were incubated at 37° for several hours. Portions were removed at intervals, diluted and boiled for 1min. before the glucose