EFFECT OF ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE ON MUSCLE PHOSPHORYLASE ACTIVITY[1]

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The phosphorylase reaction is thought to be principally rate limiting for glycogenolysis in muscle and its rate appears to depend largely on the extent to which phosphorylase b is converted to phosphorylase a (Sutherland, 1951; Krebs and Fischer, 1955; Cori, 1956; Danforth, Helmreich and Cori, 1962). The importance of other factors was suggested, however, by the observation that exposure of heart muscle to anoxia led to a more rapid and extensive breakdown of glycogen than did glucagon, although the conversion of phosphorylase to the a form was less (Table 1).

Anoxia induces a marked increase in the activity of phosphofructokinase by changing the intracellular levels of ATP, AMP and P_i (Bueding and Mansour, 1957; Passonneau and Lowry, 1962; Mansour and Mansour, 1962). It appeared possible that these changes might also account for the greater activity of the phosphorylase system with anoxia. In order to investigate this point, the isolated heart was exposed to glucagon and anoxia and measurements were made of the adenine nucleotides and P_i. Anoxia induced, within two minutes, a more than 4 fold increase in AMP, a 2-3 fold increase in P_i and a slight fall in ATP (Table 1). The changes

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Table 1. Effects of glucagon and anoxia on the glycogen content, phosphorylase percent a, adenine nucleotides and P_i in the perfused rat heart.

Substances	Aerobic		Anaerobic
	Control	Glucagon	
Glycogen content mg · g dry^{-1}	13.5 ± 1.0[c] (16)	8.0 ± 1.0[c] (15)	3.5 ± 0.5[c] (12)
Phosphorylase percent a	2 ± 2 (6)	44 ± 4 (6)	16 ± 2 (6)
AMP, mM, intracellular water	0.19 ± 0.02 (3)	0.22 ± 0.02 (3)	0.80 ± 0.17 (3)
ADP, mM, intracellular water	1.6 ± 0.1 (3)	1.7 ± 0.6 (3)	2.8 ± 0.2 (3)
ATP, mM, intracellular water	7.5 ± 0.5 (3)	8.8 ± 0.6 (3)	6.3 ± 0.5 (3)
P_i, mM, intracellular water	3.9 ± 0.5 (3)	5.8 ± 0.6 (3)	9.8 ± 1.2 (3)

Hearts were perfused with substrate-free bicarbonate buffer gassed with O_2:CO_2 (95:5, v/v) at 37° C (Morgan, Henderson, Regen and Park, 1961). The buffer passed through the heart and was collected in graduated cylinders. After 12 min., the perfusion of one group of hearts was shifted to buffer gassed with N_2:CO_2 (95:5, v/v), that of a second group to aerobic buffer containing glucagon (1 ug·ml^{-1}), whereas perfusion of a third group with aerobic buffer was continued. After 2 mins. of additional perfusion, hearts for phosphorylase assay, adenine nucleotides and P_i were rapidly frozen by clamping between blocks of aluminum at the temperature of liquid nitrogen (Wollenberger, Ristau, Schoffa, 1960) and were pulverized in a percussion mortar at the same temperatures. The tissue was extracted and assayed for phosphorylase activity by a modification of the procedures of Cori and Illingworth (1956) and Danforth et al. (1962). Percent a has been corrected for the activation of phosphorylase b resulting from the AMP content of the assay fluid arising from the heart as will be described. Pools of 3 hearts were examined for nucleotides and P_i. An aliquot from the pool was taken for estimation of percent dry weight (Morgan et al., 1961). A second and third weighed aliquot were used for the duplicate estimation of intracellular inorganic phosphate by the method of Wahler and Wollenberger (1958). A fourth aliquot (about 3 g) was weighed and dumped onto 6 ml of cold 10% trichloracetic acid (TCA) in a porcelain mortar. After mixing and grinding for about 1 min., the extracts were centrifuged and the TCA extracted with diethyl ether. AMP was estimated by absorbancy at 260 mu following separation on a Dowex 1 formate column. ADP was estimated by the pyruvate kinase-lactic dehydrogenase reactions and ATP by the phosphoglycerate kinase-glyceraldehyde phosphate dehydrogenase reactions. After 14 min. additional perfusion, hearts for glycogen content were frozen (Morgan et al., 1961). The polysaccharide was isolated, and hydrolyzed (Walaas and Walaas, 1950) and assayed by the glucose oxidase method (Huggett and Nixon, 1957).

The number in parenthesis indicates the number of hearts or pools of hearts examined. There are approximately 2.1 ml of intracellular water per gram of dry weight of tissue (Morgan et al., 1961).

c Standard error of mean

in AMP, ADP and P_i with glucagon were in the same direction but were relatively small. After a perfusion period lasting 5 minutes, a greater fall in ATP and further rises in AMP and P_i were observed with anoxia, but no further changes occurred aerobically with or without glucagon. These data, therefore, were consistent with the possibility that phosphorylase activity might be related to nucleotide and P_i concentrations.

The effects of these substances were tested using crystalline preparations of rabbit muscle phosphorylase a and b (Table II). The effects of ATP were examined at the aerobic and anaerobic levels of AMP, 0.15 mM and 0.6 mM, respectively, at different levels of P_i. The addition of 8 mM ATP, taken as representative of the concentration in aerobic tissue, induced only a 15 percent inhibition of phosphorylase a when tested in the presence of either AMP level. With phosphorylase b, however, ATP was much more strongly inhibitory particularly at the aerobic AMP concentration where inhibitions as great as 70 percent were repeatedly found at 1 and 3 mM P_i. The aerobic AMP concentration (0.15 mM) induced a near maximal rate of both enzyme forms that depended on P_i concentration. ADP did not inhibit either phosphorylase a or b activity in the presence of the aerobic AMP concentration

When the tissue is made anoxic, the changes in ATP, AMP and P_i are additive in stimulating the a and b forms of phosphorylase. Estimates of their combined effects are indicated in Table II. If the above changes in activities are considered in conjunction with conversion of b to the a form in the anoxic and glucagon treated heart (Table I), estimates of total phosphorylase activity can be made. The activities are as follows: aerobic 1.4, anaerobic 8.5, and glucagon-treated 3.0 u moles G-1-P formed· mg phosphorylase^{-1}· min^{-1}.

Table II. Effect of AMP, ATP and P_i on the activity of phosphorylase a and b.

Form of Phosphorylase	ATP, 8mM	Inorganic Phosphate, mM		
		1	3	10
		AMP, 0.15 mM		
a	0	1.8	4.0	5.4
	+	1.5	3.5[c]	4.9
b	0	2.4	4.4	11.1
	+	0.6	1.3[c]	5.7
		AMP, 0.6 mM		
a	0	1.7	4.2	5.9
	+	1.6	3.6	5.2[d]
b	0	3.3	5.9	11.4
	+	1.6	4.2	9.2[d]

c denotes simulated "aerobic" conditions

d denotes simulated "anaerobic" conditions

Phosphorylase activity was assayed by the increase in optical density at 340 mu following the addition of crystalline phosphorylase a or b to 3 ml of a solution containing imidazole 0.02 M, bovine serum albumin 0.01%, $MgCl_2$ 0.02 M, cysteine 0.015 M, potassium acetate 0.15 M, glycogen 0.8%, NADP 0.001 M, phosphoglucomutase 40 ug and glucose-6-phosphate dehydrogenase, 20 ug; pH 7.0. Results are expressed as umoles G-1-P formed · mg enzyme^{-1} · min^{-1}. AMP, ATP and inorganic phosphate were added to the above solution to give the final concentrations indicated. Quantities of phosphorylase were added so as to produce rates of G-1-P formation less than 0.015 umoles per minute, over which range the assay was linear in respect to the amount of phosphorylase added. AMP concentrations were measured at the completion of reactions in which AMP was present alone and in combination with ATP. Addition of ATP did not decrease AMP concentrations. Controls were run to show that the variables did not affect the assay system.

AMP, ATP and NADP were obtained from Sigma Chemical Co., G-6-P dehydrogenase from Boehringer and Soehne Co., phosphoglucomutase was the gift of Dr. V. A. Najjar. Phosphorylase b, 4x crystallized, was prepared from rabbit muscle by the method of Fischer and Krebs (1962) and treated with Norit a prior to use. Phosphorylase a, 10x crystallized, was prepared from a second phosphorylase b preparation by the phosphorylase b kinase reaction, recrystallized, and treated with Norit a (Krebs and Fischer, 1962).

The controlling effects of ATP, AMP and P_i on glycogenolysis may be summarized as follows: (1) In the aerobic muscle, where the enzyme appears to be almost entirely in the b form, phosphorylase activity is maintained at a low level by ATP inhibition in association with a deficiency of substrate P_i. The ATP inhibition appears to be particularly important since the tissue concentration of AMP is nearly saturating for phosphorylase

b activity. (2) On exposure to glucagon, the b form is partially converted to a. Greater phosphorylase activity results since the a form is only slightly inhibited by ATP. The small changes in intracellular concentrations of nucleotides and P_i also favor increased activity of both enzyme forms. (3) On exposure to anoxia, a smaller percent of the b form is converted to a. Much greater activity results, however, than with hormone treatment because of the marked rise in P_i and AMP concentrations, the latter counteracting phosphorylase b inhibition by ATP to a large extent. These conclusions suggest that changes in nucleotides and P_i concentrations in the cell are as important for the control of glycogenolysis as shifts between the b and a forms of the enzyme.

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