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Stimulation of the Phosphoroclastic System of *Desulfovibrio* by Nucleotide Triphosphates

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Dilworth, Subramanian, Munson & Burris (1965) reported stimulation of the pyruvic phosphoroclastic system of *Clostridium pasteurianum* by ATP, which they proposed might be due to an allosteric or cofactor-binding action. While studying cell-free preparations of normal and nitrogen-fixing strains of *Desulfovibrio* a comparable stimulation of the phosphoroclastic reaction was encountered as is reported below.

Methods. *Desulfovibrio vulgaris* strain Hildenborough (N.C.I.B. 8303) and *Desulfovibrio desulfuricans* Berre S (N.C.I.B. 8388) were grown with fixed nitrogen in batch culture in medium C (Postgate, 1966); nitrogen-fixing Berre S was grown on a modification of that medium in semi-continuous culture. Extracts were prepared by freezing in liquid nitrogen drops of a 1:1 (v/v) suspension of fresh cells in 0.85% NaCl. Phosphoroclastic activity was measured manometrically as described under Table 1. The cofactors added were those needed by the phosphoroclastic system of *Desulfovibrio* (Millet, 1955) plus α -lipoic acid, although this latter was not shown to affect the reaction rate, and acetyl phosphate, which sometimes stimulated the initial reaction rate with these preparations. The concentrations of cofactors and substrates listed in Table 1 were those that gave optimum activity: high concentrations of pyruvate, phosphate, acetyl phosphate or ATP were inhibitory so these, when used, were well below the inhibitory concentration.

Nucleotide phosphates were detected by thin-

layer chromatography on cellulose in butanol-acetone-acetic acid-aq. 5% ammonia-water (7:5:3:3:2, by vol.) (Randerath, 1962). The cellulose was purchased from Machurey und Nagel Co., Düren, Germany, and the nucleotides were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Results. The phosphoroclastic systems from nitrogen-fixing Berre S regularly showed marked stimulation by ATP; those from Hildenborough were intrinsically more active and required aging at 4° under argon to show ATP stimulation (Table 1). With Berre S extracts, ADP, ITP or IDP could substitute for ATP; with Hildenborough extracts ADP was active but ITP showed much lower ability to stimulate the phosphoroclastic reaction than ATP. IDP was not tested with Hildenborough extracts. *S*-Adenosylmethionine was inactive for Hildenborough though it has been implicated in the pyruvate metabolism of *Escherichia coli* (Knappe, Bohnert & Bruemmer, 1965). It was not tested with Berre S extracts. As well as revealing ATP stimulation, anaerobic aging led to a decline in phosphoroclastic activity (Table 1) that was more pronounced with extracts of Berre S. Some preparations showed a lag before hydrogen evolution occurred.

Extracts of both strains showed acetokinase activity measured as the disappearance of acetyl phosphate in the absence of pyruvate, and this reaction was also accelerated by ATP (Table 1, Expt. 5). ITP was also active and, in contrast with its action on the whole phosphoroclastic process, it

Table 1. *Effect of nucleotides on hydrogen evolution and acetyl phosphate concentrations during the phosphoroclastic reaction with Desulfovibrio extracts*

The reactions took place in Warburg constant-volume manometer flasks at 30° under argon. In Expts. 1, 2 and 3 the main compartments contained extract, phosphate (10mm), $MgCl_2$ (10mm), 50 μg . each of CoA, thiamine pyrophosphate and α -lipoic acid, 5 μ moles of acetyl phosphate and 10 μ moles of neutral ATP, ADP or ITP as indicated, made to a final volume of 2.3ml. with 25mm-tris buffer, pH 7.5. Extracts, prepared by freezing in liquid nitrogen, were added as follows: Expt. 1, 16.3mg. of protein from N_2 -grown Berre S; Expt. 2, 45mg. of protein from N_2 -grown Berre S stored overnight at 4° under argon; Expt. 3, 9mg. of protein from NH_4^+ -grown Hildenborough stored for 8 days at 4° under argon. The side arms contained 10 μ moles of sodium pyruvate in 0.1 ml. of water and the centre wells contained 0.1ml. of 40% (w/v) KOH. The volume of hydrogen evolved in the first 5min. was subtracted to give the values in the Table because a non-enzymic volume change (5–10 μ l.) occurred on tipping the sodium pyruvate. Conditions were similar in Expts. 4 and 5 except that double-side-arm manometer flasks with acetyl phosphate in the second side arm were used. Pyruvate or acetyl phosphate or both were tipped in to start the reaction. Each vessel contained 12mg. of protein from a Hildenborough extract. The reaction was stopped with 1% (w/v) trichloroacetic acid, and the acetyl phosphate was determined as described by Lipmann & Tuttle (1945). For the initial reading trichloroacetic acid was added to the reaction mixture in ice before tipping the acetyl phosphate.

(a) Hydrogen evolution during pyruvate phosphoroclastism

Addition	H ₂ evolved (μ l./30 min.)		
	Expt. 1	Expt. 2	Expt. 3
None	16	1	1
ATP	76	25	80
ADP		24	
ITP	74		14

(b) Acetyl phosphate concentrations during pyruvate phosphoroclastism or acetokinase action

Expt. 4 (+ pyruvate)			Expt. 5 (– pyruvate)		
Time (min.)	μ moles of acetyl phosphate		Time (min.)	μ moles of acetyl phosphate	
	+ ATP	– ATP		+ ATP	– ATP
0	7.2	7.5	0	4.9	5.1
5	0.5	7.5	4	0.5	5.3
10	0.7	7.5	8	0.4	4.4
15	1.1	7.0	12	0.4	5.3
20	1.2	5.8	16	0.3	4.6

showed equivalent activity to ATP with the Hildenborough strain.

Comparable extracts showed a strong adenosine-triphosphatase activity: a typical reaction rate was

4 μ moles of ATP hydrolysed/mg. of protein/hr. by a 4-day-old Berre S extract. Extracts from Hildenborough showed similar activity. Quantitative determination of the adenosine-triphosphatase activity during pyruvic phosphoroclastism was not possible because of the high concentration of phosphate needed for the latter reaction, but rapid conversion of ATP into ADP under conditions necessary for the phosphoroclastic reaction. However, in the absence of pyruvate or acetyl phosphate, was demonstrated qualitatively by chromatography.

Since ATP and ADP stimulated both the phosphoroclastic reaction and acetokinase activity in both strains, their action on the whole system presumably occurred at, or after, the acetokinase level. ADP, formed from ATP if the latter is added, might be the active stimulatory molecule if acetokinase action should be the rate-limiting step. In these circumstances the steady-state concentration of acetyl phosphate during pyruvic phosphoroclastism should be lower in the presence of ADP or ATP. Table 1 (Expt. 4) provides evidence that ATP had an impressive action of this kind. If acetokinase is rate-limiting then acetyl phosphate should accumulate during pyruvate phosphoroclastism in the absence of ATP. To test this possibility, an extract capable of reasonable phosphoroclastic activity in the absence of ATP was obtained from Hildenborough by thawing frozen cells, suspending in water and extruding from a French pressure cell at 6000lb./in.². After 35min. incubation with pyruvate, 11.7 μ moles of hydrogen were evolved with ATP as compared with 9.3 μ moles in the control; acetyl phosphate concentrations were 0.2 and 8.2 μ moles respectively.

Discussion. The stimulatory action of ATP on pyruvic phosphoroclastism might therefore be attributed to the formation of ADP (by adenosine-triphosphatase action), which by simple mass action, accelerates the removal of acetyl phosphate and 'pulls' the whole reaction sequence. Concentrations of ATP required to stimulate the phosphoroclastism were of the same order of magnitude as those needed to stimulate acetokinase activity in the absence of pyruvate, which supports the proposition that the action of ATP involves the acetokinase. The reported K_m for ADP with the purified acetokinase of *Desulfovibrio* is $5.4 \times 10^{-4} M$ (Brown & Akagi, 1966), only slightly less than with the concentration required for stimulation of the phosphoroclastic system (2mm for half maximal stimulation).

It is improbable that acetyl phosphate, accumulating in the absence of ATP or ADP, inhibits the phosphoroclastism by a product inhibition, since the steady-state acetyl phosphate concentrations recorded in Table 1 (Expt. 4) are one-fifth of those needed to show product inhibition. The theory that

the effect of ATP on the pyruvate phosphoroclastism is one of mass action does not account for the observation that ITP had an equal effect to ATP on acetokinase activity in Hildenborough extracts, but did not stimulate the phosphoroclastic reaction to the same extent. The best stimulation, observed with a fresh preparation of ITP, was only half of that observed with the same concentration of ATP.

The equal stimulation of the phosphoroclastic reaction with Berre S extracts by ATP or ITP (Table 1) might be connected with the presence of an ATP deaminase in the Berre S strain that is absent from the Hildenborough strain. However, attempts to show ATP production from ITP with this enzyme were not successful.

The paradox of ITP's action with Hildenborough extracts suggests that there may be a second site of nucleotide action in the phosphoroclastic system. Attempts to resolve this system from *Desulfovibrio* by the method used by Mortenson (1963) with *Clostridium pasteurianum*, to look for an effect of ATP on other component enzymes besides acetokinase, were not successful.

Unlike ATP-dependent hydrogen evolution from

sodium dithionite, which is closely associated with nitrogen fixation, and is absent from cells grown with fixed nitrogen (Burns, 1965), the hydrogen evolution from pyruvate, reported here, is, in *Desulfovibrio*, clearly not associated with nitrogen fixation since it occurs in both types of cells.

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