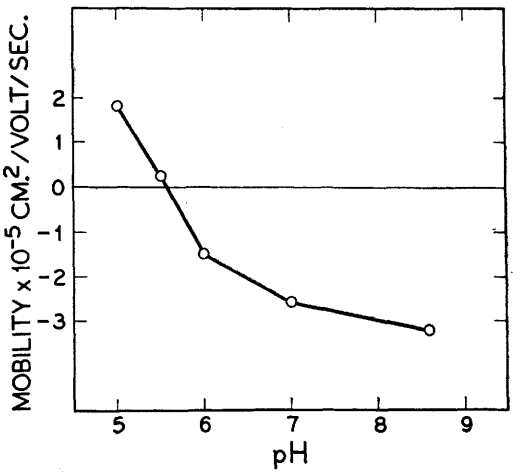

FIG. 1. Paper electrophoresis of purified thrombin with acetate, phosphate and veronal buffers, ionic strength 0.1.

material are altered as well as specific activity and ultracentrifugal properties already reported(1). In the citrate thrombin preparations previously studied it is likely that the results are in part the reflection of aggregate formation(7).

Summary. Electrophoresis on filter paper strips has been performed on purified thrombin prepared by recently developed technics of ion exchange chromatography. Mobility in veronal buffer, pH 8.6, ionic strength 0.1, was calculated to be 3.14 x 10^{-5} cm^2/volt/second. The isoelectric point was shown to be 5.6.

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Coupled Oxidative Phosphorylation in Crude Extracts of *Azotobacter agilis.* (24834)

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Oxidative phosphorylation in bacterial extracts is well authenticated(1-5), but details of this fundamental physiological mechanism are lacking. With exception of mycobacterial and corynebacterial systems of Brodie *et al.* (1), P/O ratios have been low compared to those theoretically possible, or are obtained with mammalian systems(6). It becomes desirable then to determine the influence, if any, of mode of preparation of the extract on P/O ratios. *Azotobacter agilis* (*A. vinelandii*) was employed as the test organism.

Materials and methods. Azotobacter agilis was grown with aeration in 3 L of Burk's nitrogen free mineral salts medium(7) containing 2% sucrose in 8-L pyrex glass bottle. The cells were harvested by centrifugation at 2°C and washed twice in cold distilled water. Intact cells, or disrupted ones, depending upon procedure employed, were suspended in 0.05 *M* "tris" (hydroxymethyl) aminomethane buffer, pH 8.0, containing 0.05 *M* $MgSO_4$; this solution is designated herein as TBS. Twenty-five μmoles ATP (N. B. Co.) were added to cell suspension or cell paste as stabilizer. *Sonic disintegration.* Two to 5 g of cells suspended in 5 ml of TBS were treated in 10 kc Raytheon magnetostrictive oscillator for 3 to 4 min. The homogenate was centrifuged 30 min at 20,000 X G and 2°C. The supernatant fluid (crude extract) was carefully decanted and enough phosphate buffer,

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pH 7.2, was added to give 15 to 20 μmoles of inorganic phosphate/ml. The same amount of phosphate was also employed in procedures to be described. *Grinding with alumina.* A quantity of cells (same weight as above but no fluid) was placed in mortar kept at 4°C. An equal weight of levigated alumina was added and the cells ground for 3 to 4 min at 4°C. After grinding, 5 ml of TBS were added and then centrifuged at 2,000 X G and 2°C for 10 min. The supernatant fluid was removed and recentrifuged at 2,000 X G and 2°C for 10 min before testing. Centrifugation at higher speeds removed active material. *Treatment in ball mill.* A quantity of cells equivalent to that used above was placed in a ball mill with equal weight of alumina and 15 stones and ground at 2°C for 30 min. Seven ml of TBS were then added. This material was centrifuged at 2,000 X G and 2°C for 10 min before determining activity of supernatant fluid. *Sudden release of pressure.* Nine to 13 g of cells (wet weight), suspended in 25 ml of TBS, were broken up according to method of French and Milner (see Colowick and Kaplan,8). The extract was centrifuged at 20,000 X G for 30 min at 2°C. Protein content of cell-free preparations was estimated by procedure of Stadtman *et al.*(9). Determinations of phosphate and manometric measurements were similar to those of Brodie and Gray(1). Since the extracts possessed no significant endogenous activity and there was little difference in effect of ADP added to dialyzed or non-dialyzed extracts, except to show stimulation by added acceptor with dialyzed extracts, dialysis of extracts was discontinued after first few experiments. Also, since dialyzed hexokinase showed no greater activity than undialyzed hexokinase, this treatment was also discontinued early in the investigation. Test systems consisted of 10 μmoles $MgCl_2$; 15-20 μmoles inorganic phosphate; 2 μmoles ADP (N.B. Co.); 20 μmoles mannose as the acceptor and 1 ml of crude extract in main compartment of Warburg vessels. The sidearms contained 50 μmoles of NaF, 50 μmoles of sodium succinate as substrate and 3 mg of yeast hexokinase (N.B. Co). The vessels were equilibrated for 10 min; then contents of sidearm were tipped in and oxygen uptake was measured 10 min. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid.

Results. Results recorded in Table I show that with crude extracts prepared by sonic disintegration P/O ratios are indeed low. In numerous experiments the ratios varied from 0.21 to 0.41, the highest consistent P/O ratios recorded in the literature for this organism with succinate as substrate being about 0.5 (3); and in one case, 0.8, with unwashed particles of Tissieres(5).

P/O ratios obtained under same conditions as above, but with extracts of alumina ground cells, were also low but equivalent to those observed with sonic extracts. Extracts prepared by using the French Cell also exhibited low P/O ratios, ranging from 0.15 to 0.33. Extracts prepared in ball mill were not as active oxidatively as those prepared by other methods, and were completely inactive phosphorolatively regardless of grinding time.

Time course study of oxidative phosphorylation showed, in a representative experiment, that with sonic extracts P/O ratio dropped from 0.57 at 2 min, to 0.36 at 5 min, decreasing to 0.28 at 10 min. In a representative experiment with French Cell extracts, P/O ratio dropped from 0.66 at 2 min, to 0.41 at 5 min, and to 0.25 at 10 min. These results suggest that a labile factor may be present in the extracts.

Centrifugal characteristics of extracts prepared by different methods are quite different, *e.g.*, a routine centrifugation of 20,000 X G for 30 min after breaking the cells by sonic disintegration or with French Cell, results in retention of activity in the supernatant fluid; however, centrifugation at 20,000 X G for 30 min, after breaking cells by grinding with alumina or in ball mill, separates almost all respective activities in the particulate fraction. This indicates a decided difference in size of particles obtained. Which are methodological artifacts and which truly represent the structure or structures of the intact cell await further clarification. Some measure of clarification has been offered by Bradfield(10), Tissieres *et al.*(5), and Cota-Robles *et al.*(11). In addition, the Table reveals an obvious difference with respect to specific activity (*i.e.*,

TABLE I. Oxidative Phosphorylation Ratios (μMoles Phosphate Esterified/μAtoms Oxygen Consumed) Observed with Crude Extracts of *Azotobacter agilis* under Conditions Described in Text.

System	Sonic	Alumina	Ball mill	French cell
Complete (duplicate)	2/7.5 = .26	4.2/10.2 = .41	0/3.6	1.6/10.6 = .15
	2/7.4 = .27	3.4/10.2 = .33	0/3.3	1.8/10.4 = .17
No substrate	.3/ .8 = .38	1.2/ 1.1 = 1.09	0/0.3	.8/ .9 = .89
" acceptor	2/7.1 = .28	3.2/10.1 = .32	0/3.6	2.6/11.3 = .23
Neither substrate nor acceptor	0/ .5 = —	1 / .6 = 1.67	0/ .3	1.0/ .9 = 1.11
mg protein	9.5	7.8	7.4	16.8

activity/unit of protein) of different extracts.

It is clear from our results that the common methods of preparing extracts yield preparations exhibiting low P/O ratios with this organism. It is possible, in view of accumulating evidence, that there is a poor efficiency of coupling between endergonic and exergonic activities of *A. agilis*. In conformity with this view the extent of assimilation is low and this organism lacks sensitivity to dinitrophenol and azide both with respect to oxidative phosphorylation and oxidative assimilation(12).

Results reported in Table I indicate that in the absence of added substrate, oxygen consumption is markedly reduced, although in most cases P/O ratios are as high or higher than in complete systems. It is apparent that substrate oxidation is essential for fixation of larger amounts of phosphate, although added acceptor is not. Since ball mill extracts exhibited oxidative but not phosphorylative activity and quite marked oxidative ability was noted in all extracts, it appears that low P/O ratios may in part be the result of inhibition or destruction of part of the phosphorylating or phosphate transfer system. This is supported by decrease in P/O ratios with time observed in the extracts.

Summary. 1) The effect of method of preparation of the extract, sonic disintegration, grinding with alumina, disruption in ball mill, or sudden release of pressure in the French Cell, on P/O ratio of crude extracts of *A. agilis* with succinate as substrate has been investigated. 2) The P/O ratios show a wide range of variation with different extracts prepared by same method, but with all methods used, P/O ratios were low compared to mycobacterial and mammalian systems. Some evidence is presented that a labile factor is present in extracts and is responsible, at least in part, for the low ratios observed.

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