THE RESPIRATORY ENZYME SYSTEMS OF *AZOTOBACTER VINELANDII*

A. TISSIÈRES*, H. G. HOVENKAMP AND E. C. SLATER

Molteno Institute, University of Cambridge (England) and

*Laboratory of Physiological Chemistry, University of Amsterdam (The Netherlands)***

It has been known for some time that in bacterial extracts the terminal oxidase systems are supported by particles of varying sizes. At least a part of these enzyme systems is attached to very small elements which can only be sedimented in high gravitational fields (TISSIÈRES[1]; SMITH[2]). There is evidence that in a number of bacteria, a fraction composed mostly of spherical particles with a sedimentation constant of 40 *S* and a diameter of about 15 mμ possesses succinic dehydrogenase and succinic oxidase activities (SCHACHMAN, PARDEE AND STANIER[3]; TISSIÈRES[4]). The experiments of ALEXANDER AND WILSON[5], working with *Azotobacter vinelandii* extracts, point to the same conclusion and provide quantitative data suggesting that the small particles support most of the succinic oxidase activity of the cell.

In this paper, an extract from *Azotobacter vinelandii* was fractionated by centrifugation and the fractions were studied for their ability to oxidize succinate, fumarate, reduced diphosphopyridine nucleotide (DPNH) and cytochrome c_4, and to promote respiratory chain phosphorylation with succinate, fumarate, α-ketoglutarate and DPNH as substrates. The results are discussed in the light of electron micrographs of the particulate fractions and of the bacterial cells[22].

METHODS

Preparation of extract and fractions

The strain of *Azotobacter vinelandii* and the methods of cultivation and of preparation of the cell-free extract have been described previously (TISSIÈRES[6]), the only modification being that the bacteria were washed in 0.05 *M* Sørensen phosphate buffer, pH 7.0, instead of distilled water. All manipulations were carried out between 0° and 4°. After disruption of the cells by grinding with glass powder, and addition of water, the mixture was centrifuged at 7,000 r.p.m. (3,500 *g*) for 10 min to remove most of the glass powder and intact cells. Throughout this paper centrifugal fields refer to the bottom of the tube. The supernatant was centrifuged again at the same speed and for the same time to separate from the extract the remaining intact cells and glass powder. During this centrifugation some of the largest cell debris were also removed. The supernatant, named the extract (E), was centrifuged for 30 min at 19,000 r.p.m. (22,000 *g*) giving a pink sediment of large particles and a supernatant, fraction S_1. The latter was further fractionated by centrifuging in the head No. 40 of a model L Spinco centrifuge at 40,000 r.p.m. (145,000 *g*) for 60–120 min, giving a red gelatinous sediment of small particles. The upper third of the supernatant fluid was carefully removed by pipette to give fraction S_2. Each of the particulate fractions was suspended in 0.05 *M* phosphate buffer, pH 7.0 (¼ the original volume of the extract), giving fractions LP (large particles) and SP (small particles). Alternatively, each of these two fractions was washed by resuspending in 0.05 *M* phosphate buffer, pH 7.0 (twice the original volume of the extract), sedimented by centrifugation as above and resuspended in phosphate buffer (¼ the original volume of the ex-

* Present address: Biological Laboratories, Harvard University, Cambridge, Mass., U.S.A.

** Postal address: Jonas Daniël Meyerplein 3, Amsterdam.

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tract), giving fractions WLP (washed large particles) and WSP (washed small particles). In some experiments, when specially mentioned, 0.20 *M* sucrose or 0.15 *M* lactose replaced water in the extraction of the mixture of cell debris and glass powder, and the particles of fractions LP or SP were washed and finally resuspended in the same concentration of sucrose or lactose containing 0.05 *M* phosphate buffer, pH 7.0.

Treatment with supersonic vibration

The intact cells suspended in 0.25 *M* sucrose and 0.05 *M* phosphate buffer, pH 7.0 (approx. 40 mg dry wt of cells/ml), or the fractions WLP and WSP were subjected to supersonic vibration of 25 kcyc./sec generated by a 500 W Mullard magnetostrictor oscillator. The duration of exposure varied and is given below for each experiment. The particulate fraction WLP was treated with supersonic vibration and either used as such (fraction SLP) or centrifuged first at 22,000 *g* for 30 min to remove the unbroken large particles and then at 145,000 *g* for 60 min to give a sediment of small particles which was resuspended in 0.05 *M* phosphate buffer, pH 7.0 (fraction S'LP). Similarly, fraction WSP, after the action of supersonic vibration, was either used as such (fraction SSP) or it was centrifuged at 145,000 *g* for 60 min to give a sediment of particles which was resuspended in buffer as above (fraction S'SP).

Oxidase activities

Succinic oxidase and cytochrome c_4 oxidase activities were measured in differential manometers at 30° as previously described[6]. The reaction vol. was 0.5 ml. The oxidation of fumarate was measured similarly, using 0.02 *M* fumarate as substrate. The activity of cytochrome c_4 oxidase was also estimated by following the oxidation of reduced cytochrome c_4 in the spectrophotometer as described by SMITH[7] for cytochrome *c*. The 1 cm cuvette contained the appropriate amount of reduced cytochrome c_4 and the enzyme in 0.05 *M* phosphate buffer, pH 7.0, in a total reaction mixture of 0.6 ml.

DPNH oxidase activities were estimated according to SLATER[8] with a reaction mixture of 1 ml. The cuvette (0.5 cm) contained 0.05 *M* phosphate buffer, pH 7.0, and $1.6 \cdot 10^{-4}$ *M* DPNH (Sigma Chemical Co.). The decrease in optical density at 340 mμ was followed in the spectrophotometer for 5 min. It was linear with time over this period. Under the conditions of the experiments, the changes in optical density were found to be proportional to enzyme concentrations over a 5-fold range.

Oxidative phosphorylation

Oxidative phosphorylation with succinate, fumarate, and α-ketoglutarate was determined as previously described (TISSIÈRES AND SLATER[9]). The procedure with DPNH as substrate was as follows. The DPNH content of the stock solutions (prepared from Sigma "Cozymase 90"[10]) was determined by measuring the extinction at 340 mμ of an aliquot, before and after the addition of pyruvate and lactic dehydrogenase. The oxidation of DPNH in the presence of the *Azotobacter* extract was followed spectrophotometrically at 340 mμ, until a steady reading was obtained, corresponding to complete oxidation of the DPNH.

The reaction mixture in the test cuvette contained: phosphate, pH 7.4, 0.03 *M*; glucose, 0.007 *M*; ethylenediaminetetraacetate (EDTA), pH 7.4, 0.001 *M*; NaF, 0.04 *M*; $MgCl_2$, 0.005 *M*; adenosine monophosphate, $2 \cdot 10^{-4}$ *M*; adenosine diphosphate, $2 \cdot 10^{-4}$–$6 \cdot 10^{-4}$ *M*; DPNH, $3 \cdot 10^{-4}$–$6 \cdot 10^{-4}$ *M*; yeast hexokinase, 100 units[11]. The reaction volume was 2.4 ml. When the concentration of DPNH exceeded $5 \cdot 10^{-4}$ *M*, the solution was oxygenated before the beginning of the experiment, so that it contained sufficient oxygen to react with the DPNH. The reference cuvette contained the same reaction mixture, except that the DPNH was omitted. At zero time, 0.1 ml *Azotobacter* fraction was added to each cuvette. When the oxidation was complete (between 1 and 4 min was usually sufficient), 0.4 ml trichloroacetic acid (40% w/v) was added and the mixture centrifuged. The ~ P (reactive groups of adenosine diphosphate and triphosphate) and hexose monophosphate (HMP) contents of the neutralized supernatants were determined by the enzymic procedure described by SLATER[10].

The amount of phosphate esterified coupled to the oxidation of DPNH was calculated as Δ esterified P (corr.) = $(\sim P_{test} + HMP_{test})$ *minus* $(\sim P_{ref.} + HMP_{ref.})$, where the suffixes "test" and "ref." refer to the solutions obtained from the corresponding cuvettes. This method of calculation compensates for any phosphorylation due to the oxidation of endogenous substrate, but in fact this was negligible. The considerable amount of myokinase found in the soluble fractions of *Azotobacter* have no influence on these measurements, since (HMP + ~P) is not changed by the combined action of myokinase and hexokinase on ADP.

Control experiments showed no phosphorylation when DPN was used instead of DPNH, or when KCN (10^{-3} or 10^{-2} *M*) was added to inhibit the oxidation of DPNH (Table I).

Protein was estimated in the cell-free extracts by the biuret method[12].

Cytochrome c was purified according to MARGOLIASH[13].

Cytochrome c_4 was prepared as already described[6].

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TABLE I

PHOSPHORYLATION COUPLED WITH THE OXIDATION OF DPNH BY *Azotobacter* EXTRACTS

In expt. 1, the extract was prepared by two centrifugations at 10,000 *g* for 10 min, and the particles sedimented at 20,000 *g* for 30 min. The particles are thus intermediate in size between those described in this paper as large particles (LP) and as small particles (SP), and are described as medium particles (MP).

Expt.	*Fraction*	*Pyridine nucleotide (μmoles)*	*KCN (M)*	*Δesterified P (corr.) (μmoles)*
1	MP	DPNH (1.46)	0	1.01
	MP	DPN (1.5)	0	−0.03
	S_1	DPNH (1.46)	0	1.04
	S_1	DPN (1.5)	0	−0.02
2	S_1	DPNH (1.56)	0	0.68
	S_1	DPNH (1.56)	10^{-3}	0.04
	S_1	DPNH (1.56)	10^{-2}	0.04
	none	DPNH (1.56)	0	0.05

RESULTS

Oxidation of succinate and fumarate

Figs. 1 and 2 show that the washed small particles (WSP) are the most active fraction for the oxidation of succinate. The supernatant fraction (S_2) has little or no activity, provided that it is prepared by centrifugation for 120 min at 145,000 *g*. There was an appreciable activity if the centrifugation proceeded for only 60 min.

With succinate as substrate, the addition of S_2 inhibited the rate of oxygen uptake with WSP, but stimulated the oxygen uptake with SP. These effects are explained by the inhibitory action of oxaloacetate on succinic dehydrogenase (*cf.* STONE AND WILSON[14]) and by the behaviour of the various fractions with fumarate as substrate (Fig. 3). The fraction WSP did not oxidize fumarate at a detectable rate in the experiment shown in Fig. 3 (nevertheless the progressive inhibition of the oxidation

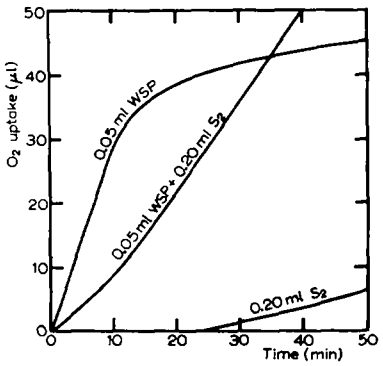

Fig. 1. Oxidation of succinate by fractions WSP and S_2 (supernatant after 90 min centrifugation at 145,000 *g*). The manometric flasks contained the enzyme preparation as shown with 0.02 *M* succinate and 0.05 *M* phosphate buffer, pH 7, in a total reaction volume of 0.5 ml. Temperature: 30°C.

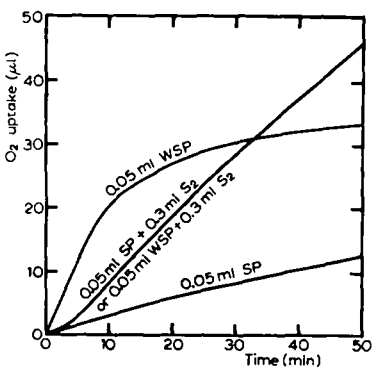

Fig. 2. Oxidation of succinate by fractions WSP, SP and S_2 (supernatant after 120 min centrifugation at 145,000 *g*). The manometric flasks contained the enzyme preparation as shown, with 0.02 *M* succinate and 0.05 *M* phosphate buffer, pH 7, in a total reaction volume of 0.5 ml. Temperature: 30°C.

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of succinate by WSP shown in Fig. 1 is probably due to the building up of small concentrations of oxaloacetate by a very slow oxidation of the fumarate; see Table II).

The addition of S_2 to WSP promoted a rapid oxidation of fumarate, and the appreciable activity with SP alone (Fig. 3) is undoubtedly due to the small amount of S_2 contaminating this fraction. Table II shows that the O_2 uptake greatly exceeds the amount of (oxaloacetate + pyruvate) formed, indicating that the oxidation proceeds beyond that stage.

Thus, when succinate is substrate, the addition of S_2 has three different effects on the oxygen uptake: (1) the further oxidation past fumarate results in an increased oxygen uptake; (2) the formation of oxaloacetate causes an inhibition of the oxidation

TABLE II

CONCENTRATION OF OXALOACETATE IN THE REACTION MIXTURE DURING THE OXIDATION OF SUCCINIC ACID BY WSP

The reaction mixture was as described in Table I of TISSIÈRES AND SLATER[9]. 30 min, 25°. Δ(oxaloacetate + pyruvate) measured as in SLATER[10]. Much of the pyruvate is probably derived by decarboxylation of the oxaloacetate, either during the experiment, or after the deproteinization.

Expt.	*Substrate*	*Fraction*	*Protein* (mg)	O_2 *uptake* (μatoms)	Δ (*oxaloacetate* + *pyruvate*) (μmoles)
1	Succinate	WSP	1.5	2.10	0.13
		S_2	12	0.67	0
		WSP + S_2	13.5	6.94	0.08
2	Fumarate	WSP	1.2	0.16	0.06
3	Succinate	WSP + S_2	5.7	3.35	0.05
	Fumarate	WSP + S_2	5.7	6.90	2.25

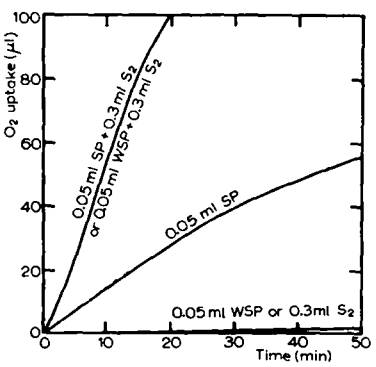

Fig. 3. Oxidation of fumarate by fraction WSP, SP and S_2 (supernatant after 120 min centrifugation at 145,000 *g*). The manometric flasks contained the enzyme preparation with 0.02 *M* fumarate and 0.05 *M* phosphate buffer, pH 7, in a total reaction volume of 0.5 ml. Temperature: 30°C.

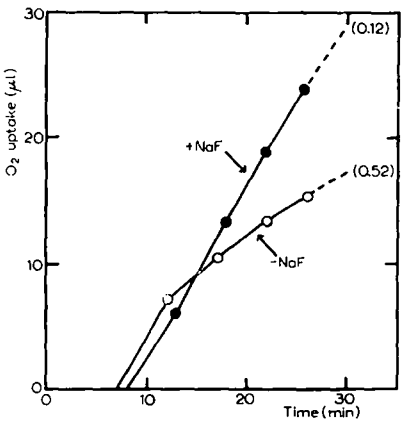

Fig. 4. Course of O_2 uptake with LP oxidizing succinate in the presence and absence of 0.04 *M* NaF. Reaction mixture: phosphate, pH 7.4, 0.03 *M*; glucose, 0.03 *M*; ADP, $6 \cdot 10^{-4}$ *M*; AMP, $6 \cdot 10^{-4}$ *M*; succinate, 0.02 *M*; EDTA, 0.001 *M*; $MgCl_2$, 0.005 *M*; hexokinase, 137 units; LP, 1.5 mg protein; reaction vol., 1 ml; 25°C. The LP fraction was added at zero time and the flasks immediately attached to the manometers and placed in the bath. The points on the abscissa represent the first reading of the manometer. The figures in brackets represent the amount of (oxaloacetate + pyruvate).

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of succinate; (3) the ability of (S_2 + WSP) to oxidize oxaloacetate relieves this inhibition. The actual result depends upon the balance of these three effects. Thus, in Fig. 1, the addition of S_2 causes a considerable inhibition of the initial rate of oxygen uptake, due to the rapid building up of a steady-state concentration of oxaloacetate. Once this steady-state is reached, however, the oxygen uptake proceeds at a uniform rate. With WSP, the oxaloacetate formed slowly is not removed, so that there is a steadily decreasing oxygen uptake. After 30 min (earlier in Table II), the O_2 uptake with (WSP + S_2) exceeds that with WSP alone. Fig. 2 suggests that SP contains sufficient of S_2 to promote the formation of appreciable amounts of oxaloacetate, but these are rather slowly removed. Thus, the predominating effect of the addition of S_2 is to decrease the steady-state concentration of oxaloacetate and to promote the oxygen uptake. It should be noted that with both substrates, (SP + S_2) behaved exactly the same as (WSP + S_2). There is, therefore, probably no difference between SP and WSP, except that the former contains a little S_2.

That the rapid fall off of the rate of O_2 uptake was due to inhibition by oxaloacetate is shown by the effect of fluoride (Fig. 4). The addition of fluoride to LP lowered the concentration of oxaloacetate and, at the same time, allowed a practically uniform rate of O_2 uptake. Fumarate was oxidized by this preparation of unwashed particles, in the absence of fluoride, with the production of the corresponding amount of oxaloacetate ($\Delta O = -0.85$ μatom; Δ[oxaloacetate + pyruvate] = + 0.86 μmole). It was also found that fluoride decreased the production of oxaloacetate by SP oxidizing succinate, and decreased the rate of oxidation of fumarate.

Qualitatively, the large particles (fractions LP and WLP) behaved in the same way as SP and WSP. However, the specific activity (Q_{O_2}) of the large particles is considerably less than of the small (see Table III). Doubling the concentration of succinate (0.04 *M* instead of 0.02 *M*) did not change the activity.

TABLE III

SUCCINIC, CYTOCHROME c_4 AND DPNH OXIDASE ACTIVITIES IN FRACTION WLP AND WSP

Q_{O_2} = μl O_2/h/mg protein

Experiment No.	*Fraction WLP*			*Fraction WSP*		
	succinic oxidase Q_{O_2} 30°C	*cytochrome oxidase* Q_{O_2}	*DPNH oxidase* Q_{O_2} 22°C	*succinic oxidase* Q_{O_2} 30°C	*cytochrome oxidase* Q_{O_2}	*DPNH oxidase* Q_{O_2} 22°C
1	248	203[m]	714	990	173[m]	2760
2	208	357[m]	691	966	165[m]	3330
3	223	—	—	805	—	1970
4	—	447[m]	—	—	280[m]	—
5	—	—	870	—	—	2910
6	—	425[s]	—	—	180[s]	—
7	295	310[m]	1024	1080	205[m]	2600

[m] Manometric estimation (Q_{O_2} at 30°C).
[s] Spectrophotometric estimation (Q_{O_2} at 22°C).

Cytochrome c_4 oxidase

Table III shows that the large particles have a higher cytochrome oxidase activity than the small. It should be noted that the Q_{O_2}'s of the succinic oxidase and cyto-

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chrome c_4 oxidase are not directly comparable because the latter is dependent upon the concentration of cytochrome c_4 which was chosen arbitrarily in these experiments. Thus the cytochrome c_4 oxidase activity was about $\frac{1}{4}$ the activity of succinic oxidase in fraction WSP. S_2 alone did not have any activity, nor did the addition of this fraction have any effect on the cytochrome c_4 oxidase activity of the particulate fractions.

DPNH oxidase

The DPNH oxidase system was found to be entirely located in the particles of fractions LP or SP. Washing the particles in 0.05 *M* phosphate buffer did not modify the total activity; the specific activity (Q_{O_2}) was increased as a consequence of removing contaminating soluble proteins. Fraction S_2, obtained after 120 min centrifugation at 145,000 *g*, had no measurable activity, nor did the addition of S_2 to either LP or SP have any effect. The addition of cytochrome c_4 or cytochrome *c* in concentrations varying from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-1}$ *M* had no effect. KCN ($1 \cdot 10^{-3}$ *M*) produced more than 95% inhibition. Fraction WSP has 3-4 times the activity of WLP (Table III).

Oxidative phosphorylation

Oxidative phosphorylation in *Azotobacter* extracts has been demonstrated by HYNDMAN, BURRIS AND WILSON[15], TISSIÈRES AND SLATER[9] and ROSE AND OCHOA[16]. Our results, obtained with succinate and fumarate as substrate, which have been reported in a preliminary communication[9], are included in Table IV, which also shows experi-

TABLE IV

OXIDATIVE PHOSPHORYLATION IN VARIOUS FRACTIONS OF *Azotobacter vinelandii*

Expt.	*Substrate*	*Fraction*	*Protein (mg)*	*ΔO (μatoms)*	*Δ est. P (μmoles)*	*P : O*
209	Succinate	E	–	4.31	1.37	0.32
		LP	0.3	1.07	0.55	0.52
		SP	0.35	1.37	1.19	0.87
210	α-Ketoglutarate	S_1	–	1.10	0.97	0.88
		SP	–	0.11	0.12	–
		SP + S_2	–	3.28	2.70	0.82
212	Succinate	WSP	1.2	3.78	0.52	0.14
	fumarate	WSP	1.2	0.16	0.02	–
214	Succinate	WSP	1.5	2.10	0.49	0.23
219	Succinate	WSP	0.7	1.80	0.45	0.25
		S_2	5.0	0.61	0.14	0.22
		WSP + S_2	5.7	3.35	1.41	0.42
	fumarate	WSP + S_2	5.7	6.90	2.83	0.41
H 21	DPNH	E	–	1.46	0.63	0.43
		S_1	–	1.46	1.05	0.72
		SP	–	1.46	1.14	0.78
H 46	DPNH	WLP	1.1	1.30	0.43	0.33
		S_1	–	1.30	0.80	0.62
		WSP	0.4	1.30	0.62	0.48
H 19	DPNH	LP	–	0.72	0.24	0.33
		S_1	–	0.72	0.46	0.64
H 43	DPNH	S_1	3.2	1.32	0.60	0.46
		SP	0.68	1.32	0.54	0.41
		WSP	0.27	1.32	0.46	0.35
		WSP + S_2	1.77	1.32	0.41	0.32
H 50	DPNH	WLP	–	1.79	0.41	0.23
		S_1	–	1.79	0.62	0.34
		WSP	–	1.79	0.69	0.38

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ments with α-ketoglutarate and DPNH as substrates. With succinate and fumarate as substrate, the conditions were not completely comparable with those in Figs. 1, 2 and 3, because the reaction medium contained various substances necessary for the demonstration of oxidative phosphorylation (adenine nucleotide, magnesium, fluoride).

Oxidative phosphorylation was demonstrated with succinate, DPNH, fumarate and α-ketoglutarate as substrates. For the last two substrates, it was necessary to add S_2 to SP (or WSP) in order to obtain an appreciable oxidation. The isolation of SP and WSP from S_1 sometimes caused a lowering of the P:O ratio (expts. H 43 and H 46, Table IV), but not always (expts. H 21 and H 50). The addition of S_2 to WSP increased the P:O ratio with succinate as substrate (expt. 219), but had no effect on the ratio with DPNH (expt. H 43).

The small particles have a considerably higher P:O ratio than the large particles, with both DPNH and succinate as substrate (this point was not tested with α-ketoglutarate or fumarate).

Activities of particles prepared in the presence of sucrose and lactose

Experiments were performed in order to find out whether particles isolated in sucrose or lactose solutions have different enzymic activities, or whether the amount of protein in each particulate fraction differs under those conditions. The concentration of the sucrose (0.20 *M*) was chosen arbitrarily to be the same as that used by WEIBULL[17] to prepare protoplasts from *Bacillus megatherium*. The concentration of lactose (0.15 *M*) was the same as that used by ALEXANDER AND WILSON[5] in their work on the succinic dehydrogenase of particulate preparations from *Azotobacter vinelandii*.

The isolation in either 0.20 *M* sucrose or 0.15 *M* lactose had no effect on the amount of protein present in each fraction. The succinic and DPNH oxidase activities, measured in the presence of sucrose or lactose, were 20 to 30% lower than that of the same preparation suspended in 0.05 *M* phosphate buffer, pH 7.0, after the last centrifugation, or of a preparation made up in the usual way by extracting with distilled water and finally suspending in 0.05 *M* phosphate buffer. The isolation in either sucrose or lactose had no appreciable effect on the P:O ratios with LP or S_1 oxidizing DPNH.

TABLE V

EXPOSURE OF PARTICLES TO SUPERSONIC VIBRATION

WLP = washed large particles WSP = washed small particles
SLP = WLP treated with supersonic vibration SSP = WSP treated with supersonic vibra
S'LP = small particles prepared from SLP S'SP = small particles prepared from SSP
P:O ratios measured with DPNH as substrate

Expt.	Time of exposure (min)	WLP			SLP			S'LP			WSP		
		Succinic oxidase (Q_{O_2})	DPNH oxidase (Q_{O_2})	P:O	Succinic oxidase (Q_{O_2})	DPNH oxidase (Q_{O_2})	P:O	Succinic oxidase (Q_{O_2})	DPNH oxidase (Q_{O_2})	P:O	Succinic oxidase (Q_{O_2})	DPNH oxidase (Q_{O_2})	P:
1	5	332	980	—	—	—	—	1070	3310	—	885	2920	—
2	2	296	778	—	—	—	—	690	1765	—	765	1965	—
3	2	—	1234	—	—	—	—	1440	1960	—	900	2160	—
4	2	—	1000	0.14	—	—	—	—	2640	0	—	2440	0.4
5	0.5	—	1288	0.23	—	—	—	—	2160	0.04	—	2460	0.3
6	1	—	940	0.16	—	1910	0.16	—	2300	—	—	2800	0.2

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Effect of supersonic vibration

The results of experiments where the particles were treated with supersonic vibration for 0.5 to 5 min are shown in Table V. The succinic and DPNH oxidase activities of the large particles of fraction WLP were increased to the level of those found for the small particles. There was no appreciable liberation of soluble proteins (supernatant after centrifugation at 145,000 *g* for 60 min) after supersonic vibration. When the treatment with supersonic vibration exceeded 5 min the succinic and DPNH oxidase activities were found to be decreased. These two enzyme systems were almost completely inactivated by 15 min exposure to supersonic vibration. The DPNH oxidase activity of small particles (WSP) after treatment with supersonic vibration for 0.5–1 min (SSP or S'SP) did not increase. Either it remained unchanged or it decreased by as much as 25%. The P:O ratios dropped by about 25% in two experiments under the same conditions.

DISCUSSION

Small particles

The washed small particles isolated from *Azotobacter* extracts contain a very active respiratory chain system, capable of catalysing the oxidation of succinate to fumarate, of DPNH to DPN, and of coupling these oxidations with the synthesis of ATP. With respect to these reactions, the particles correspond to mitochondria isolated from animal, plant and yeast cells. There are, however, important differences between the *Azotobacter* particles and isolated mitochondria.

(1) The diameter of the *Azotobacter* particles (approx. 10 mμ) is only about 1% that of mitochondria. This means that their volume is only about $1/10^6$ that of mitochondria.

(2) Mitochondria contain all the enzymes necessary for the oxidation of pyruvic acid to CO_2 and H_2O, by way of the KREBS cycle, and do not lose the ability to carry out this oxidation, even after repeated washings by centrifugation. The washed small particles of *Azotobacter* are unable to carry out more than one step of the KREBS cycle, unless supplemented with the supernatant fraction remaining after centrifugation at 145,000 *g* for 1–2 h. Even when supplemented with this fraction, succinate is not completely oxidized to CO_2 and H_2O. The unwashed small particles contain sufficient of this supernatant fraction to promote the oxidation of fumarate.

(3) The specific activity (Q_{O_2}) of the *Azotobacter* particles for the oxidation of succinate or DPNH is considerably greater than that of mitochondria, or even of particles derived from mitochondria (see below).

(4) The P:O ratios found with the isolated *Azotobacter* particles are much less than in mitochondria. Also, unlike mitochondria, the phosphorylation is relatively insensitive to dinitrophenol (TISSIÈRES AND SLATER[9]; ROSE AND OCHOA[16]; HOVENKAMP, unpublished).

SSP			S'SP		
inic ase (Q_{O_2})	*DPNH oxidase* (Q_{O_2})	*P:O*	*Succinic oxidase* (Q_{O_2})	*DPNH oxidase* (Q_{O_2})	*P:O*
–	—	—	....	—	—
–	.	. .	—	—	. .
.	—	—	—	—	....
-	. .	—	—	—	—
–	1820	0.31	. .	—	—
–	2770	0.22	. .	2660	0.18

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In some respects, the small *Azotobacter* particles resemble the small particles which can be derived from the mitochondria of animal tissues. The KEILIN AND HARTREE heart-muscle preparation, which consists of fragments of heart-muscle mitochondria (sarcosomes), oxidizes succinate and DPNH rapidly, but has no action on the other components of the KREBS cycle. The particles studied by CRANE, GLENN AND GREEN[18], which are about 50 mμ diameter, are similar in composition and enzymic activity to the KEILIN AND HARTREE heart-muscle preparation (SLATER[19]). Neither of these preparations is able to bring about oxidative phosphorylation. The small particles obtained by KIELLEY AND BRONK[20] by sonic disintegration of liver mitochondria appear to be even more like the *Azotobacter* particles, in that they phosphorylate with either DPNH or succinate as substrate. COOPER AND LEHNINGER[21] have isolated from liver mitochondria small particles (particle weight about $5 \cdot 10^7$), which oxidize β-hydroxybutyrate to acetoacetate and succinate to fumarate, and are able to bring about oxidative phosphorylation. These particles do not oxidize other substrates of the KREBS cycle. Unlike the *Azotobacter* particles, the oxidation of DPNH by COOPER AND LEHNINGER'S preparation is not coupled with phosphorylation, and the phosphorylation obtained with β-hydroxybutyrate is sensitive to dinitrophenol.

These considerations raise the possibility that the small granules isolated from the *Azotobacter* extracts are liberated, during the isolation procedure, from some larger granule corresponding to the mitochondria in animal and plant cells. However, this appears most unlikely. BRADFIELD[22], after a critical examination of the evidence, has concluded that nothing corresponding in shape, size or structure to mitochondria has been detected in bacteria. On the other hand, particles of a diameter of about 10 mμ have been detected by electron microscopy in the bacterial cytoplasm, and in the small particle fraction isolated from the extract[22].

There appears, then, to be a real difference between the organization of respiratory enzyme systems in animal and plant cells on the one hand, and in *Azotobacter* on the other. In the former, all the enzymes of the KREBS cycle, and ancillary enzymes, are localized within a relatively large granule, the mitochondrion, which is bound by a semi-permeable membrane and possesses a considerable degree of internal structure. The exact localization of the various enzymes within the mitochondrion is not yet known, but it seems likely that the respiratory chain is localized in the osmiophilic external membrane or in the internal cristae, or both, while the more easily detached dehydrogenases are perhaps more uniformly distributed throughout the mitochondria (see SLATER[24] for a discussion of this point). In the *Azotobacter* extracts, however, the respiratory chain is localized in independent small particles, with which the dehydrogenases and other soluble enzymes of the KREBS cycle are only loosely associated. When the cells are disrupted, there is no highly organized structure to keep all the enzymes of the KREBS cycle closely associated with one another, as in mitochondria (see also BRADFIELD[22]). In this connexion, it is necessary to keep in mind the different dimensions of the animal and bacterial cells; many of the latter are, in fact, of about the same size as mitochondria.

The P: DPNH ratios found with S_1, SP or WSP varied between 0.29 and 0.78 in 28 experiments (mean, 0.45). This is greater than the values found by ROSE AND OCHOA[16] for extracts, which probably contained LP as well as SP. Further studies of this phosphorylating system in the small particles will be reported in a separate paper.

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Comparison of the two particulate fractions

The small and large particles differ in a number of ways. (1) An electron micrograph of the small particles[22] shows that they are approximately spherical and have a diameter of about 10 mμ. The same value can be obtained from the sedimentation constant of the major component present in this fraction (see SCHACHMAN, PARDEE AND STANIER[3]). The diameter of the large particles is about 10 times greater[23]. (2) The small particles are 2–3 times more active in the oxidation of succinate and DPNH than the large particles. Also the P:O ratios obtained with small particles oxidizing succinate or DPNH are higher than those given by the large particles. (3) The large particles contain more cytochrome of the "*c*" type and less cytochrome b_1 than the small particles (TISSIÈRES[6]). (4) The two kinds of particles also differ in some properties revealed by the examination with the electron microscope[23]. These differences make it very unlikely that the larger particles are formed exclusively of a number of small ones. However, it is conceivable that part of fraction LP is formed of aggregated small particles or large pieces of the cell membrane (see below), the rest being composed of yet another structure present in the extract, for example volutin granules (see below) or pieces of cell wall. The experiments with sonic vibration are in accordance with this view, as they can be interpreted to mean that by this treatment small particles, with respect to size and oxidase activities, were liberated from the large ones. The phosphorylating system is less stable to supersonic treatment than the oxidases.

The structures known to be present in bacterial cells have been discussed by BRADFIELD[22]. According to this author, the major part of the cytoplasm in each of the species investigated consists of small granules about 10–20 mμ in diameter. Those granules, rather smaller than in other species, are clearly visible on electron micrographs of either sections of *Azotobacter vinelandii* or of the WSP fraction[22]. In the latter preparations, clumps containing 10–20 small granules each are also visible. Such clumps will sediment faster than the small granules and are therefore likely to contaminate the fraction LP. The volutin granules are the only other granules visible in the cytoplasm of *Azotobacter*. They are about 30–150 mμ in diameter, approximately spherical, and are the most electron opaque material of the cell. It was shown in the case of *Aerobacter aerogenes* that their formation, which varies with the composition of the culture medium, is accompanied by an increase of the total phosphorus of the cell and by the appearance of substantial amounts of metaphosphates (SMITH, WILKINSON AND DUGUID[25]). The presence of metaphosphates has led to the idea that the volutin granules might play a role in the storage of energy (SCHMIDT[26]) and thus, although we have little precise information about their function, it would not be surprising to find cytochromes closely associated with them. These granules are visible in electron micrographs of the fraction WLP[23]. Although the 10 mμ particles which are present in the fraction SP can be seen in any part of the cytoplasm, it is not possible yet to decide whether the respiratory granules of the same size are also distributed in the whole cytoplasm or whether they are located at one particular point, for instance near or even in the cell membrane. Indeed, when the latter which is probably only 5 mμ thick (BRADFIELD[22]; MITCHELL AND MOYLE[27]) disintegrates, it is not unlikely that its pieces would coil up to form spherical particles with a diameter of about 10 mμ. The particles in the SP fraction are all approximately of the same size but they may represent a number of elements differing in their function and structure. For instance, it is known that the particulate fraction in bacterial extracts contains the bulk of the ribonucleic

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acid (RNA) of the cells and we can imagine that there is one kind of particle formed mostly of RNA and corresponding to the animal-cell microsome (PALADE AND SIEKEVITZ[28]).

The "ghost" fraction, after lysis of protoplasts from *Bacillus megatherium*, has been found to contain cytochrome pigments (WEIBULL[29]). If the "ghosts" are exclusively formed of cytoplasmic membranes, it follows that the cytochrome system is located in this membrane. This is the conclusion drawn by MITCHELL AND MOYLE[30] from their observation that "plasma membranes" isolated from *Staphylococcus aureus* by controlled autolysis contained the cytochrome system. However, the possibility that some cytoplasmic constituents adhere to the thin membrane cannot so far be excluded.

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SUMMARY

1. An extract from *Azotobacter vinelandii* was fractionated by centrifugation into a large particle fraction (WLP) and a small particle fraction (WSP), isolated respectively in a field of 22,000 *g* for 30 min and 145,000 *g* for 60–120 min, and the supernatant (S_3) from the latter centrifugation.

2. The small particles have a greater respiratory activity than the large particles, and also a higher P:O ratio with both succinate and DPNH as substrate. If prepared by centrifugation for 120 min at 145,000 *g*, S_2 has no respiratory activity.

3. The washed small particles do not oxidize α-ketoglutarate or fumarate unless S_2 is added. Under these conditions, oxidative phosphorylation was demonstrated with both substrates.

4. The effects of added S_2 on the rate of oxidation of succinate can be explained in terms of inhibition by oxaloacetate formed during the oxidation.

5. Treating the large particles with supersonic vibration for 0.5 to 5 min raised the specific activity of the DPNH oxidase or the succinic oxidase systems to the value characteristic of the small particles.

6. The respiratory chain in extracts of *Azotobacter vinelandii* is localized in the small particles. It is possible that these small particles are identical with granules of about the same size which can be seen in the cytoplasm in electron micrographs of the whole cell. It cannot be excluded, however, that they might be derived by the disintegration of the cell membrane. In any case, these granules differ in several respects from mitochondria, which do not appear to be present in bacterial cells.

7. The fraction WLP probably consists of a mixture of large particles (possibly volutin granules) and aggregated small particles or large pieces of the cell membrane. The small particles are liberated by the action of supersonic vibration.

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Addendum added in proof (July 17th, 1957). Since submitting the above paper, we have seen the paper by J. H. BRUEMMER, P. W. WILSON, J. L. GLENN AND F. L. CRANE [*J. Bacteriol.*, 73 (1957) 113] also describing the preparation of small particles (called electron-transporting particle) from extracts of *A. vinelandii*. The DPNH oxidase activity of these particles (prepared by alcohol fractionation) is about the same as that of the WSP described in the above paper when correction is made for the different temperatures employed for the measurements.

THE MECHANISM OF THE REACTION BETWEEN CYSTINE IN KERATIN AND SULPHITE/BISULPHITE SOLUTIONS AT 50° C

PART I

P. T. SPEAKMAN

Department of Textile Industries, University of Leeds (England)

INTRODUCTION

Many experiments have been described in which a keratin fibre — wool, human hair, etc. — has been stretched and then treated in some way so that, after boiling slack in distilled water, its final length differs from its initial length. If the final length is greater than the initial length, the phenomenon has been called "permanent set"; if the fibre is shorter after the treatment, it has been called "supercontraction". The physical mechanism of these changes is well-established[1]: cross-linkages between the protein chains of the keratin are broken by chemical treatment, and the supercontraction or permanent set of the fibre after treatment depends on the degree to which cross-linkages are re-formed between the protein chains in the extended fibre. There is evidence that the cross-linkage breakdown is a dynamic equilibrium, and that under the conditions of the initial chemical treatment described in this paper, the number of unbroken cystine disulphide cross-linkages at any time is very small.

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