The rate of cytochrome *c* reduction across the membrane can be controlled either by the surface area or by the thickness of the membrane. Buffer, substrate (succinate), submitochondrial particles and cyanide are required in order to produce the reduction.

In addition to reduction of cytochrome *c* across the membrane, a number of phenomena have been found to be associated with this reaction: (a) calcium ions increase the rate of the reaction only in the presence of a semipermeable membrane; (b) pH changes occur on both sides of the membrane and follow the kinetics of cytochrome *c* reduction; (c) gramicidin, a specific membrane potential-collapsing reagent[4], has an inhibitory effect on the reduction of cytochrome *c*. No inhibition has been observed in normal reduction.

In order to explain the possible mechanism of this reduction across the membrane, we have investigated the possibility of some soluble and dialysable component that could be released from the particles in our conditions. In fact a soluble factor capable of reducing cytochrome *c* has been detected after dialysis of the particles in the same conditions used for the assay, and its features are now under extensive investigation.

It is possible that such a soluble dialysable factor might be responsible for a shuttle transport of electrons across the membrane.

Otherwise the succinate-cytochrome *c* reductase activity in the presence of a semipermeable membrane seems to share certain features of a chemioosmotic process such as the one postulated by MITCHELL[5], namely the proton-translocating respiratory chain and the exchange-diffusion system coupling proton translocation to that of anions and cations.

Further experiments are in progress to utilize extensively this apparatus and system in order to evaluate the basic transfer of electrons in the respiratory chain and to investigate the characteristics of dialysable soluble factors that could play a fundamental role in such a process.

Riassunto. La riduzione del citocromo *c* può aver luogo anche quando sia separato dalla catena respiratoria mitocondriale da una membrana. L'azione del calcio e della gramicidina sono caratteristiche distintive tra la riduzione attraverso la membrana e la riduzione diretta.

L. GALZIGNA and L. SARTORELLI

Istituto di Chimica Biologica, Università di Padova (Italy), 31 July 1967.

[4] J. B. CHAPPELL and A. R. CROFTS, Biochem. J. *95*, 393 (1965).
[5] P. MITCHELL, Publ. No. 66/1 of Glynn Res. Ltd. (1966).

A Lytic Factor Associated with Brucellaphage Causing 'Lysis-from-without'

Concentrations of *Brucella abortus* phage, greater than the routine test dilution, cause lysis or inhibitory reactions on lawns of *Brucella suis*, *Brucella neotomae*, and some *Brucella melitensis* cultures, although the phage does not replicate on these cultures. An attempt was made to associate this phenomenon with a the activity of a lytic enzyme causing 'lysis-from-without'[1–4]. The present report describes the tube-test developed with *B. abortus* cells and demonstrates that lytic activity is bound to the infective phage particle. Studies relating the specificity of the lytic factor with phage typing reactions on other *Brucella* species will be reported elsewhere.

Materials and methods. The source and characteristics of the brucellaphage and its host propagating strain *B. abortus* R19 have been described previously[5]. Trypticase soy broth (BBL) was employed for growth of cells. High titer phage was obtained by centrifuging phage infected broth cultures before lysis occurred, resuspending cells in a small volume of *Tris* buffer and allowing lysis to proceed. The phage used in this study had a count of 2×10^{12} plaque-forming units (PFU)/ml and was stored at -20 °C. For demonstration of the lytic reaction, early log phase broth cultures incubated at 37 °C on a shaker were used within a few min of removal from the shaker. Glycine and ethylenediamine tetra acetic acid (EDTA) were added at final concentrations of 0.3 *M* and 0.0013 *M* respectively. The reaction mixture consisted of 1.6 ml broth culture ($2–3 \times 10^9$ cells/ml), 0.3 ml of 3 *M* glycine, 0.1 ml of 15% EDTA, 1 ml phage dilution in 0.1 *M* *Tris* buffer, pH 8. The change in optical density (O.D.) was measured in a spectrophotometer at intervals during incubation without shaking in a 37 °C waterbath. The % of the initial O.D. was determined at each time interval and plotted on semi-log paper.

Results. Figure 1 shows the decrease in O.D. with time, observed with various ingredients of the reaction mixture singly and in combination. Glycine and phage, or EDTA and phage do not cause as great a decrease as glycine, EDTA and phage.

Citrate (0.3 *M*) could be substituted for EDTA, and phosphate buffered saline (0.1 *M*, pH 8) could be substituted for *Tris* buffer. The rate of the reaction was greater at pH 7.8–8 than at pH 6.5–7, and at 37–40 °C than at 25–32 °C. No reaction occurred at 5 °C. Cells killed by toluene, heat, acetone, ether or formalin were not lysed. Chloramphenicol treated cells (25 µg/ml broth culture for 30 min at 37 °C) were lysed. Other genera (*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureas* and *Micrococcus lysodeikticus*) were not lysed.

Figure 2 shows the kinetics of the reaction using constant substrate and varying amounts of phage. One hundred PFU/cell caused 50% drop in O.D. in about 25 min. An excess of 400 PFU/cell did not speed the reaction time. If fewer than 25 PFU/cell were employed, the time required for lysis approached the latent period of the bacterium-phage system (e.g. 100 min). At least 5×10^{10}

[1] J. S. MURPHY, Virology *4*, 563 (1957).
[2] J. S. MURPHY and L. PHILIPSON, J. gen. Physiol. *45*, 155 (1962).
[3] H. STOLP and M. P. STARR, A. Rev. Microbiol. *19*, 79 (1965).
[4] W. WEIDEL, A. Rev. Microbiol. *12*, 27 (1958).
[5] C. R. MCDUFF, L. M. JONES and J. B. WILSON, J. Bact. *83*, 324 (1962).

PFU/tube were required to cause a decrease in O.D. greater than that of the glycine-EDTA control.

Neither lytic activity nor PFU count were reduced with dialysis against phosphate buffer at 5 °C for 48 h, nor treatment at 50 °C for 30 min but both were lost after treatment at 75 °C for 30 min. All lytic activity was lost by treatment with trypsin (100 μg/ml at 37° C for 30 min), most activity was lost by similar treatment with papain, and a drop in PFU also followed these treatments. Micrococcal nuclease (100 μg/ml) had no effect on lytic activity or PFU. Treatment with anti-phage serum greatly reduced lytic activity. The lytic activity was sedimented with phage following centrifugation at 20,000 *g* for 2 h.

Density gradient centrifugation was performed by dissolving cesium chloride in concentrated phage to a final density of 1.49, centrifuging for 20 h at 5 °C at 30,000 rpm in a Spinco SW39 swinging bucket rotor, collecting fractions in *Tris* buffer and assaying for PFU and lytic activity expressed as:

$$K = \frac{\log \text{O.D. at 30 min} - \log \text{O.D. at 60 min}}{30} \times 100 .$$

The close correlation between lytic activity and PFU can be seen in Figure 3.

Figure 4 shows the effect of aeration on lysis of phage infected cultures. Phage was added to one of the early log phase broth cultures to give a phage:cell input ratio of 1:1. The addition of phage reduced the growth rate but overt lysis did not occur as long as the culture remained on the shaker, whereas rapid lysis of stationary samples occurred 4 h after addition of phage. Samples taken from the flask without phage did not change their O.D. during stationary incubation. When available oxygen is reduced, *Brucella* cells stop multiplying and phage infected cells lyse rapidly. The samples of lysed cultures were immediately tested for lytic activity in the tube test (i.e.

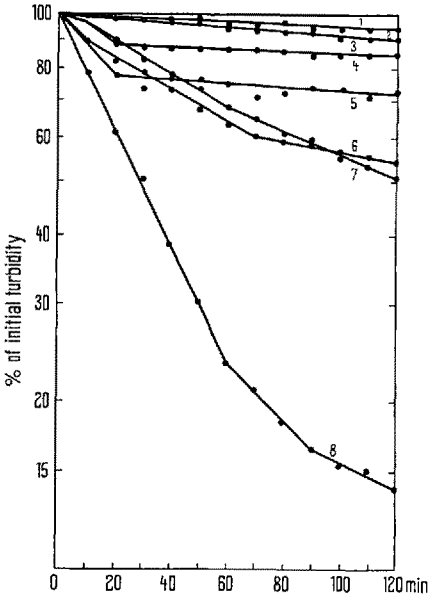

Fig. 1. Change in optical density of a broth culture of *B. abortus* in presence of: (1) *Tris* buffer (turbidity unchanged), (2) EDTA, (3) phage, (4) glycine, (5) EDTA + glycine, (6) glycine + phage, (7) EDTA + phage, (8) EDTA + glycine + phage. Phage:cell ratio, 68:1 in (3), (6), (7) and (8).

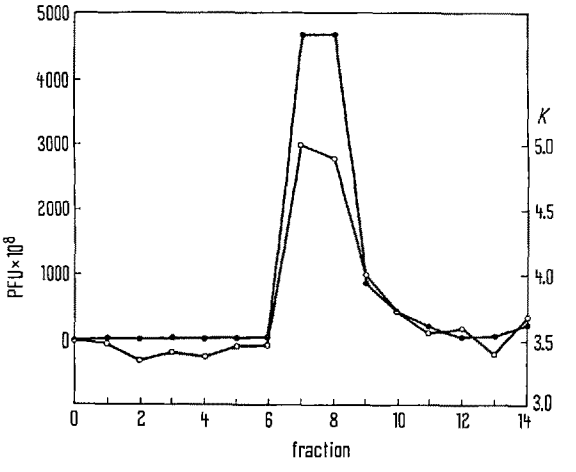

Fig. 3. Cesium chloride density gradient centrifugation of brucella-phage. Correlation of lytic activity, *K*, (open circles) and PFU (closed circles) in each fraction.

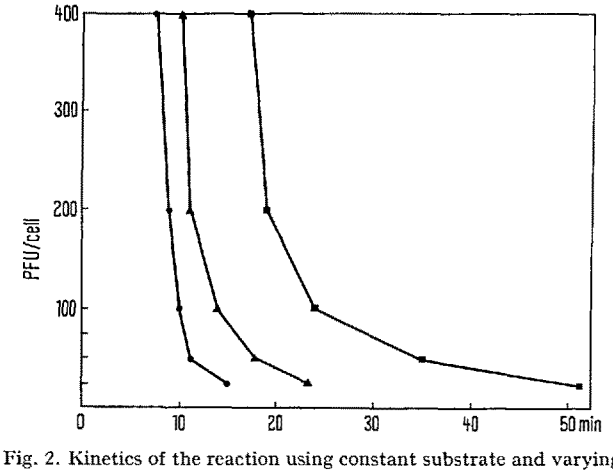

Fig. 2. Kinetics of the reaction using constant substrate and varying amounts of phage. The number of PFU/cell plotted against time required for 25% (circle), 33% (triangle) and 50% (square) decrease in initial optical density.

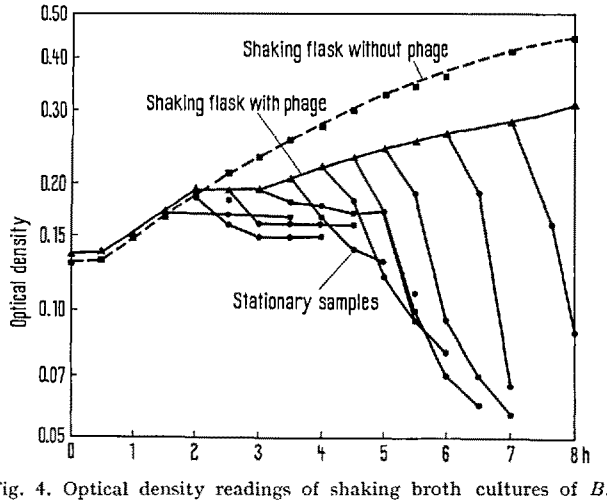

Fig. 4. Optical density readings of shaking broth cultures of *B. abortus* with and without phage and stationary samples taken at intervals from the broth culture with phage.

non-infected broth cultures + glycine + EDTA). Their lytic activity was as great as, but not greater than could be accounted for on the basis of PFU count. Free lytic enzyme is apparently not released in the brucellaphage system.

Discussion. The phenomenon of 'lysis-from-without' occurs when a large number of phage particles simultaneously attack the bacterium, and the phage enzyme damages the cell wall faster than the cell can repair it[3,4]. Both EDTA[6] and glycine[7] have been used to sensitize gram-negative cells to the action of lysozyme, and EDTA was employed to sensitize *E. coli* K 12 cells to the action of endolysin from lambda phage[8]. These substances also sensitize *Brucella* cells to 'lysis-from-without' by a lytic agent associated with brucellaphage.

The similarities between this lytic agent and the lysozyme associated with other bacteriophage are: (1) its inactivation by proteolytic enzymes, (2) the kinetics of the reaction which show that the decrease in turbidity of the substrate/unit time varies directly with enzyme concentration, and (3) its specificity. The lytic agent could not be separated from the brucellaphage particle by differential centrifugation, anti-phage serum inactivation or by cesium chloride density gradient centrifugation. MURPHY and PHILIPSON[2] described a lytic enzyme which was bound to the *Bacillus megaterium* phage G particle. In contrast, other phage lysins[1,3,4,9] were separable from the phage particle[10].

Résumé. Des bactériophages de Brucella se sont montrés capables de provoquer une lyse extra-cellulaire, en cultures liquides de *B. abortus*, en présence de glycine et d'acide éthylène-diamine-tétraacétique. Des traitements au sérum anti-bactériophage, aux enzymes protéolytiques, ou la centrifugation différentielle dans le chlorure de césium n'ont pu séparer l'activité lytique de la particule infectieuse elle-même.

L. M. JONES, G. S. MERZ[11] and J. B. WILSON

Department of Bacteriology, University of Wisconsin, Madison (Wisconsin 53706, USA), 21 July 1967.

[6] R. REPASKE, Biochim. biophys. Acta *30*, 225 (1958).
[7] D. J. RALSTON, B. S. BAER and S. S. ELBERG, J. Bact. *82*, 354 (1961).
[8] N. B. GROMAN and G. SUZUKI, J. Bact. *86*, 187 (1963).
[9] D. J. RALSTON and M. MCIVOR, J. Bact. *88*, 667 (1964).
[10] This study was supported by Public Health Service research grant No. AI 06161 from the National Institute of Allergy and Infectious Diseases and by a World Health Organization research grant. The authors are grateful to Dr. D. PRATT for helpful suggestions and assistance with the density gradient centrifugation.
[11] Present address: The Wistar Institute, Philadelphia, Pennsylvania.

Two $NADH_2$ Dehydrogenases in the Basidiomycete *Oudemansiella mucida*

During our study of oxidation-reduction systems in higher fungi, we followed enzymatic oxidation and dehydrogenation of nicotinamide adenine dinucleotide ($NADH_2$) as the first step in the respiratory chain, using the basidiomycete *Oudemansiella mucida* (Schr. ex Fr.) Höhnel.

The experiments were carried out with the acetone powders of the mycelium grown for 6 days at 25 °C in a laboratory fermenter at 350 rpm of the stirrer, the intensity of aeration being 25 mmoles O_2/1000 ml medium/h. The nutrient medium contained glucose 5.0%, corn-steep liquor (50% dry weight) 1.5%, $MgSO_4$ cryst. 0.15%, dissolved in tap-water, initial pH 5.5

The first results showed a very slow course of the $NADH_2$ oxidation in the presence of aerial oxygen. It was considered that the acetone treatment could remove some lipophilic limiting factors necessary for the undisturbed transfer of electrons to oxygen. In order to test this possible explanation, the enzymatic dehydrogenation of $NADH_2$ was followed using 2 artificial electron acceptors – 2,6-dichlorophenol-indophenol (DIF) and 2,3,5-triphenyltetrazolium chloride (TTC).

For the estimation of $NADH_2$ dehydrogenase activity the following system was adopted: $NADH_2$ 0.31 μmoles, phosphate buffer 150 μmoles, DIF 1.5 μmoles or TTC 3.0 μmoles, aqueous suspension of the acetone powder or its cell-free extract, total volume 4.0 ml. As the enzyme source, a homogenized suspension of 80 mg acetone powder in 5.0 ml distilled water (preparation A) was used, or the respective cell-free extract prepared by eluting 80 mg acetone powder with 5.0 ml of distilled water for 3 h at 4 °C and centrifuging 2 min at 800 *g* (preparation B), or cell-free extract B diluted 1:5 with distilled water (preparation C). The period of incubation at 27 °C depended on the sort of terminal electron acceptor used and on the concentration of the respective enzyme preparation. It is specified in the Tables.

Continual colorimetry at 600 nm was used for the estimation of DIF reduction, whereas the amount of the red formazane formed by the reduction of TTC was followed using the colorimetry at 480 nm according to a modification of the procedure introduced by LINDEMANN[1].

The results of experiments dealing with $NADH_2$-DIF reductase have shown considerable activity of this enzyme which could be readily eluted from the acetone powder by distilled water (Table I). Its optimal pH was

Table I. Elution of the $NADH_2$-dichlorophenol-indophenol reductase from the acetone powder of *O. mucida*

Enzyme preparation (0.2 ml)	Protein content mg	Extinction at 600 nm after the incubation period (sec)					
		0	1	15	30	45	60
A (suspension)	1.203	0.25	0	0	0	0	0
B (extract)	0.455	0.25	0.15	0.05	0	0	0
C (diluted extract)	0.091	0.25	0.25	0.22	0.19	0.16	0.14

[1] J. LINDEMANN, Schweiz. Z. allg. Path. Bakt. *17*, 311 (1954).