BBA 45 565

ATP PRODUCTION IN THE LIGHT AND THE DARK BY VESICLE PREPARATIONS ISOLATED FROM *CHLOROBIUM THIOSULPHATOPHILUM* L660

J. SYKES AND J. A. GIBBON
Department of Biochemistry, The University, Sheffield (Great Britain)
(Received January 2nd, 1967)

SUMMARY

The "chromatophore fraction" isolated from *Chlorobium thiosulphatophilum* and physico chemically characterised by SYKES, GIBBON AND HOARE[6] is shown to be identical with the vesicular elements described in an electron microscope study of this organism by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1]. These "Chlorobium vesicles" may therefore be defined as membrane-bounded sub-cellular organelles (300–400Å wide, 1000–1500Å long), free in the cytoplasm, carrying the bulk of the Chlorobium chlorophyll, sedimenting with an $s^0_{20,w}$ of 116 S and predominantly lipoprotein in nature.

The present report additionally demonstrates that these well defined cellular elements will catalyse active photophosphorylation under defined conditions and this reaction may be strongly inhibited by 5 μM carbonyl-*m*-chlorophenylhydrazone (CCCP). Other features of this photophosphorylating system are also described.

A high level of ATP production in the dark (control) systems is invariably observed. This ATP production is insensitive to CCCP. Experiments are described which demonstrate that vesicle or crude cell-free extract preparations from *C. thiosulphatophilum* contain inorganic pyrophosphates and these are metabolised in a light-independent ADP-requiring reaction to form ATP.

The rates of ATP production in the light and in the dark are correlated with the batch growth characteristics of this organism.

INTRODUCTION

The structural basis for the photosynthetic activity of the green- and purple-sulphur bacteria has been more clearly defined in recent studies employing the electron microscope[1–5] and by physicochemical techniques[6]. In the green bacterium *Chlorobium thiosulphatophilum* COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] have observed distinct vesicles in the fine structure of the cells. The vesicles are elongated bodies, 300–400 Å wide and 1000–1500 Å long, located directly under the cytoplasmic membrane. The membrane surrounding the vesicles appears to be distinct from the cytoplasmic membrane (*cf.* HOLT AND MARR[4]). These Chlorobium vesicles were reported to be associated with a centrifugal fraction isolated from cell-free extracts having a high

Abbreviation: CCCP, carbonyl-*m*-chlorophenylhydrazone.

specific chlorophyll content. SYKES, GIBBON AND HOARE[6] also isolated and physicochemically characterised a fraction containing the bulk of the Chlorobium chlorophyll. This fraction which was referred to as "the chromatophore fraction", sedimented at an $s^0_{20,w}$ of 116 S and its sedimentation was strongly dependent on concentration, suggesting an asymmetric particle. Furthermore the kinetics of release of the chromatophore fraction from the cells indicated that it was a true cytological element and not derived by comminution of a larger structure (*cf.* HOLT AND MARR[4]). The present work confirms the identity of the chromatophore fraction isolated by SYKES, GIBBON AND HOARE[6] with the "Chlorobium vesicles" of COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] and describes the properties of ATP-generating systems found in these preparations. Photophosphorylation has not previously been demonstrated in well defined vesicular structures from green sulphur bacteria.

MATERIALS AND METHODS

Organism and culture

A strain of *C. thiosulphatophilum* L660 was used. The organism was maintained and cultured by methods previously described[6]. For the experiments recording the changes in cell composition and metabolic activity during growth, the cultures were grown in 1-l wash bottles fitted with rubber tubing and screw clips. These bottles were filled with media, inoculated and flushed out with N_2 gas. The N_2 was freed from traces of O_2 by passing it through a reducing mixture containing anthraquinone-2-sulphonic acid, NaOH and sodium dithionite. Samples of the culture were withdrawn at recorded intervals by blowing out with this O_2-free N_2, the culture being shaken vigorously prior to sampling. The turbidity of the sample was measured at 540 mμ after filtration through Whatman No. 42 filter paper.

Preparation of extracts

Growing cultures were harvested and the cell paste suspended in 0.1 M Tris–HCl containing 5 % sucrose (pH 7.8) in the ratio 1 part paste to 5 parts buffer. The suspension was then subjected to 60–90 sec sonic oscillation at 0–5° with a precooled titanium probe fitted to a 60-W M.S.E. sonic disintegrator (Measuring & Scientific Instruments Ltd., London). The dispersed cell homogenate was then centrifuged at 8000 $\times$ *g* (max.) for 30 min in an M.S.E. Mistral centrifuge (high-speed head No. 69402) to give a debris fraction and crude extract. Further centrifugation of the crude extract at 68475 $\times$ *g* (max.) for 90 min in the SW 39 head of a Spinco Model L2A preparative ultracentrifuge gave a pigmented fraction similar to the P_1 fraction prepared by SYKES, GIBBON AND HOARE[6]. Chromatophore fractions were prepared exactly as described in this earlier paper.

Estimation of photophosphorylation activity

Photosynthetic phosphorylation was measured anaerobically (gas phase, O_2-free N_2), in the light in Thunberg tubes. Identical tubes were set up to act as dark controls, and these were incubated in a similar manner completely wrapped in aluminium foil to exclude light. Each tube contained per ml, 100 μmoles D-(+)-glucose; 50–70 μmoles Tris–HCl buffer (pH 7.8) containing 150–210 μmoles sucrose; 10 μmoles phosphate (buffered at pH 7.6); 15 μmoles $MgCl_2$; and 20 μg hexokinase (E.C. 2.7.1.1) containing

approximately 2.8 I.U. of activity. 30 μg Chlorobium chlorophyll contained within a sample of vesicle preparation or crude cell-free extract was added last to the tubes. The side arm contained 15 μmoles ADP (Sigma Chemical Co.). The tubes were incubated at 20° usually for 15 min and illuminated by two 100-W tungsten bulbs placed at a distance of 30 cm from the tubes. The reaction was initiated by tipping the ADP from the side arm, allowed to continue for 15 min and stopped by opening the tube and making the reaction mixture 0.5 M with respect to HCl. The tubes were then chilled in ice, and the precipitates which formed removed by centrifugation. The supernatants were then carefully brought to pH 7–8 with 10 % NaOH. Samples of the supernatant were assayed for glucose 6-phosphate spectrophotometrically, with glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49). The assay cuvettes contained 200 μmoles Tris–HCl buffer (7.8); 5 μmoles NADP and the sample to assayed. The reaction volume was 3 ml. The reaction was started by the addition of 5 μg glucose-6-phosphate dehydrogenase (approx. 0.7 I.U. of activity). The absorbance change at 340 mμ was read on completion of the reaction at ambient temperature (approx. 20°). In all cases the cuvettes were read against the appropriate blanks. Hexokinase and glucose-6-phosphate dehydrogenase were obtained as $(NH_4)_2SO_4$ suspensions from Boehringer Chemical Co. For assays the $(NH_4)_2SO_4$ suspensions were routinely diluted with 1 % bovine serum albumin.

Metachromasy measurements

Substances having the property of causing spectral shifts in certain basic dyes *e.g.* toluidine blue, are known as metachromic substances[7]. This property of metachromasy has been used as a diagnostic test for certain substances particularly polyphosphate. Metachromasy measurements were made on a Beckman DB recording spectrophotometer equipped with a Sargent recorder. Each cuvette contained 5 μmoles polyphosphate or cell extract; 2 μg toluidine blue and water up to a volume of 3 ml. The blank cuvette contained distilled water.

Electron microscopy

Fixation, dehydration and staining of cells for examination in the electron microscope was performed as described by RYTER AND KELLENBERGER[8], except that the main fixation was reduced to 6 h. Araldite (Ciba, A.R.L. Ltd., Duxford, Cambridge, England) was used as the embedding material. All sections were post-stained with lead citrate according to the procedure of REYNOLDS[9]. Thin sections were cut with a Porter–Blum microtome. Negatively stained preparations were made by the method of HUXLEY AND ZUBAY[10] and stained with potassium phosphotungstate (pH 6.0). All specimens were examined on an A.E.I. Model 6 electron microscope (Associated Electrical Industries Ltd., England).

Chemical determinations and chemicals

Phosphate was determined by the method of SUMNER[11]. Polyphosphate was extracted and estimated according to the method described by COLE AND HUGHES[12]. Synthetic polyphosphate was prepared by the method of PFANSTIEL AND ILER[13]. Chlorobium chlorophyll was measured spectrophotometrically on methanolic extracts of whole cells or fractions derived from them, assuming the extinction coefficients reported by STANIER AND SMITH[14].

Protein was estimated by the method of Lowry *et al.*[15] for the experiments determining changes in protein during batch growth and also by the biuret method[16]. For both estimations bovine serum albumin (Armour & Co. Ltd., Chicago, U.S.A.) was used as a standard. The pigment associated with the fractions for analysis was first removed from a measured sample by successive treatments with hot ethanol.

Carbonyl-*m*-chlorophenylhydrazone (CCCP) was obtained from California Co. for Biochemical Research, Los Angeles, U.S.A. The water-soluble sodium salt of CCCP was prepared fresh daily by dissolving the solid in dilute NaOH.

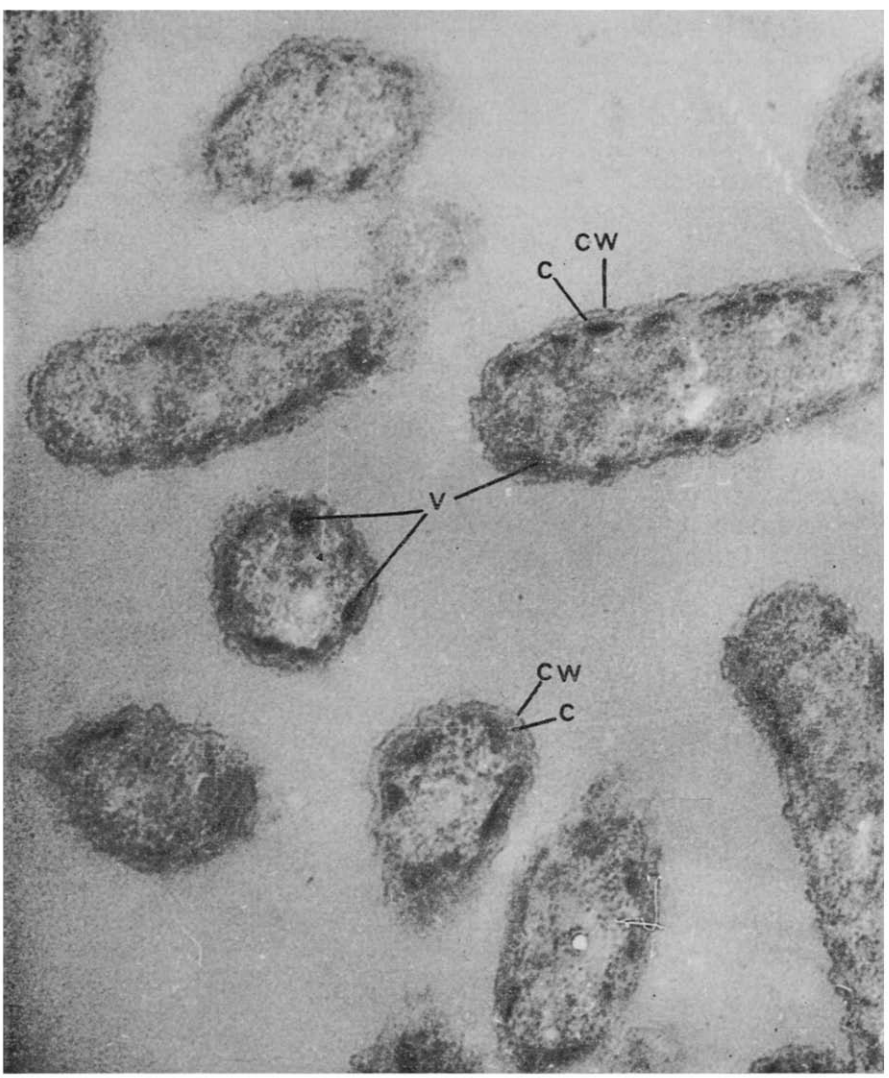


Fig. 1. Electron micrograph of a thin section of *C. thiosulphatophilum* Araldite embedded and post-stained with REYNOLDS' lead stain. C, cytoplasmic membrane; V, vesicles; CW, cell wall. Magnification × 64000.

RESULTS

Electron microscopy of whole cells and chromatophore fractions

Fig. 1 is an electron micrograph of a thin section of *C. thiosulphatophilum* whole cells embedded in Araldite. The structures (V) visible under the cytoplasmic membrane are similar in their position in the cell and their shape to those found by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] in sections of Epon-embedded specimens of this organism. Fig. 2 is a negatively stained preparation of the 'chromatophore fraction', isolated by the procedure of SYKES, GIBBON AND HOARE[6], and described as being essentially homogeneous in the ultracentrifuge and on density gradient analysis. This plate shows that the fraction consists predominantly of elongated structures 670–1200 Å long by 230–370 Å wide. Ribosomal contaminants (R) are clearly visible as found by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] and as would be expected from the data given by SYKES, GIBBON AND HOARE[6] (see Fig. 2a and Table I). From these plates it can be concluded that the chromatophore fraction as prepared and characterised by SYKES, GIBBON AND HOARE[6] exactly corresponds to the 'Chlorobium vesicles'

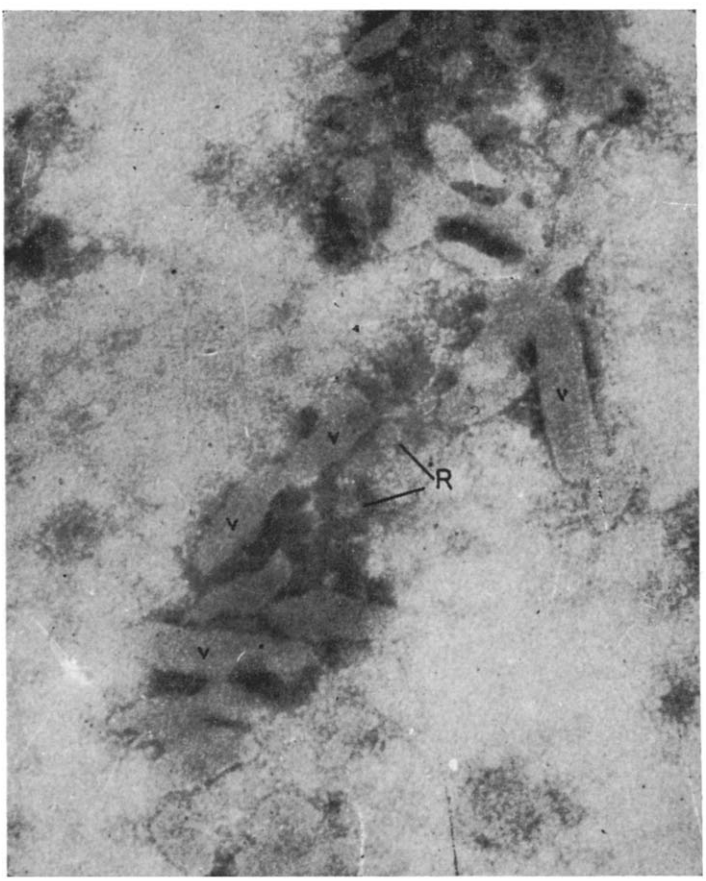


Fig. 2. Purified vesicles negatively stained with phosphotungstate. V, Chlorobium vesicles; R, ribosomes. Magnification × 114000.

described by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] for this organism. Henceforth the term Chlorobium vesicles will be used to describe this fraction.

Photophosphorylation by Chlorobium crude extracts and vesicles

Table 1 shows the rates of photosynthetic phosphorylation achieved with pigment fraction P_1 (*cf.* SYKES, GIBBON AND HOARE[6]) under a variety of experimental conditions. The rates of ATP produced per mg chlorophyll per h in the preparations varied within the range 10–43 μmoles ATP, with most of the preparations falling in the range 20–30 μmoles. In crude cell-free extracts rates of photosynthetic phosphorylation as high as 200 μmoles ATP per mg chlorophyll per h were obtained, and in general, rates in crude extracts were always higher than those in more purified preparations. Presumably some factor was removed during further purification of the vesicles. The results in Table I clearly demonstrate that the reaction is dependent on the presence of ADP, light and phosphate. The dependence on added phosphate was found to be variable and presumably was a reflection of the endogenous level of phosphate in the preparation used. O_2 does not appear to strongly inhibit the reaction since the aerobic photosynthetic production of ATP was 85 % of the anaerobic rate. However, preparations stored under aerobic conditions at 0–4° lost 43 % of the photosynthetic activity in 24 h while similar preparations stored under anaerobic conditions at 0–4° remained active for 2–3 days without loss of activity. A large production of ATP in the dark controls was invariably observed and this phenomenon will be discussed later. Tests on the efficiency of the hexokinase trap showed that under conditions of the assay quantitative recovery of added ATP could be made. Furthermore, added glucose 6-phosphate could also be totally recovered from the assay system, indicating that this compound was not metabolised to any appreciable extent by the extract under the conditions of the assay. Fig. 3 shows the variation of photophosphorylation with pH. The range of pH tested was limited to 7–9 as yeast hexokinase has a broad pH optimum from 6–9 (*cf.* ref. 17) and is thus most active at these pH values. Therefore, the variation of activity is a true response by the photophosphorylation system to pH and not a reflection of the activity of the hexokinase trap. The amount of ATP produced in the light varied with the concentration of added

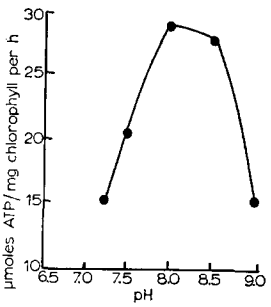
TABLE I

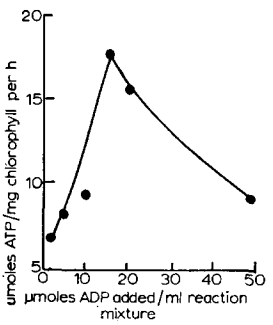
DEMONSTRATION OF PHOTOSYNTHETIC PHOSPHORYLATION BY CHLOROBIUM VESICLES

Vesicles were prepared as described in the text, and photophosphorylation measured as described in MATERIALS AND METHODS.

Experimental conditions	*μmoles ATP per mg chlorophyll per h*
1. Illuminated anaerobic	43.6
2. Dark anaerobic	18.6
3. Illuminated aerobic	37.2
4. Dark aerobic	22.4
5. As condition 1 *minus* phosphate	28.0
6. As condition 1 *minus* ADP	0.4
7. As condition 1 *minus* extract	0.3

ADP in the manner shown in Fig. 4. This has been observed for other photophosphorylating systems[18]. In all subsequent photophosphorylation experiments 15 μmoles ADP/ml reaction mixture were used. Fig. 5 shows that the response of the reaction was nearly linear with time, and Table II shows that the amount of ATP produced increased with the amount of chlorophyll added to the reaction mixture.





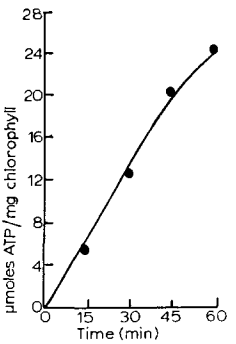


Fig. 3. Variation of net (illuminated — dark) photophosphorylation ability of Chlorobium vesicles with pH.

Fig. 4. Variation of the photophosphorylation ability (illuminated — dark produced ATP) of Chlorobium vesicles with differing levels of ADP.

Fig. 5. Variation of net ATP (illuminated — dark) produced with time by Chlorobium vesicles prepared as in MATERIALS AND METHODS.

TABLE II

VARIATION OF THE PHOTOPHOSPHORYLATION REACTION WITH VESICULAR CHLOROPHYLL CONCENTRATION

Chlorobium vesicles and method of ATP estimation as described in the text.

Amount of vesicular chlorophyll added (μg)	*Net ATP* (μmoles) *produced per h*
47	0.2
97	0.4
194	0.7

The inhibition and stimulation of ATP formation in the light and in the dark

HEYTLER[19] found that CCCP was an effective uncoupler of oxidative phosphorylation in plant, animal and insect mitochondria. He also found that at the low concentration of 5 μM it inhibited cyclic photophosphorylation by 80 % in spinach chloroplasts. CCCP was therefore added to the usual Chlorobium vesicle phosphorylation mixture (MATERIALS AND METHODS) at two final concentrations (5 and 1 μM). The inhibitor was added to both light and dark reaction systems. The net light reaction (*i.e.* light—dark) was inhibited 75 % by 5 μM and 13 % by 1 μM CCCP. In contrast, the dark reaction was only inhibited 15 % by 5 μM and was not inhibited at all by 1 μM CCCP. Whilst these results are those expected for a system carrying out cyclic photophosphorylation, and confirm that the Chlorobium vesicles carry out this

reaction, they strongly indicate that the mechanism for ATP production in the dark was probably not an oxidative phosphorylation reaction.

The levels of phosphorylation activity in the light and dark reactions were invariably lower in purified vesicle preparations compared with the cell-free extracts. Ascorbate (final concn. 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} M), phenazine methosulphate (final concn. 1 mM), and crude preparations of *Clostridium pasteurianum* ferredoxin all failed to stimulate the vesicle photophosphorylating system. However, stimulation of ATP formation was obtained with a fraction prepared from cell-free extracts as follows. The supernatant remaining after the removal of the vesicles from the cell-free extract (*i.e.* S_1 fraction of Scheme I in SYKES, GIBBON AND HOARE[6]) was saturated with solid $(NH_4)_2SO_4$ at 0–5° with continuous stirring during the addition of the salt. The precipitate was allowed to form over a period of 1 h in the cold and it was collected by centrifugation, resuspended in 0.1 M Tris–HCl buffer (pH 7.8) and dialysed in the cold against distilled water. The solution (labelled AS_1) was diluted (approx. 8 ×) with water so that the ratio of absorbances at 260/280 mμ was 0.69, *i.e.* approx. 0.2 mg protein/ml (*cf.* ref. 20). A 1.4-fold stimulation of the rate of vesicle ATP formation was obtained when the fraction was added at the level of 0.1 ml per ml assay mixture (Table III). However, the dark reaction was also enhanced by a similar amount (1.5 ×) and AS_1 alone exhibited a comparable level of activity to the dark reaction (Table III). In a further experiment a concentrated preparation, AS_2, was used (0.1 ml per ml of assay mixture). AS_2 alone showed an activity increase (6.6 ×) approximately equal to the increase in protein added (7 ×). The slight additional reaction (*cf.* dark in Table III) in the illuminated AS_2 alone is probably accounted for by the small residual vesicle content of this fraction since it was pigmented and found to contain 10.1 μg chlorophyll per ml (*cf.* vesicle preparation used in this experiment which contained 1250 μg chloroplyll per ml, 50 μg used in this assay). The ATP production in the dark with the vesicles and the observation that the supernatant fraction equally stimulates ATP production in the light and the dark reaction systems therefore appears to be due

TABLE III

EFFECTS OF ADDITION OF VARIOUS SUPPLEMENT FACTORS ON THE PHOTOPHOSPHORYLATION ACTIVITY OF CHLOROBIUM VESICLE PREPARATIONS

Preparation of the $(NH_4)_2SO_4$ precipitates and vesicles as described in MATERIALS AND METHODS. AS_1, $(NH_4)_2SO_4$-treated supernatant in dilute form; AS_2, $(NH_4)_2SO_4$-treated supernatant in concentrated form; AS_3, $(NH_4)_2SO_4$-treated supernatant after further treatment with DEAE-cellulose.

Reaction conditions	*Supplement*	*μmoles ATP/assay tube per h*			
		Vesicle prep., alone	*Vesicles + supplement*	*Vesicles + boiled supplement*	*Supplement, alone*
Illuminated	AS_1	0.34	0.48	0.17	0.17
Dark		0.15	0.23	0.13	0.21
Illuminated	AS_2	0.44	2.1	1.5	1.7
Dark		—	1.3	—	1.4
Illuminated	AS_3	0.34	0.39	0.30	0.19
Dark		0.15	0.22	0.20	0.16
Illuminated	Ferredoxin	0.39	—	—	—
Dark		0.21	—	—	—

to the addition of an ATP-generating enzyme system, together with its substrate, which is insensitive to CCCP and which is found in both the vesicles and supernatant fractions. A likely candidate is the inorganic polyphosphatase of *C. thiosulphatophilum*[12] which catalyses the following reaction:

$$(\text{polyphosphate})_n + \text{ADP} = \text{ATP} + (\text{polyphosphate})_{n-1}$$

Coupled with an efficient hexokinase ATP trap, as used in this assay system, total conversion of the polyphosphate to glucose 6-phosphate would then take place. This trap would minimise the subsequent breakdown of ATP by ATPases reported to be present in this organism[12].

Polyphosphate is undoubtedly present in the strain of *C. thiosulphatophilum* used in these studies since samples of the organism taken from various stages in the batch growth cycle and analysed for polyphosphate by the procedure described in MATERIALS AND METHODS gave the results shown in Table IV.

TABLE IV

POLYPHOSPHATE DISTRIBUTION IN CELLS OF *C. thiosulphatophilum*

Polyphosphate was extracted and then estimated as inorganic phosphate according to the procedures described by COLE AND HUGHES[12]. The '5% trichloroacetic acid' values represent a straight estimation of phosphate extractable by 5% trichloroacetic acid *i.e.* low molecular weight polyphosphates and inorganic phosphate. The 10% trichloroacetic acid values represent extracted polyphosphates which have been selectively precipitated with barium salts and estimated as hydrolysable phosphate.

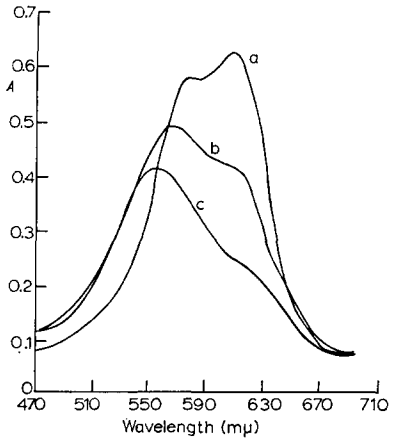
Age of culture	*Inorganic polyphosphate (μmoles/10 mg protein)*	
	5% trichloroacetic acid	*10% trichloroacetic acid*
Early logarithmic phase	3.86	1.3
Stationary phase	4.15	0.69
Stationary phase	4.65	0.75

These values may be low estimates of the level of polyphosphate since the methods of extraction in current use have been shown to give incomplete extraction of all polyphosphates[21].

Enzymic estimation (see below) gave values of 1.1 μmoles polyphosphate (expressed as inorganic phosphate) per mg protein in a crude extract of the cells. Polyphosphate may also be detected by its metachromic reaction with toluidine blue. Fig. 6 demonstrates this point, Fig. 6a showing the absorption spectrum of toluidine blue alone, Fig. 6b toluidine blue with added Chlorobium extract, the extract being in the form of the supernatant fraction (AS_1) and Fig. 6c shows toluidine blue with added synthetic polyphosphate. From the spectral shifts observed there is a clear indication of polyphosphate in AS_1.

Attempts to remove the polyphosphate from AS_1 by dialysis against 0.1 M Tris–HCl + 0.001 M EDTA (pH 7.8) followed by stirring with DEAE-cellulose were not successful as the data in Table III show. This preparation, AS_3, was tested with the same Chlorobium vesicle preparation as AS_1 and added at the same protein concentra-

tion. This treatment has clearly produced little differences in the light and dark ATP production with the vesicles and in AS_3 alone. Polyphosphate is known to be bound extremely strongly to proteins[22].



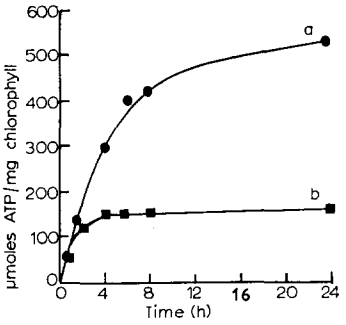


Fig. 6. The effect of added synthetic polyphosphate (c), Chlorobium supernatant AS_1 (b) upon the absorption spectrum of toluidine blue (a).

Fig. 7. Rate of production of ATP in the dark by crude extracts of Chlorobium with and without synthetic polyphosphate. (a) Chlorobium extract + 5 μmoles synthetic polyphosphate per ml reaction mixture; (b) control experiment Chlorobium extracts alone. The system for the production of ATP and the assay for the ATP are described in MATERIALS AND METHODS.

As a further indication of the inorganic polyphosphatase activity, the addition of additional synthetic polyphosphate substrate should lead to increased production of ATP over the unsupplemented (control) system. Fig. 7 shows the results obtained from this type of experiment. The figure shows that the addition of synthetic polyphosphate to a crude extract of cells did not increase the initial rate of the dark reaction, but rather extended its linearity. This type of result indicates that the polyphosphatase was already saturated with polyphosphate present in the crude extract. The K_m for polyphosphate for *Escherichia coli* inorganic polyphosphatase is 47 μM (based on total phosphate residues). In this experiment 5.5 μmoles synthetic polyphosphate per ml of reaction mixture were added and 6.0 μmoles ATP per ml reaction mixture produced in excess of the control level. This result would indicate that for every μmole polyphosphate consumed a μmole ATP is produced. This would agree with the scheme postulated by COLE AND HUGHES[12] for the production of ATP from polyphosphate. A consideration of Fig. 7 shows that, as the initial rates in experiments (a) (supplemented) and (b) (control), are the same, the mechanism for the production of ATP in the dark must be the same. A different mechanism for the utilisation of the added polyphosphate would give an enhanced initial rate of ATP production in system (a) and this was never observed even in identical experiments with frequent sampling during the initial (1 h) period of incubation. From curve (a) it can be seen that in the first hour 50 μmoles of ATP per mg chlorophyll are produced in the dark via the polyphosphatase enzyme. Since 15 % of the dark reaction has been shown to be sensitive to CCCP, the residual 85 %, *i.e.* 42.5 μmoles ATP per mg chlorophyll, can be said to represent the maximum yield due to polyphosphate in the dark during

the first hour of the reaction. From a consideration of the typical values obtained in dark controls, this value is sufficient to account for most of the ATP produced in the dark by crude cell-extracts.

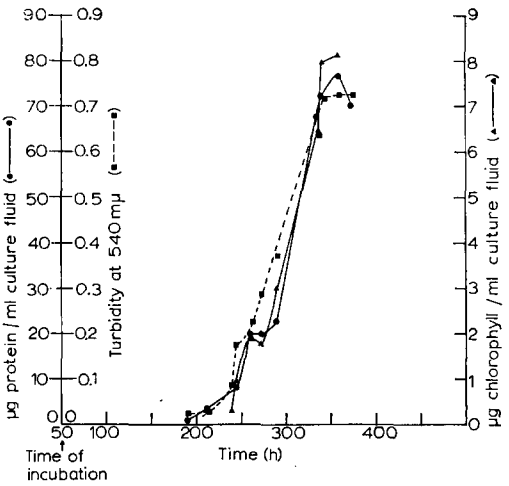


Fig. 8. Cell turbidity, protein and chlorophyll changes during growth of *C. thiosulphatophilum* in batch culture, the sampling and the chemical estimations being carried out as described in MATERIALS AND METHODS. ■—■, turbidity at 540 mμ; ●—●, protein; ▲—▲, chlorophyll.

Levels of ATP production and other sub-cellular constituents during growth

Fig. 8 shows the variation of levels of pigment, total protein and cell density during growth in batch culture. The inoculum for these experiments was a culture which had been sub-cultured every 24 h to ensure that the inoculum was predominantly young cells. From this figure it is obvious that all of the above-mentioned constituents increase in parallel fashion until there is some limiting factor in the medium; they then reach a maximum and decline. The production of chlorophyll at the end of the growth becomes extremely rapid and similar results have been found with *Euglena gracilis*[24]. This rapid increase may be a reflection of an effort by the cells to maintain energy production as the medium becomes limiting, or it may be that, as

TABLE V

PRODUCTION OF ATP BY PHOTOSYNTHETIC PHOSPHORYLATION DURING GROWTH OF A CULTURE OF *C. thiosulphatophilum*

ATP was estimated as described in MATERIALS AND METHODS; crude extracts were used, the extracts also being prepared as described in MATERIALS AND METHODS.

Time of sampling after inoculation of culture (h)	*Absorbance of culture*	*μmoles ATP per mg chlorophyll per h*	
		Light	*Dark*
91	0.140	37.0	13.8
116	0.270	46.1	18.4
120	0.285	40.8	22.4
144	0.400	27.6	11.1

the differential rate of chlorophyll synthesis is an inverse function of light intensity, this rapid production of chlorophyll is a direct consequence of light limitation[25] following upon the increase in turbidity of the culture.

Table V shows the results of a similar experiment in which the levels of ATP produced in the light and dark and the cell density were measured during growth in batch culture. It can be seen that the cells are most active in producing ATP in the early exponential phase, and that this activity falls as the stationary phase is reached.

DISCUSSION

The results presented in this paper show that the chromatophore fraction isolated from *C. thiosulphatophilum* by SYKES, GIBBON AND HOARE[6] is probably identical with the large peripheral, chlorophyll-containing vesicles described by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] in their electron microscope study of sections of this organism. Thin sections embedded in Araldite were found to show the electron-dense structures described by COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] in Epon-embedded sections. Fractions consisting of concentrates of these vesicles readily carried out the esterification of ADP and inorganic phosphate to ATP in the light. This reaction was proportional to the amount of added chlorophyll (*i.e.* vesicular preparation), had a pH optimum of 7.9, and was inhibited by CCCP to a similar extent as spinach chloroplasts[19]. The Chlorobium vesicles are therefore capable of active photophosphorylation reactions. Furthermore, the photophosphorylation activity of the vesicles varies with the cultural age of the cells from which they were prepared. The results show that preparations from young cells are most active (*cf.* FRENKEL[26]). Thus Chlorobium vesicles must be regarded as a major site of the photosynthetic activity in the green sulphur bacterium *C. thiosulphatophilum*.

From work on the purple-sulphur bacteria it is now clear that the photosynthetic apparatus of these organisms is part of an extensive structure which includes the cytoplasmic (*i.e.* peripheral membrane). Previous results by SYKES, GIBBON AND HOARE[6] and COHEN-BAZIRE, PFENNIG AND KUNISAWA[1] on the fine structure of the green-sulphur bacteria *C. thiosulphatophilum* and *C. limicola*, show that in these organisms the photosynthetic apparatus consisted of elongated, vesicular structures. These structures do not appear to be connected to the cytoplasmic membrane. Recent work by HOLT, CONTI AND FULLER[2] on another green-sulphur bacterium, *Chloropseudomonas ethylicum*, has substantiated the uniqueness of this group in this connection. These workers found that the photosynthetic apparatus was associated with large vesicles 'which may exist as part of a more extensive structure, independent of or only loosely associated with the bacterial cytoplasmic membrane'. HOLT, CONTI AND FULLER[2] found some evidence for interconnections between vesicles and kinetic release studies indicated that the vesicles might not be totally independent from each other. It is apparent that further work on the vesicular structures of the green-sulphur bacteria will be required to establish the precise relationship between the vesicles themselves and also between the vesicles and the peripherpal membrane. It may well be that the photosynthetic vesicles of the green bacteria represent an evolutionary step between the extensive membrane structure of the purple bacteria[25] and the complex photosynthetic apparatus of algae and green plants[27].

The results presented here show that there was invariably a substantial pro-

duction of ATP in the dark even from fractions containing a negligible amount of pigment. Experiments have been described which suggest that this is due to an independent energy production system involving the utilisation of polyphosphate and ADP, as described by COLE AND HUGHES[12] for *C. thiosulphatophilum*. ATP could also have been produced by a myokinase-type of reaction involving ADP, the detection of such an enzyme in the presence of the polyphosphate/polyphosphatase system was not attempted. It is interesting to note that although polyphosphate was detected in *C. thiosulphatophilum*, electron micrographs obtained from thin sections of this organism in this laboratory have failed to show more than the occasional polyphosphate granule (*cf.* electron micrographs in refs. 1 and 28). However, KORNBERG[23] noted that although *E. coli* contained an active polyphosphatase, capable of producing ATP from polyphosphate and ADP, the organism did not exhibit the metachromic granules usually associated with the presence of polyphosphate in bacteria[7].

Quite recently BALTSCHEFFSKY *et al.*[29] have identified inorganic pyrophosphate as a major product of photophosphorylation by isolated chromatophores of *Rhodospirillum rubrum* in the absence of added nucleotides. These authors suggested that inorganic pyrophosphate is formed directly (no ATP involved) by a photophosphorylation process. Although in isolated chromatophores they could not find any evidence for inorganic pyrophosphate being involved in the formation of polyphosphate, this work has indicated that phosphate polymers may well have an active role as intermediates in photophosphorylation reactions.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. H. WOOLHOUSE and Miss CHRISTINE SMITH, Department of Botany, University of Sheffield, Great Britain, for help with the preparation of electron micrographs, and Miss EMILIA DEL MISTRO for technical assistance. We are also indebted to the Science Research Council for a research studentship (to J.A.G.).

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