Physiology. — *Phosphate-exchanges in purple sulphur bacteria in connection with photosynthesis.* By E. C. WASSINK, J. E. TJIA and J. F. G. M. WINTERMANS. (From the Laboratory of Plantphysiological Research, Agricultural University, Wageningen.) (21st Communication on Photosynthesis) *). (Communicated by Prof. A. J. KLUYVER.)

(Communicated at the meeting of March 26, 1949.)

Introduction.

Since the time VOGLER and his collaborators made the important discovery that in cultures of the chemo-autotrophic sulphur bacterium *Thiobacillus thiooxidans* the shift from the energy-producing to the energy-consuming phase of metabolism is accompanied by a phosphate exchange (1), suggestions as to something analogous in photo-autotrophic metabolism have not been lacking (2—7). Already VOGLER himself put the question: "..... is it possible to irradiate photosynthetic organisms in the absence of CO_2 and to store at least a portion of the radiant energy within the cell in a form which can later be used for CO_2 fixation in the dark?" (2). A certain uptake of CO_2 in the dark by photosynthetic organisms had already been demonstrated by sensitive methods (8), so that there appeared to be a fair chance that VOGLER's question would be answered in the affirmative. Nevertheless, the attempts undertaken so far to furnish direct proof herefor cannot be said to have shown very definite results (5, 9).

These attempts all made use of green cells. Now, in our opinion, there are some good reasons to give preference to purple sulphur bacteria for these studies. In the first place they are more closely related to the organisms VOGLER used. Some strains of purple bacteria even have been shown to be capable of an anaerobic, photosynthetic mode of life as well as of a heterotrophic, oxydative one. In the second place, in purple bacteria, both carbon dioxide and the hydrogen donor can be supplied at will. In the third place, previous studies had given some general idea of the kinetics of the metabolism of at least one strain (*Chromatium,* strain D).

We, therefore, decided to look for possible connections of photosynthesis and phosphate exchange in suspensions of *Chromatium,* strain D.

The method of phosphate determination was worked out chiefly by the second author, the results reported below were collected by the last mentioned author.

Methods.

The bacteria were grown in the medium, described earlier as "combination 23",

*) 20th Comm.: Ann. Rev. Biochem. **17**, 559—578 (1948).

containing 0.24% sodium-l-malate and 0.16% sodium thiosulphate in an inorganic stock-solution (10). In the reported experiments, cultures from a small amount of dense inoculum were used after 1 day of incubation at about 27° in a light cabinet. They were centrifuged, suspended in the medium used in the experiments (see below), and centrifuged again. After this, the bacteria were resuspended in the same medium, and the experiments were started.

The general trend of an experiment was very simple. A suspension of bacteria in a glass cylinder of about 20 cm height and about 50 ml. contents was ventilated with a suitable gas stream, mostly consisting of oxygen-free nitrogen with either hydrogen or carbon dioxide added, and exposed either to light or to darkness. At the moment of changing conditions, or at intervals during an exposition, inorganic phosphate-P in the suspension medium was determined.

To this purpose, it was deemed advisable to separate the cells from the suspension medium as quickly as possible, and under sensibly the same conditions as during the experiment. In view of the experiments in light, centrifuging appeared unsuitable in this respect. At first, an experimental set-up was made in which the bottom of a glass cylinder was replaced by a cylindrical funnel with glass filter plate. Suspension liquid could be removed by suction during the fully undisturbed experiment.

Unfortunately, however, bacteria entered into the pores of the filter plate — of about 1 μ width — soon preventing rapid filtering. After a few trials the following simple procedure was found satisfactory. Cylindrical glass funnels were made, about 5 cm in diameter, and 6 cm in height, with a nearly flat bottom and a tube of about 1 cm width. A stiffly rolled strip of ordinary filter paper, about 2 cm broad and 30 cm long, was pressed into the upper end of the tube. About 5 ml. of suspension were now removed from the glass cylinder in which the bacteria were exposed to the experimental conditions, introduced into the funnel — which was either illuminated or in darkness — and ventilated with the same gas stream as used in the experiment. Then suction was quickly applied, upon which the suspension medium rapidly went through clear.

In order to have a chance upon measurable relative changes in PO_4–P, solutions of low phosphate contents had to be used; in general about 10 μ g P/ml was taken. Initially, dilute phosphate buffers of pH ~ 8.0, according to CLARK and LUBBS, and to SÖRENSEN were used. Since the bacteria soon became inactive in these dilute media, 1% NaCl was added. A remaining drawback was the weak buffering capacity of the medium. In case of ventilation with gas containing CO_2, the buffering capacity could be increased by addition of sodium bicarbonate, which, however, had the disadvantage of preventing subsequent removal of CO_2 — *e.g.*, when applying $N_2 + H_2$ afterwards — and, moreover, turned out to have an influence upon the phosphate determination.

In search for other suitable buffering systems it has to be observed that systems containing organic acids, as *e.g.*, acetate, citrate, *etc.* are less advisable since these substances are likely to be used as sources of hydrogen and/or of CO_2 by the bacteria. Finally, a borate mixture was found suitable and was stood very well by the bacteria; PALITZCH' mixtures were used. Ten ml. solution containing 3 ml. M/20 borax, and 7 ml. M/5 boric acid + M/20 NaCl, pH ~ 8.0 were added to 40 ml. of a phosphate mixture containing about 10 μ g P/ml. When ventilated in dark with $N_2 + 1\%\ CO_2$, a decrease of pH was observed which, after about 3 hours, remained at about 7.15. B-concentrations used did not interfere with P-determination.

Phosphate-P was determined colorimetrically, according to the phospho-molybdic acid method first devised by OSMOND (1887). BELL and DOISY (1920) first used this method in connection with an organic reductant. Several modifications, differing in the sort of reductant, have since been described. We used that of LOWRY and LOPEZ (11), with ascorbic acid as reductant. Advantages of this method are the operation at moderate pH and the use of a rather dilute solution of molybdate.

Two ml. of suspension medium sucked through the filter (*cf.* above) were introduced into a measuring flask of 25 ml., and some acetate buffer, pH 4.0, was added. Subsequently,

2 ml. of a solution containing 1 % of ammonium molybdate in 0.05 N. sulfuric acid, and 2 ml. of a 1 % solution of ascorbic acid were added, and filled up to 25 ml. with acetate buffer. A stopwatch is started after addition of the reductant, and the blue color, gradually developing, is measured after 5 and 10 minutes with a "lumetron, model 400 A" colorimeter, using the red filter, and a solution without molybdic acid as reference. In general, the readings after 5 and 10 minutes turned out to be the same, so that each moment between was suitable for the determination. About 10 readings were taken from each sample, yielding one measurement. The phosphate content according to the reading was computed from a calibration curve obtained by submitting solutions of known phosphor contents to the same procedure.

The accuracy obtained may be indicated by the following figures: 20 measurements of blank P + B-solution (before introducing bacteria into it) yielded $P = 8.9 \pm 0.05$; $\sigma = 0.23\ \mu$ g P/ml.

The following experimental details still have to be mentioned. In the earlier experiments glass cylinders were used to take up the bacteria in the experiments (*cf.* above). As a rule two of these were used in each experiment, submitted to different conditions (*e.g.*, light *versus* darkness, $N_2 + H_2$ *versus* $N_2 + CO_2$). The suspensions contained about 5 cmm bacteria/ml. The cylinders stood in a thermostatic water bath with glass sides, at about 29°, illuminated from 2 opposite sides with a 100 Watt incandescent lamp, yielding on either side an intensity of 8—9000 lux. In later experiments flat glass boxes of about $20 \times 10 \times 0.6$ cm were used instead of the cylinders.

During the experiments a flow-meter-controlled gas stream from a bomb was passed through the suspensions. Either N_2 + 30 % H_2, N_2 + 30 % H_2 + 5 % CO_2, or N_2 + 1 % CO_2, obtained by mixing pure nitrogen with nitrogen containing 5 % of carbon dioxide, were applied. The gasses were freed from oxygen by passing them over electrically heated copper gauze. With the aid of flow meters the gas streams in both cylinders or glass boxes were adjusted to equal velocity. At the top the vessels were closed except inlet and outlet of gas, the first ending in the suspension near the bottom. Before entering into the vessel with the bacteria, the gas passed a washing bottle with water at room temperature, to control evaporation of the suspension.

Unfortunately, so far, we were not in a position to follow simultaneously the gas exchange of the cells used, owing to the lack of suitable apparatus herefor. This is planned for a subsequent part of this investigation.

Experimental results and their preliminary discussion.

The first experiments tended to "translate" VOGLER's crucial experiments in terms of photosynthesis as closely as possible. A suspension of *Chromatium*, Strain D, in a borate mixture with about 10 μg PO_4–P/ml., pH ~ 8.0, under N_2 + 30 % H_2 was submitted to a short dark period for "adaptation", and then illuminated. During the illumination period, a decrease of phosphate in the external medium is observed. After some hours, the light is turned off, and the gas phase replaced by N_2 + 5 % CO_2. Then, an increase in external phosphate is observed. The procedure may be repeated with the same result (fig. 1, curve *a*). It is obvious that these observations are concordant with a concept, analogous to the one proposed by VOGLER for the chemo-autotrophic sulphur bacteria. In the light, hydrogen may be taken up, and fixed in some form, involving phosphate uptake. If this form is an "energy rich phosphate", able to aid in the reduction of carbon dioxide in the dark, release of phosphate in a subsequent dark period may be expected.

It may be remarked already now that, in a M/15 phosphate buffer of pH ~ 8.0, WASSINK and KUIPER observed a long-lasting uptake of gas from $N_2 + H_2$, in the absence of CO_2, even in cells submitted to special "starvation" treatments (in an investigation on the relation between redox potential and photosynthesis [12—14]). So far studies

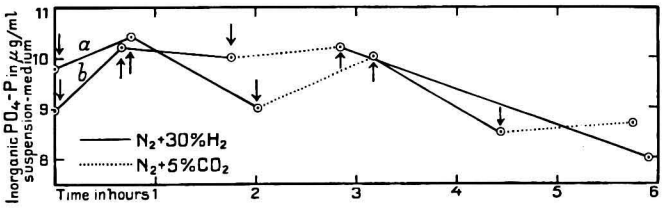

Fig. 1. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness, and under various gas phases. Exp.s of 13, 14. 10. 48.
↓ : shift to darkness. ↑ : shift to light.

as to a "dark pick-up" of CO_2 have not yet been made. In the course of the mentioned investigation some incidental indications were obtained that cells, allowed to take up hydrogen in the light for a long time, already quickly thereafter produce considerable amounts of CO_2 in the dark.

Not always, however, the cells showed clear responses as to phosphate in the various phases of the above mentioned type of experiment (*cf.*, *e.g.*, fig. 1, curve *b*). This forced us to a more systematic study of the various phenomena involved.

The next observation made was a release of PO_4 in the dark (increase in the external medium) when freshly harvested, and washed cells were suspended as described. This PO_4-liberation starts at once, and shows a tendency to decrease with time. In N_2 + 30 % H_2, and in N_2 + 1 % CO_2 it was studied in greater detail and only little difference between these two gas phases was found (fig. 2, 3, 4, 5, 6, 8, 10; Table I). This release

TABLE I.

Release of PO_4–P (increase of P in suspension medium) by *Chromatium*, strain D, in darkness under various gas phases.
Suspension medium: borate buffer, pH ~ 8.0, with ~ 10 µ g P/ml.

Ventilated with	Time	Total PO_4–P released	Number of observations
a $N_2 + 30\%\ H_2$	35′	0.53 ± 0.05	15
	120′	1.1 ± 0.13	12
	180′	1.8 ± 0.14	5
	285′	2.15 ± 0.25	2
b $N_2 + 1\%\ CO_2$	120′	1.08 ± 0.18	12
	270′	2.19 ± 0.55	5

of PO_4 probably may be compared with that accompanying "endogenous respiration" in VOGLER's experiments. Notwithstanding the fact that, owing to the absence of oxygen, the *Thiorhodaceae* can not show a respiration-proper, some form of energy liberating metabolism is likely to continue in darkness, which may well be accompanied by a release of PO_4.

Since neither hydrogen nor carbon dioxide are likely to be active in the dark in these bacteria — unless, eventually, after special treatments — it is perhaps not very surprising that the rate of release is fairly independent of the gas phases applied.

Next, the behaviour of the cells after admitting light, in $N_2 + 30\ \% H_2$, was studied in greater detail. The phosphate release is now replaced by phosphate uptake (fig. 1, 2, 3, 4, 7*b*, 8*b*); after a short dark period, when the tendency to release phosphate apparently still was strong, the uptake at first was sometimes small or even slightly negative still (*cf.* fig. 4, 7*b*) especially in dense suspensions (low average light intensity). Some typical experiments are represented in fig. 2 and 3; in each case, after a short dark period, the two halves of the same suspension were submitted oppositely to light and darkness, whereas, some 2 hours later, the conditions were reversed (fig. 2) or continued (fig. 3) without changing the gas phase.

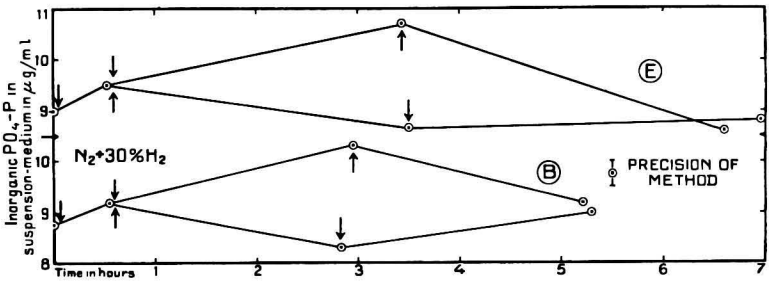

Fig. 2. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness. Flushed with $N_2 + 30\ \% H_2$. E: Exp. of 17.11.48; B: Exp. of 10.11.48.
↓ : shift to darkness. ↑ : shift to light.

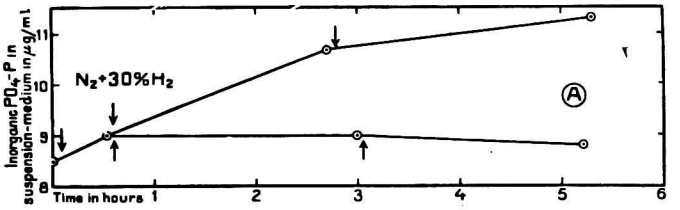

Fig. 3. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness. Flushed with $N_2 + 30\ \% H_2$. Exp. of 9. 11. 48.
↓ : shift to darkness. ↑ : shift to light.

A number of experiments of the type-fig. 2 is summarized in fig. 4, showing the different behaviour in light and darkness very clearly. It may be concluded too, provisionally, that the rate of phosphate uptake in the light is higher after a longer dark period (fig. 4, Table II, *a*). The reaction upon renewed darkness will be discussed below.

Also in $N_2 + 1\ \% CO_2$, illumination as a rule causes uptake of phosphate, replacing the release occurring in the dark (fig. 5, a few experiments are summarized in fig. 6). Here, too, after a prolonged dark period the rate of uptake appeared increased (*cf.* Table II, *b*).

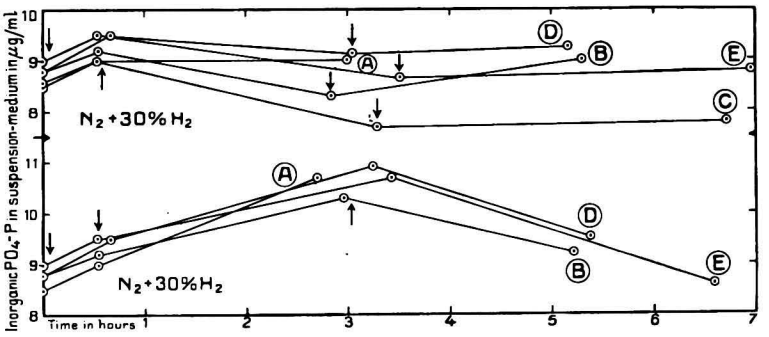

Fig. 4. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness. Flushed with $N_2 + 30\,\% H_2$. Aliquots of the same culture indicated by equal characters. Exp.s of various dates.
↓ : shift to darkness. ↑ : shift to light.

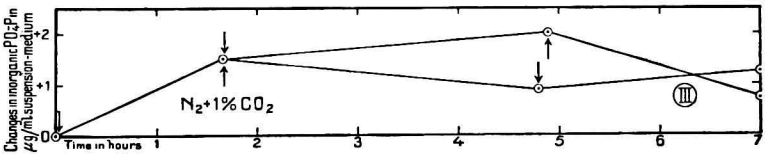

Fig. 5. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness. Flushed with $N_2 + 1\,\% CO_2$. Exp. of 2. 12. 48.
↓ : shift to darkness. ↑ : shift to light.

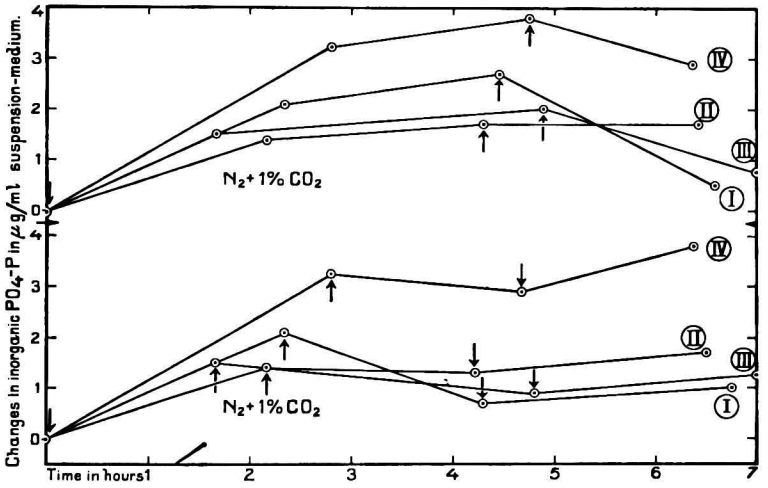

Fig. 6. Phosphate changes in suspension medium with *Chromatium*, strain D, in relation to light and darkness. Flushed with $N_2 + 1\,\% CO_2$. Aliquots of the same suspension indicated by equal numbers. Exp.s of various dates.
↓ : shift to darkness. ↑ : shift to light.

TABLE II.

Uptake of PO_4–P (decrease of P in suspension medium) by *Chromatium*, strain D, in illumination under various gas phases.

Suspension medium: borate buffer, pH ~ 8.0, with ~ 10 μ g P/ml.

	Condition during dark period	Duration of dark period	Condition during subsequent illumination	Time of exposure to light	Total PO_4–P taken up in light	Number of observations
a	N_2 + 30% H_2	30′	N_2 + 30% H_2	75′	0.29 ± 0.1 μg/ml.	12
				135′	0.49 ± 0.15 „	12
				195′	0.80 ± 0.32 „	5
				235′	1.38 ± 0.70 „	3
	same	2 hours	same	120′	0.80 ± 0.26	6
				240′	1.11 ± 0.16	5
	same	3 hours	same	120′	1.20 ± 0.10	3
	same	4.5 hours	same	120′	2.40 ± 0.2	2
b	N_2 + 1% CO_2	2 hours	N_2 + 1% CO_2	120′	0.31 ± 0.16	11
				240′	0.20 ± 0.17	5
	same	4.5 hours	same	120′	1.0 ± 0.4	5
c	N_2 + 30% H_2	30′	N_2 + 30% H_2 + 5% CO_2	150′	0.0 ± 0.14	5

However, another feature is very obvious, *viz.*, that the uptake in light under N_2 + 1 % CO_2 is much smaller than under N_2 + 30 % H_2 (fig. 8). In some cases with CO_2 it even hardly reaches significantly positive values (*cf.* Table II, *b*). The same can be said, comparing $N_2 + H_2 + CO_2$ with $N_2 + H_2$, also here the presence of CO_2 reduces the phosphate uptake in the light practically to zero, and the difference in the general trend of the curves is very obvious, notwithstanding some incidental exceptions (fig. 7, Table II, *c*).

It should be emphasized that most of the curves of *a* and *b* in fig. 7 and 8 have been obtained one by one in the same experiment so that the variable "activity" of the cells to take up and give off phosphate cannot have interfered with these results.

In the type of experiment as shown in fig. 2 and 5 (*cf.* also fig. 4 and 6) darkness was again given after light in one of the two aliquots of suspension. The number of these observations is still too small to draw definite conclusions. It is obvious, however, that phosphate uptake stops or is converted into release. It would seem that in $N_2 + CO_2$ the release is somewhat more definitely pronounced than in $N_2 + H_2$ (*cf.* fig. 4 and 6). The reaction upon darkness after light without changing the gas phase throws some doubt upon the meaning of the release of phosphate after changing from H_2 to CO_2. More and quantitative studies, taking into account the gas exchange, are required here. In general, however, it may be admitted that under all conditions the cells are provided with organic

substances, which may be converted under release of phosphate and probably of CO_2, as soon as conditions for active rebuilding of ~ph are absent, especially when the source of energy: light, is lacking.

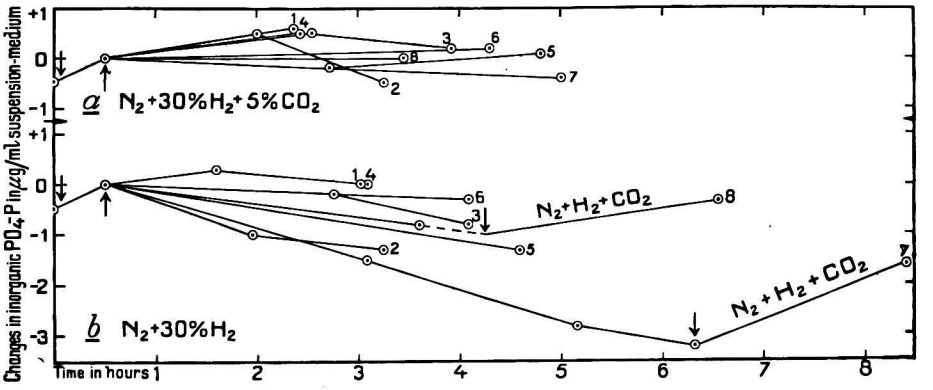

Fig. 7. Phosphate changes in suspension medium with *Chromatium*, strain D. Influences of various gas mixtures during illumination and eventually subsequent darkness. Aliquots of suspensions were separated after ± ½ hour adaptation in dark with $N_2 + 30\% H_2$. (Numbers indicate parallels with the same culture.) Exp.s of various dates.
↓ : shift to darkness. ↑ : shift to light.

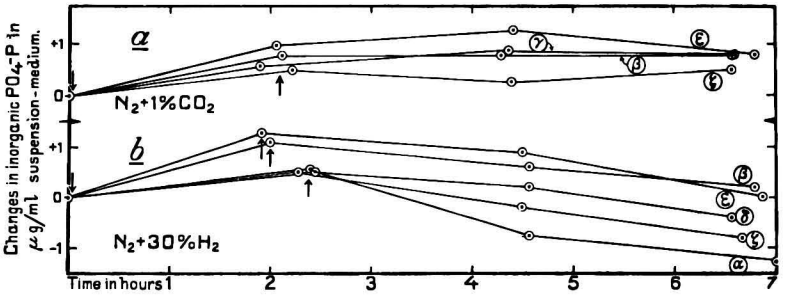

Fig. 8. Phosphate changes in suspension medium with *Chromatium*, strain D. Influence of various gas mixtures in dark and in light in parallel experiments (indicated by characters.) Exp.s of various dates.
↓ : shift to darkness. ↑ : shift to light.

General Discussion.

In the foregoing, uptake and release of phosphate have been observed. It is obvious, *a priori*, that neither of the two can last infinitely with the same speed, even under as such favorable conditions. It is obvious too, therefore, that both uptake and release will show an asymptotic course in relation to time. Furtheron, it may be admitted that the rate of reversion upon a change of conditions also depends upon the nature and duration of the condition before the change. Finally, if indeed there is a relation between phosphate metabolism and photosynthesis of the type VOGLER found in *Thiobacillus thiooxidans*, we may expect that in the light, in the

absence of carbon dioxide, cells will accumulate phosphate to a higher degree than in its presence. In darkness, we may expect phosphate bond energy to be released as part of the energy reserve of the cell, used up in darkness.

From these general statements, and founded upon the results of VOGLER and UMBREIT, we would expect purple sulphur bacteria to influence the phosphate content of a limited amount of medium in which they are suspended, in a way schematically represented in fig. 9.

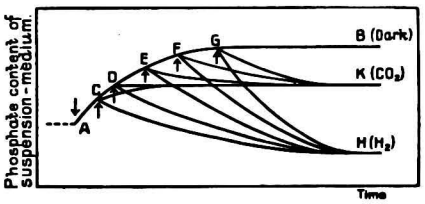

Fig. 9. Schematical representation of expected PO_4-shifts under different conditions. See text.

↓ : shift to darkness. ↑ : shift to light.

Cells brought into darkness from a well-fed condition will start releasing phosphate from a level A, which phosphate accumulates in the medium. Since neither hydrogen nor carbon dioxide are known to act as energy sources in the dark for *Chromatium*, there is no reason to expect that the release of phosphate will depend either on the presence of hydrogen or of carbon dioxide. The release will show an asymptotic course (AB). Upon illumination, *e.g.* in $N_2 + H_2$, an uptake of phosphate will occur which may be expected to start at higher rate, the more phosphate has been released before, so, *e.g.*, the longer the dark period has lasted. The curves starting at C, D, E, *etc.*, will be expected to strive towards virtually the same level, indicated by H. The probability that this level is, *e.g.*, dependent on light intensity, will not be discussed here. Under $N_2 + CO_2$ we may expect a behaviour fundamentally analogous to that under $N_2 + H_2$, only pointing to a significantly higher ultimate phosphate content in the external medium (level K).

In fig. 10 we have collected from our total experimental material, the

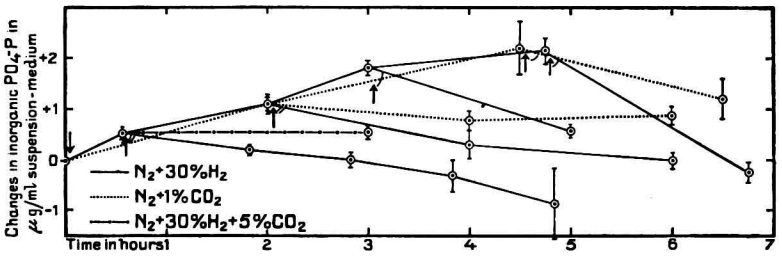

Fig. 10. Summarized representation of actual PO_4-shifts from table I and II. Cf. fig. 9 and text.

↓ : shift to darkness. ↑ : shift to light.

data pertaining to the points outlined above. Notwithstanding the fact that on various points the material still is somewhat scanty (expressed by the large mean errors of some points and the lack of data on shorter intervals) it is clear that, in general, fig. 10 shows a very distinct analogy with the scheme of fig. 9. This would seem to indicate that, in general, the situation answers to the outline given.

It should, however, not be overlooked that the experiments made so far, do not *prove* that, in photosynthesis of *Chromatium*, in the absence of CO_2, part of the energy of the light actually is accumulated as phosphate bond energy. To prove this would require recovery of these energy rich phosphates from the cells which determinations have not yet been attempted. The findings merely are very well in accordance with the supposition that indeed light energy *is* accumulated in this form.

It may be useful still to emphasize a rather fundamental difference between VOGLER's case and that of the photosynthetic bacteria. In *Thiobacillus*, the oxidation of the hydrogen donor is achieved by oxygen, and is, or at least may be conceived to be fundamentally independent of carbon dioxide. This easily leaves room for a storage of energy from the oxidation process in some form which may, or may not, be used for the reduction of carbon dioxide. In *Chromatium*, on the other hand, carbon dioxide itself is the ultimate oxidant of the hydrogen donor; for the transfer of hydrogen light is essential. This, however, would seem to leave much less room for an independent storage of (light) energy at the expense of the hydrogen donor, and requires a much more specialized mechanism. The observation, alluded to above, that excessive hydrogen uptake may occur without supply of carbon dioxide (13, 14), indicates that indeed under certain conditions — *e.g.* pH $\sim$ 8.0 — hydrogen may be assimilated more or less independent of carbon dioxide. It is interesting in this respect that so far no indications were found for an appreciable CO_2-uptake without hydrogen during prolonged illuminations. The results reported in the present paper suggest that the mentioned uptake of hydrogen — which was not measured in the present study — is connected with phosphate uptake. It is perhaps most plausible to assume, that phosphate-shifts are connected with the transfer of energy from the pigment-protein system (*cf.* [15, 16, 12]).

In view of an estimation of the quantitative importance of the phosphate shifts observed, it may be of interest to compare their magnitude with the presumable phosphate content of the cells. If we suppose a "normal" phosphor content in *Chromatium*-cells, this may be fixed at $\sim$ 5 % of the dry weight, as P_2O_5; the dry weight may be 15 % of the fresh weight. This would mean that the bacteria contain 0.75 % of their fresh weight as P_2O_5, or 0.33 % P. The suspension density as a rule was about 5 cmm/ml., or about 5 mg/ml. Thus, the bacteria in 1 ml. contain about 17 μg P. On exposure to $N_2 + H_2$ in the light, an uptake of 1.5 μg P/ml is not rare. It thus appears that the bacteria may underly shifts in phosphate amounting to about 10 %. In order to achieve that these shifts will definitely surpass

the limits of the method of determination, rather dense bacterial suspensions are advisable. In order to obtain sufficient illumination inside the suspension, in our more recent series we used glass boxes instead of cylinders to suspend the bacteria.

Summary.

In suspensions of *Chromatium*, strain D, in borate buffer, pH ~ 8, containing some phosphate, shifts in phosphate content of the medium were found correlated with shifts from light to darkness, and from a gas phase consisting of $N_2 + H_2$ to one containing $N_2 + CO_2$.

The principal observations were as follows. Bacteria taken from the culture medium ("comb. 23", see [10]) release phosphate in darkness, at about equal rates under $N_2 + H_2$ as under $N_2 + CO_2$. Shift to light results in a marked uptake of phosphate under $N_2 + H_2$ and in a much smaller one under $N_2 + CO_2$. Shift from light under $N_2 + H_2$ to darkness under $N_2 + CO_2$ results in a marked release of phosphate. Change from light to darkness without change of gas phase results in a certain release, too.

As a whole the data collected so far show a marked similarity with VOGLER's findings with *Thiobacillus thiooxidans*, if $N_2 + H_2$ in light is parallelized with sulphur oxidation. They thus form a preliminary support for the hypothesis that *Chromatium* in the light is capable of building up energy rich phosphate bonds which are broken down in part when CO_2 becomes available and/or light is withdrawn. The phosphate exchanges observed so far can amount to 10 % of the phosphate content of the bacteria.

Correlations with gas exchange were not made so far. The investigation is being continued.

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