BIOCHEMICAL AND STRUCTURAL CORRELATIONS IN ISOLATED SPINACH CHLOROPLASTS UNDER ISOTONIC AND HYPOTONIC CONDITIONS

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Summary

Methods are described for obtaining preparations of isolated spinach chloroplasts which are predominantly either "whole", i.e. retain the outer limiting membrane, or "naked", i.e. with the outer membrane removed. The whole chloroplasts are shown to retain more of their soluble components as evidenced by higher endogenous capacities for CO_2-fixation, photophosphorylation, and light-dependent reduction of triphosphopyridine nucleotide. The isolation is also described of whole chloroplasts whose peripheral jackets have retained the property of mobility seen in living cells.

Chloroplast fragments, obtained by mechanically rupturing intact chloroplasts, exhibited a higher Hill reaction activity than did intact chloroplasts.

Parallel microscopic and biochemical observations of isolated chloroplasts under increasingly hypotonic conditions revealed a marked decline in photophosphorylating capacity along with a progressive swelling of the chloroplast. In contrast, Hill reaction capacity is increased threefold at sucrose concentrations below 0·005M. Enhancement of Hill reaction and loss of photophosphorylating capacity coincided with osmotic fragmentation of the chloroplast mass and formation of many small, discrete, balloon-like structures. Low concentrations of Mg^{2+} suppress to a large extent both structural and biochemical effects of hypotonicity.

Water-treated preparations must be restored to a higher solute concentration (0·1M) before the enhanced rate of Hill reaction is elicited. The Hill reaction of such water-activated chloroplasts was compared with that of non-swollen chloroplasts with respect to light intensity relationships, temperature coefficient, and action spectra. The Hill reaction of manganese-deficient plants was not activated by water treatment.

I. INTRODUCTION

Recent observations by phase-contrast and fluorescence microscopy have documented the appearance of isolated spinach chloroplasts, and their structural changes under a range of hypotonic conditions (Spencer and Wildman 1962). Preparations of isolated "whole" chloroplasts in 0·4M sucrose commonly contain two classes of chloroplast, namely those which retain the outer jacket and its limiting membrane, and those in which this membrane is missing. Dilution of the suspending medium to 0·1M sucrose causes the outer, limiting chloroplast membrane to rupture. Below this concentration extensive swelling and distortion and finally fragmentation of the remaining lamellar system occurs. This paper reports two groups of experiments arising from the above observations. The first concerns methods of obtaining

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preparations of isolated chloroplasts which are either predominantly whole (i.e. with outer membrane intact) or predominantly lacking in this outer membrane, together with some comparisons of the endogenous activities of these two types of preparations. The second group of experiments seeks to correlate the structural changes of isolated chloroplasts under a variety of hypotonic conditions with changes in their functional capacity.

II. Methods

Spinach (*Spinacia oleracea*) plants were grown in nutrient solutions using procedures described earlier (Possingham and Spencer 1962), and harvested 2–3 weeks after transplanting.

Hill reaction measurements were made with 2,3′,6-trichlorophenolindophenol (TCPIP) by measuring the extent of dye reduction at 620 mμ (Spencer and Possingham 1960). Chlorophyll was assayed according to Arnon (1949).

Action spectra for TCPIP reduction were determined using a series of narrow-band interference filters from Schott & Co. (Mainz) and Carl Zeiss (Jena). The voltage at the tungsten light source was adjusted for each interference filter so as to give equal energy of transmitted light as determined by a cadmium sulphide photocell. Intensity of transmitted light was well below saturation level for TCPIP reduction. Illumination was carried out for 2 min.

Triphosphopyridine nucleotide (TPN) reduction was determined in a reaction mixture containing 0·5 ml of chloroplasts (≡ 285 μg chlorophyll) in 0·4M sucrose, 0·05M tris(hydroxymethyl)aminomethane (Tris, pH 7·8), 0·01M NaCl, and 0·0001M $MgCl_2$, together with TPN (0·5 μmole). Where required, a saturating level of photosynthetic pyridine nucleotide reductase (PPNR) was added. The final volume was 0·8 ml. After incubation for 10 min at 15°C and 4240 f.c. the reaction mixture was stopped by the addition of 1·0 ml of saturated Na_2CO_3 followed by immersion in boiling water for 0·5 min. The reaction mixture was then centrifuged at 12,000 *g* for 10 min and the optical density of the supernatant solution determined at 340 mμ. Activity is expressed as the increase in optical density (ΔO.D.) at 340 mμ/mg chlorophyll/10 min relative to a parallel, non-illuminated reaction mixture. PPNR was prepared by the method of San Pietro and Lang (1958), the purification being taken to the stage of the dialysed, acetone-precipitated fraction.

For measurement of endogenous CO_2 fixation, the reaction mixture (0·507 ml) consisted of 0·5 ml of chloroplasts (≡ 0·1 mg chlorophyll) suspended in 0·4M sucrose, 0·05M Tris, pH 7·3, 0·01M NaCl, and 0·0001M $MgCl_2$, together with 7 μl of a solution of $NaH^{14}CO_3$ (≡ 0·85 μmoles and $9{\cdot}8 \times 10^4$ counts/min). Reaction mixtures were gassed with N_2 for 3 min preceding, as well as during, the incubation which was at 15°C for 30 min either in the dark or at 4240 f.c. The reaction was stopped with 0·25 ml of 1N HCl and the extent of $^{14}CO_2$ fixation determined by plating 0·05-ml aliquots, which were then dried and then counted in a New England Nuclear gas flow counter with a Micromil window. CO_2 fixation is expressed as μmole CO_2 fixed/hr/mg chlorophyll.

For measurement of endogenous photophosphorylation 0·25 ml of chloroplasts (≡ 0·1 mg chlorophyll) in 0·4M sucrose, 0·05M Tris, pH 7·8, 0·01M NaCl, and

0·0001M $MgCl_2$ were incubated with 0·1 ml adenosine diphosphate (ADP, 2×10^{-2}M) and 0·1 ml potassium phosphate, pH 7·8 (0·0125M) for 1 hr at 15°C either in the dark or at 4240 f.c. Photophosphorylation in the presence of cofactors was assayed as described earlier (Spencer and Possingham 1961) with a final volume of 0·75 ml.

It should be noted that for all comparisons between class I and class II chloroplasts, precautions were taken to ensure that both preparations underwent the same degree of dilution during isolation, and the two classes were assayed at equal chlorophyll concentration.

Microscope observations were made under phase contrast with a Zeiss GFL Research Model microscope.

III. Results and Discussion

(*a*) *Microscopic Appearance of "Whole" Chloroplast Preparations*

Most standard procedures for the isolation of chloroplasts in buffered isotonic solution yield two microscopically distinct kinds of chloroplast. For example Plate 1, Figure 1, shows the appearance under phase contrast of a chloroplast suspension prepared by hand-grinding leaves in a buffered 0·4M sucrose solution as in the procedure of Jagendorf and Avron (1958). One class (class I) consists of bright, highly reflecting chloroplasts of somewhat irregular outline in which the grana are not clearly resolved in any plane of focus. The other class (class II) consists of saucer-shaped chloroplasts of more regular oval or circular form in which the grana are very distinct. From their behaviour under hypotonic conditions it has previously been shown that class I chloroplasts are those which have retained, during isolation, the outer membrane which surrounds the chloroplast and its stromal jacket in the living cell. The isolation procedure causes shrinkage of the outer membrane, which in turn causes a distortion of the regular saucer shape of the lamellar system into a highly folded form. Class II chloroplasts are those which lack the outer surrounding membrane and consist only of the naked lamellar system (Spencer and Wildman 1962).

(*b*) *Preparation of Suspensions of Class I and Class II Chloroplasts*

In general, procedures for the isolation of chloroplasts fall into one of two groups. In one group the leaves are ground by hand in 1–2 volumes of isotonic solution, and in the other, leaves are homogenized in 5–10 volumes of isotonic solution in a mechanical blender. After filtration through muslin, the fraction of the extract which sediments between 200 and 1000 *g* is used as the isolated chloroplast preparation.

These two procedures have been used as the basis of methods for obtaining chloroplast preparations which are predominantly of either class I or class II.

(i) *Preparation of Class I Chloroplast Suspensions.*—This is essentially the procedure of Jagendorf and Avron (1958) with modifications to the centrifuging step. Leaves were scissored into small pieces and ground by hand in a mortar (without sand) with 1½ volumes of buffered sucrose solution containing 0·4M sucrose, 0·05M Tris, pH 7·8, and 0·01M NaCl. The slurry was then squeezed through four layers of fine cloth, the mesh (110 to the inch) of which was such that virtually all unbroken

cells and clusters of cells were filtered out. Microscopically the crude cell-free extract was seen to contain a major proportion of chloroplasts of class I. Chloroplasts were sedimented from the cell-free filtrate by three successive, brief, low-speed centrifugations. The centrifuge (Servall RC-2 with SS-34 rotor) was accelerated to 200 *g* and immediately shut off. The resulting pellet was retained and the supernatant suspension was similarly brought briefly to 325 *g* and, in turn, the supernatant suspension from this step was brought briefly to 580 *g*. Microscopic examination showed that the three pellets obtained in this way were highly enriched with class I chloroplasts and were therefore resuspended in buffered sucrose and combined. Occasionally higher-speed centrifugations were carried out and the resulting chloroplasts also used if found microscopically to be rich in class I. A typical preparation of class I is shown in Plate 1, Figure 2. It is estimated to contain at least 80% class I chloroplasts. In some experiments a single centrifugation at 121 *g* for 3 min was substituted for the repeated, brief, low-speed centrifugations.

(ii) *Preparation of Class I Chloroplasts with Mobile Jackets.*—In the living leaf cell, chloroplasts are frequently seen to be surrounded by a peripheral layer of non-chlorophyllous material which undergoes amoeboid movement (Wildman, Hongladarom, and Honda 1962; Spencer and Wildman 1962). Honda, Hongladarom, and Wildman (1962) have reported the isolation of chloroplasts in which this outer mobile jacket is retained using a buffered sucrose–Ficoll–dextran medium. We have observed that the crude leaf extract obtained by grinding leaves in buffered sucrose and filtering through cloth as described in Section III(*b*)(i) contains many chloroplasts with prominent jackets. When these are centrifuged down and resuspended in fresh buffered sucrose as in Section III(*b*)(i) they are transformed into bright class I chloroplasts in which the outer jacket has contracted and deformed the normal, open, saucer shape (Plate 1, Fig. 2). If, however, a boiled and cleared solution of the same leaf extract is used for resuspension, the jackets of many chloroplasts are preserved and with them the property of mobility. Plate 2, Figures 1–12, shows a time sequence of photomicrographs of a single isolated chloroplast from such a preparation showing the mobile property of the jacket material. While their jackets are not as prominent as in the living cell, these chloroplasts correspond to those in the cell far more closely than either the folded type of class I chloroplast seen in Plate 1, Figure 2, or the class II chloroplasts seen in Plate 1, Figure 3.

(iii) *Preparation of Class II Chloroplasts.*—For this purpose the procedure of Jagendorf and Evans (1957) was found to be most suitable. Approximately 5 *g* of leaves are homogenized for 90 sec in 60 ml solution, containing 0·4M sucrose, 0·05M Tris, pH 7·8, and 0·01M NaCl, in an Omnimix blender (Ivan Sorvall Inc., Connecticut, U.S.A.) at 40% of line voltage. The homogenate is filtered through cloth, centrifuged at 1000 *g* for 10 min, and the resulting pellet resuspended in 30 ml of buffered sucrose, recentrifuged, and finally resuspended in more buffered sucrose. Such preparations contain 80–90% of chloroplasts of class II as seen in Plate 1, Figure 3. Occasionally potassium phosphate and potassium chloride were substituted for Tris and sodium chloride in the buffered sucrose solution without affecting the appearance of the preparations.

In experiments where a comparison was to be made of endogenous activities of class I and class II chloroplasts the preparative procedure was modified in order

that class II chloroplasts underwent the same dilution as class I chloroplasts during isolation, thereby equalizing the degree of leaching to which both classes were subjected. Spinach Leaves (10 *g*) were scissored finely and then blended with 15 ml of buffered sucrose solution in an Omnimix blender at top speed for 70 sec. After filtration through cloth, this suspension was centrifuged at 121 *g* for 2 min, the pellet discarded, and the supernatant fraction again centrifuged at 480 *g* for 3 min. The resultant pellet, which contained approximately 80% of class II chloroplasts was resuspended in fresh buffered sucrose.

(c) *Endogenous Chloroplast Activities*

It might be anticipated that, because of their intact outer membrane, class I chloroplasts would suffer less loss of soluble constituents during isolation than the class II type of chloroplast. If so, class I chloroplasts would be useful tools for studying integrated reactions *in vitro* involving both soluble and structural chloroplast components. This possibility was tested by comparing the endogenous capacity of class I and class II chloroplasts for a range of photochemical activities.

(i) *TPN Reduction.*—Light-dependent reduction of TPN by chloroplasts is known to involve two soluble enzymes, ferredoxin and a flavoprotein. Davenport (1963) has reported that ferredoxin is very readily lost from isolated spinach chloroplasts, while the flavoprotein component is bound more firmly. The results in the following tabulation show that class I chloroplasts reduce TPN without supplement, and that this activity is not enhanced by the addition of ferredoxin in the form of a crude preparation of the PPNR of San Pietro and Lang (1958). Incubation conditions were as described in Section II.

Chloroplast Preparation	Additions	TPN Reduction (ΔO.D.$_{340}$ mμ/mg chlorophyll/10 min)
Class I	TPN	1·23
	TPN+PPNR	1·29
Class II	TPN	0·10
	TPN+PPNR	1·02

Assays were carried out at high chloroplast concentration ($\equiv$ 0·35 mg chlorophyll/ml) so as to avoid dilution of the highly diffusible endogenous ferredoxin. Class II chloroplasts at an equivalent chlorophyll concentration reduce TPN at about one-tenth the rate, and show a tenfold stimulation with added PPNR. The ease with which endogenous ferredoxin diffuses out of, and exogenous ferredoxin diffuses into, class I chloroplasts is indicated by the fact that if they are assayed at a lower chloroplast concentration (0·02 mg chlorophyll/ml) endogenous TPN reduction is negligible and a marked response to added PPNR is seen.

(ii) *Photophosphorylation.*—The endogenous capacity of class I chloroplasts for photophosphorylation was more than twice that of class II chloroplast preparations. The endogenous activity of class I preparations was lower in nitrogen than in air, and was 97% inhibited by low concentrations (5×10^{-5}M) of 1-(*p*-chlorophenyl)-3, 3-dimethylurea (CMU) (Table 1). Since these are the characteristics of non-cyclic photophosphorylation this result argues against the existence of a cyclic form of photophosphorylation *in vivo*. This is in accord with the findings and conclusions of Forti and Jagendorf (1961) who used a spinach chloroplast preparation which,

judged by their method of preparation, would contain a significant proportion of class I chloroplasts.

(iii) CO_2 *Fixation.*—The rate of endogenous CO_2 fixation by class I preparations was about 10 times that of class II preparations (Table 2). The rate of CO_2 fixation

TABLE 1

ENDOGENOUS PHOTOPHOSPHORYLATION BY CLASS I AND CLASS II CHLOROPLAST PREPARATIONS

Incubation conditions as described in Section II

Expt. No.	Chloroplast Preparation	Gas Phase	Additions	Endogenous Photophosphorylation (μmoles phosphorus esterfied/mg chlorophyll/hr)
1	Class I	Air	—	2·33
	Class II	Air	—	1·06
2	Class I	Air	—	1·98
	Class I	Nitrogen	—	1·29
3	Class I	Air	—	2·40
	Class I	Air	5×10^{-5}M CMU	0·07

by class I preparations was doubled if the chloroplasts were suspended in the supernatant fraction obtained by centrifuging the original leaf homogenate at 20,000 *g* for 30 min. When this supernatant fraction was boiled for 2 min, clarified by

TABLE 2

ENDOGENOUS CO_2 FIXATION BY CLASS I AND CLASS II CHLOROPLAST PREPARATIONS

Incubation conditions as described in Section II

Expt. No.	Chloroplast Preparation	CO_2 Fixed (μmoles CO_2/mg chlorophyll/hr)		
		Dark	Light	Light–Dark
1	Class I	0·26	2·88	2·62
	Class II	0·02	0·23	0·21
2	Class I in sucrose/Tris/KCl	0·45	2·32	1·87
	Class I in extract supernatant	0·20	5·02	4·82
	Class I in boiled extract supernatant	0·24	10·80	10·56

centrifugation, and the resultant solution used for resuspension of class I chloroplasts, CO_2 fixation by the latter was five times greater than that of class I in fresh buffered sucrose. The form of class I chloroplast which is most active in CO_2 fixation is the one which morphologically resembles most closely the chloroplast in the living cell

(cf. Plate 2, Figs. 1–12). Further work is required to determine whether this effect is due to a direct stimulation of CO_2 fixation by non-protein substances in the leaf extract, or whether it is primarily due to the better preservation of the original form of the chloroplast resulting, in turn, in a more complete retention of the components of the CO_2 fixation process.

(*d*) *Photochemical Activity of Chloroplast Fragments*

Whilst the intact class I chloroplasts provide a potentially useful tool for studying integrated, endogenous processes, it might be expected that class II chloroplasts and chloroplast fragments would exhibit higher activity under standard assay conditions where soluble reactants are added back to the chloroplasts. In the

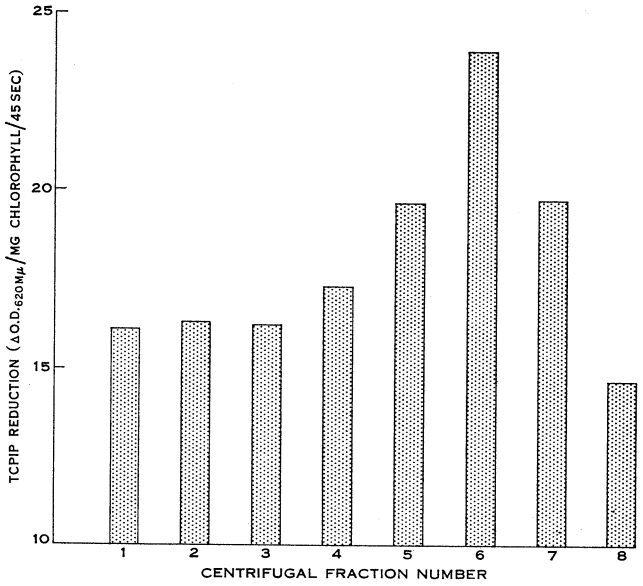

Fig. 1.—Hill reaction activity of a series of chloroplast fractions collected at increasing centrifugal speeds from a cell-free homogenate. Fraction number 1 is the lowest-speed fraction. The fractions are specified in detail in Section III(*d*).

more fragmented material, there would be less of a diffusion barrier between the soluble reactants and those components bound to particulate structures which are responsible for the primary reactions. The data of Figure 1 show that this is the case. A class I chloroplast preparation in buffered 0·4M sucrose was disrupted for 30 sec in an Omnimix blender at top speed, and successive pellets were collected from the homogenate by a series of centrifugations at increasing speeds. Successive pellets were collected by accelerating up to 170, 270, 480, and 1085 *g* and further fractions by centrifuging at 1085 *g* for 7 min, 4340 *g* for 10 min, 12,100 *g* for 10 min, and 27,000 *g* for 30 min. These centrifugal fractions are designated 1–8 in Figure 1. Pellets were resuspended in fresh buffered sucrose and their capacity for Hill reaction was determined. This activity rose to a maximum in the 4340 *g* pellet with a con-

sistent decline in fractions collected at higher speeds. Microscopically the first pellet was seen to contain a mixture of class I and class II chloroplasts. Subsequent fractions become progressively more enriched with class II and then with chloroplast fragments (cf. Plate 2, Figs. 13–20). The pellet with maximum activity contained no class I or class II chloroplasts but consisted of fragments with up to four clear grana. The same relationship between particle size and activity was seen for cyclic photophosphorylation when pyocyanin was used as the redox mediator.

Higher-speed fractions which consistently showed a marked decline in activity, were made up of small fragments with 1–3 recognizable grana. It is likely that these latter fractions also contained fragments which were below the limit of resolution of the light microscope.

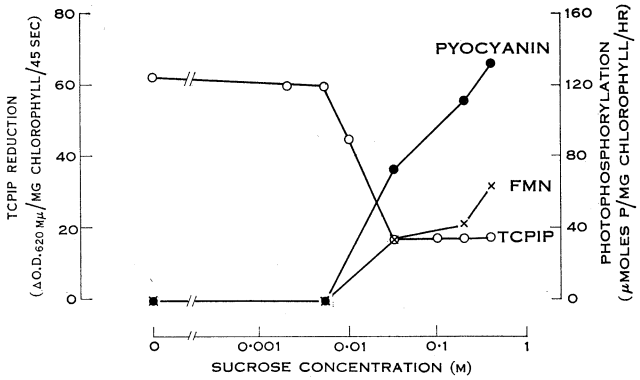

Fig. 2.—Effect of increasing hypotonicity on the Hill reaction (TCPIP reduction) and cyclic and non-cyclic photophosphorylation. Chloroplasts initially prepared in buffered 0·4M sucrose were resuspended in a series of lower sucrose concentrations.

(e) *Effect of Hypotonicity on Chloroplast Reactions and Structure*

It has been shown that progressive dilution of the suspending medium causes firstly the rupture of the outer limiting membrane of the chloroplast, followed by extensive distortion, swelling, and finally fragmentation of the remaining lamellar system (Spencer and Wildman 1962).

In the present experiments class II chloroplasts were prepared in buffered 0·4M sucrose, resuspended in a range of lower sucrose concentrations and in water, and all samples then assayed under standard assay conditions in which the solute concentration was 0·1M or greater. Figure 2 shows that in such preparations there is a roughly inverse relationship between the trends in Hill reaction and photophosphorylation activity with change in tonicity. Dye reduction is unaffected by decreasing sucrose concentration down to 0·02M, but between 0·02 and 0·005M the rate is increased more than threefold. No further change in activity occurs at lower sucrose concentrations or in water. On the other hand both cyclic and non-cyclic

photophosphorylation decrease steadily with reduction of the sucrose concentration and at 0·005M and lower these activities are reduced to nil.

The appearance of these preparations at these various sucrose concentrations is shown in Plate 3, Figures 1–8. The point of activation of the Hill reaction by dilution coincides with the fragmentation of the chloroplast mass and the appearance of many small, isolated, swollen balloons approximately 1–2 μ in diameter. These are sometimes found in clumps but largely as separate structures. At this same point the capacity for photophosphorylation is low. The structural requirements for coupling of photophosphorylation to electron flow are thus more complex than for the uncoupled Hill reaction. Indeed the latter is markedly activated under conditions of extreme swelling and disruption of the original structure, whilst any modification of the original form of the chloroplast by osmotic swelling appears to be detrimental to photophosphorylation.

TABLE 3

EFFECT OF 10^{-4}M Mg^{2+} ON TCPIP REDUCTION AND PHOTOPHOSPHORYLATION

Incubation conditions as described in Section II

Suspending Medium	TCPIP Reduction (ΔO.D.$_{620m\mu}$/mg chlorophyll/45 sec)		Photophosphorylation (μmoles phosphorus esterified/mg chlorophyll/hr)	
	2500 f.c.	8000 f.c.	Flavin Mononucleotide	Pyocyanin
Sucrose, 0·4M	19·4	20·4	56	130
Sucrose, 10^{-4}M	57·4	75·8	—	—
Water	60·4	82·4	Nil	Nil
$MgCl_2$, 10^{-4}M	23·0	23·5	28	82

(*f*) *Effect of Mg^{2+} on Chloroplast Reactions and Structure under Hypotonic Conditions*

Jagendorf and Smith (1962) have shown that low concentrations of divalent ions such as magnesium or calcium offset the effects of hypotonicity on phosphorylation and Hill reaction. We have confirmed these effects under our conditions, and Table 3 shows that 10^{-4}M $MgCl_2$ partially protects the chloroplasts against loss of phosphorylative ability, and at the same time negates the activating effect of treatment with water on both absolute rates and saturating light intensity of the Hill reaction. We have observed a striking parallel effect of 10^{-4}M $MgCl_2$ on chloroplast structure. Plate 4, Figure 3, is a photomicrograph of chloroplasts suspended in 10^{-4}M $MgCl_2$. These can be compared with Plate 4, Figure 2, which shows chloroplasts suspended in water. The low concentration of magnesium ions appears to have stabilized the membranes of both grana and intergrana regions, so that swelling has not been so disruptive. Grana are more clearly defined in 10^{-4}M $MgCl_2$.

(*g*) *Characteristics of the Hill Reaction of Water-treated Chloroplasts*

The above findings are consistent with the recent reports of Jagendorf and Smith (1962) that water-treated chloroplasts suffer loss of phosphorylative capacity

and enhancement of ferricyanide-reducing capacity. In addition we have found that TPN reduction, in the presence of added PPNR, was also stimulated 2·5-fold by water treatment. This marked change in both structure and Hill reaction activity of water-treated chloroplasts raised the possibility that a qualitative change may have occurred in the Hill reaction. For this reason the characteristics of this reaction in water-treated chloroplasts were examined in more detail.

(i) *Requirement for Subsequent Shrinkage.*—Water-treated chloroplasts must be returned to a medium of higher osmotic pressure before the activating effect of water treatment on the Hill reaction is exhibited (Fig. 3). In the standard assay

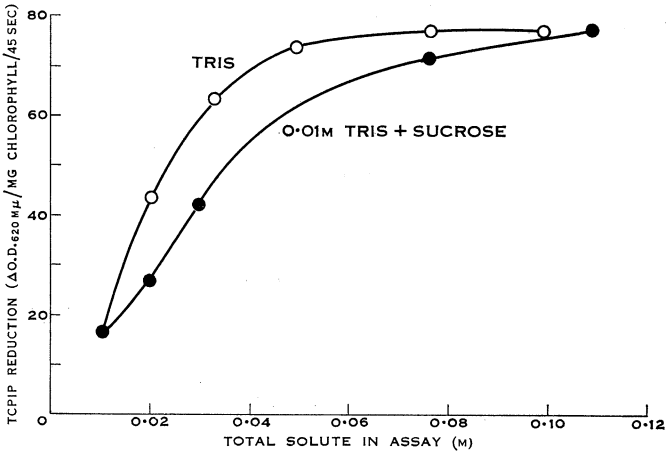

Fig. 3.—Effect of solute concentration on the Hill reaction activity (TCPIP reduction) of water-treated chloroplasts. Chloroplasts were isolated in buffered 0·4M sucrose, resuspended in water, and assayed at a range of Tris and sucrose concentrations.

chloroplasts are suspended in 0·1M Tris, and under these conditions water-treated chloroplasts exhibited the enhanced dye-reducing activity (cf. Fig. 2). If, however, water-treated chloroplasts are assayed in 0·01M Tris no enhancement is seen. Figure 3 demonstrates that there is definite requirement for a higher solute concentration (0·05–0·10M) in the assay medium for maximum Hill activity of water-treated chloroplasts. The effect is similar whether solute concentration is raised with Tris or with sucrose. Microscopically it can be seen that in water-treated chloroplasts transferred to 0·1M sucrose, extensive collapse of the small balloon-like structures has occurred.

(ii) *Light Intensity Relationships.*—Figure 4 shows the relationship between dye-reducing activity and light intensity for chloroplasts prepared in 0·3M sucrose (class II) and for the same chloroplasts subsequently suspended in water. The saturating light intensity for control chloroplasts was approximately 2000 f.c. while that of water-treated chloroplasts was 6000 f.c. However, while water-treated chloroplasts showed greatly enhanced activity at high light intensities, at lower

intensities (<400 f.c.) the control chloroplasts consistently showed greater activity than the water-treated preparation.

Manganese deficiency is known to cause an impairment of the oxygen-evolving sequence in chloroplasts (Spencer and Possingham 1961), and to bring about marked changes in the internal fine structure of chloroplasts, both intergrana lamellae and grana being affected (Mercer, Nittim, and Possingham 1962). Manganese-deficient chloroplasts did not react to water treatment in the same way as chloroplasts from healthy plants (Fig. 4). Neither the saturating light intensity nor the absolute rate of TCPIP reduction by manganese-deficient chloroplasts was increased by water treatment, although microscopically the general appearance of water-treated

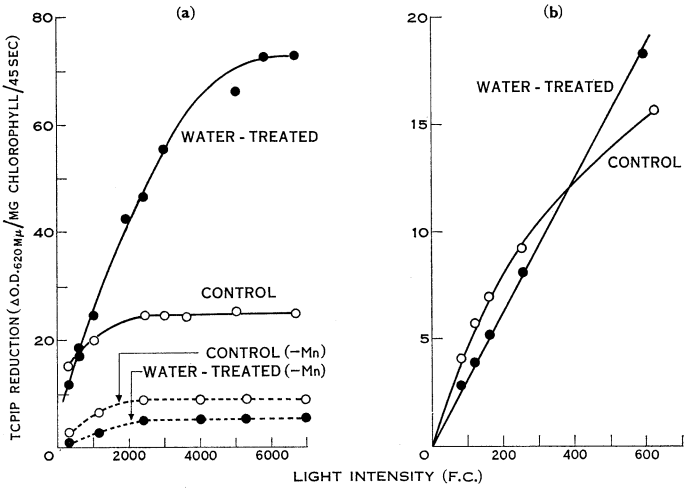

Fig. 4.—Relationship between light intensity and Hill reaction activity (TCPIP reduction) for both water-treated chloroplasts and chloroplasts in sucrose. (*a*) Comparison of water-treated and control chloroplasts both from healthy and from manganese-deficient plants. (*b*) Comparison of water-treated and control chloroplasts from healthy plants over a range of low light intensities.

chloroplasts from manganese-deficient plants was not distinguishable from corresponding healthy plants. Apparently the structural changes resulting from manganese deficiency are such as to prevent the subsequent modifications responsible for an enhanced rate of Hill reaction. It follows that the structural changes in healthy chloroplasts associated with the enhanced Hill reaction rate are below the limits of resolution of the light microscope.

(iii) *Temperature Coefficient.*—The temperature coefficient for dye reduction between 10 and 20°C was similar for both control chloroplasts in sucrose and for water-treated preparations, being 1·48 and 1·42 respectively. Unlike chloroplasts in sucrose the water-treated chloroplasts showed an actual decrease in rate at 30°C as compared to 20°C. Since the illumination period is only 45 sec and the total

exposure of the chloroplasts to 30°C conditions approximately 2 min, this result indicates that the structural changes brought about by water treatment have induced a big increase in heat lability of the photochemical apparatus.

(iv) *Action Spectra.*—The possibility that water-treatment may have exposed a new site of reduction of TCPIP prompted a study of the action spectrum of water-treated chloroplasts. However, it was found that with TCPIP as the electron acceptor the action spectrum for both untreated and water-treated chloroplasts was the same (Fig. 5). The major peak was found at 671 mμ, presumably due to chlorophyll *a*, with a shoulder at 649 mμ due to chlorophyll *b*. A similar action spectrum was found by Chen (1952) with chard chloroplasts extracted in 15% methanol and assayed with 2,6-dichlorophenolindophenol.

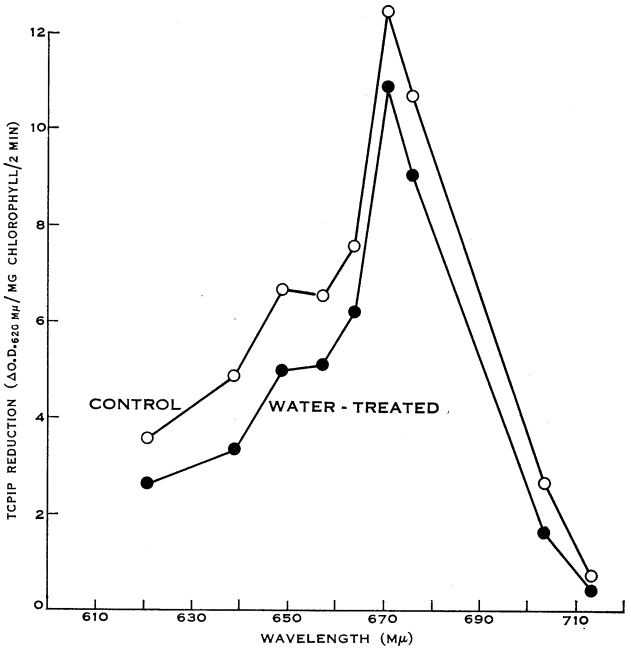

Fig. 5.—Action spectrum for the Hill reaction (TCPIP reduction) by water-treated chloroplasts compared with that of control chloroplasts in sucrose.

IV. Conclusions

The results reported in this paper present a strong argument for a more widespread monitoring, by means of phase-contrast microscopy, of chloroplast preparations intended for biochemical studies. It has been shown that the state of the chloroplast can be greatly modified by the particular isolation procedure employed, and that these modified chloroplast types vary greatly in their capacity to carry out chloroplast reactions.

All the above observations were made with spinach chloroplasts isolated in buffered sucrose. Preliminary microscopic observations showed that spinach chloroplasts isolated in sodium chloride were less well preserved and deteriorated more rapidly during storage at 0°C. Such chloroplasts quickly took on an osmotically shocked appearance with extensive swelling and loss of definition of the grana (Spencer, unpublished observations). No biochemical measurements were made on chloroplasts in sodium chloride, but the above behaviour makes a rapid loss of soluble components seem likely.

Exposure of spinach chloroplasts to low sucrose concentrations or to water brought about marked changes in both structure and function. In particular the enhanced Hill reaction capacity of water-treated chloroplasts was examined in detail to determine whether qualitative changes had also been induced. However, in all respects except the light-intensity relationships, the enhanced rate showed the same characteristics as the reaction in the unmodified chloroplasts. The simplest explanation would seem to be that hypotonic treatment has caused an uncoupling of the electron transport sequence, resulting in an increased rate of electron flow. Avron (1964) has recently produced evidence that exposure of chloroplast fragments to ethylenediaminetetraacetate removes a coupling factor and concomitantly causes stimulation of uncoupled electron transport. Our observations on light-intensity relationships are not in agreement with those of Jagendorf and Smith (1962) on one point. These authors found that the capacity for reduction of ferricyanide was higher in water-treated chloroplasts than chloroplasts in sucrose at all light intensities. We observed a crossover at about 400 f.c. below which water-treated chloroplasts consistently showed a lower capacity for both ferricyanide (Spencer, unpublished observations) and TCPIP reduction.

The precise nature of the changes in the chloroplast membranes associated with the changes in activity of water-treated chloroplasts cannot be resolved by light microscopy. It is possible that more precise correlations could be made by employment of electron microscope techniques.

V. References

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Explanation of Plates 1–4

All material illustrated is from spinach leaves, and all photomicrographs have been taken under phase contrast

Plate 1

Fig. 1.—Chloroplast suspension prepared by the method of Jagendorf and Avron (1958). The preparation contains approximately equal proportions of class I (highly refractive without clear grana) and class II (more regular shape with clearly defined grana) chloroplasts.

Fig. 2.—Chloroplast suspension enriched with class I chloroplasts prepared as described in Section III(*b*)(i).

Fig. 3.—Chloroplast suspensions enriched with class II chloroplasts prepared by the procedure of Jagendorf and Evans (1957).

Plate 2

Figs. 1–12.—Time sequence of a single isolated chloroplast showing the amoeboid movements of the peripheral chloroplast jacket material. Photographs were taken at irregular intervals in the course of 1 hr.

Figs. 13–20.—Appearance of successive fractions sedimented at increasing speeds from a cell-free extract of spinach leaves. These correspond respectively to centrifugal fractions Nos. 1–8 in Text-figure 1. Specific details of centrifugation conditions are given in Section III (*d*).

Plate 3

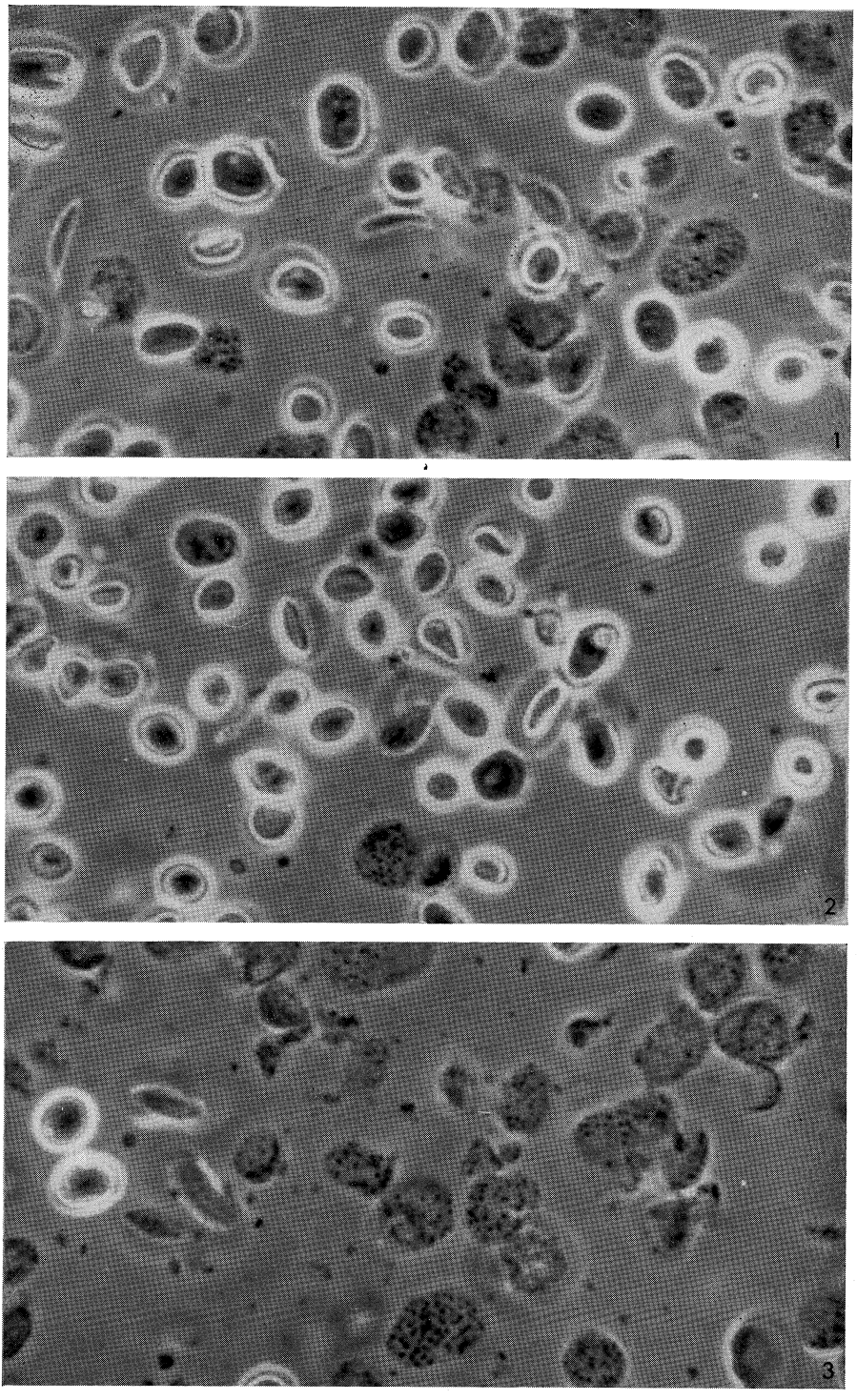
Figs. 1–7.—Showing, respectively, class II chloroplasts which have been resuspended in sucrose in the following series of decreasing concentrations: 0·4, 0·2, 0·1, 0·05, 0·01, 0·005, and 0·002M.

Fig. 8.—Class II chloroplast resuspended in water.

Plate 4

Figs. 1–3.—Effect of low concentrations of Mg^{2+} on chloroplast structure. Figure 1 shows a class II chloroplast preparation resuspended in 0·3M sucrose, 0·05M potassium phosphate, pH 7·2, and 0·01M KCl. Figures 2 and 3 show the same preparation resuspended in water and 10^{-4}M $MgSO_4$. Although swelling occurs in the magnesium sulphate series, this is more restricted and, in general, the chloroplast mass does not fragment and the grana are still well defined.

BIOCHEMICAL AND STRUCTURAL CHANGES IN CHLOROPLASTS



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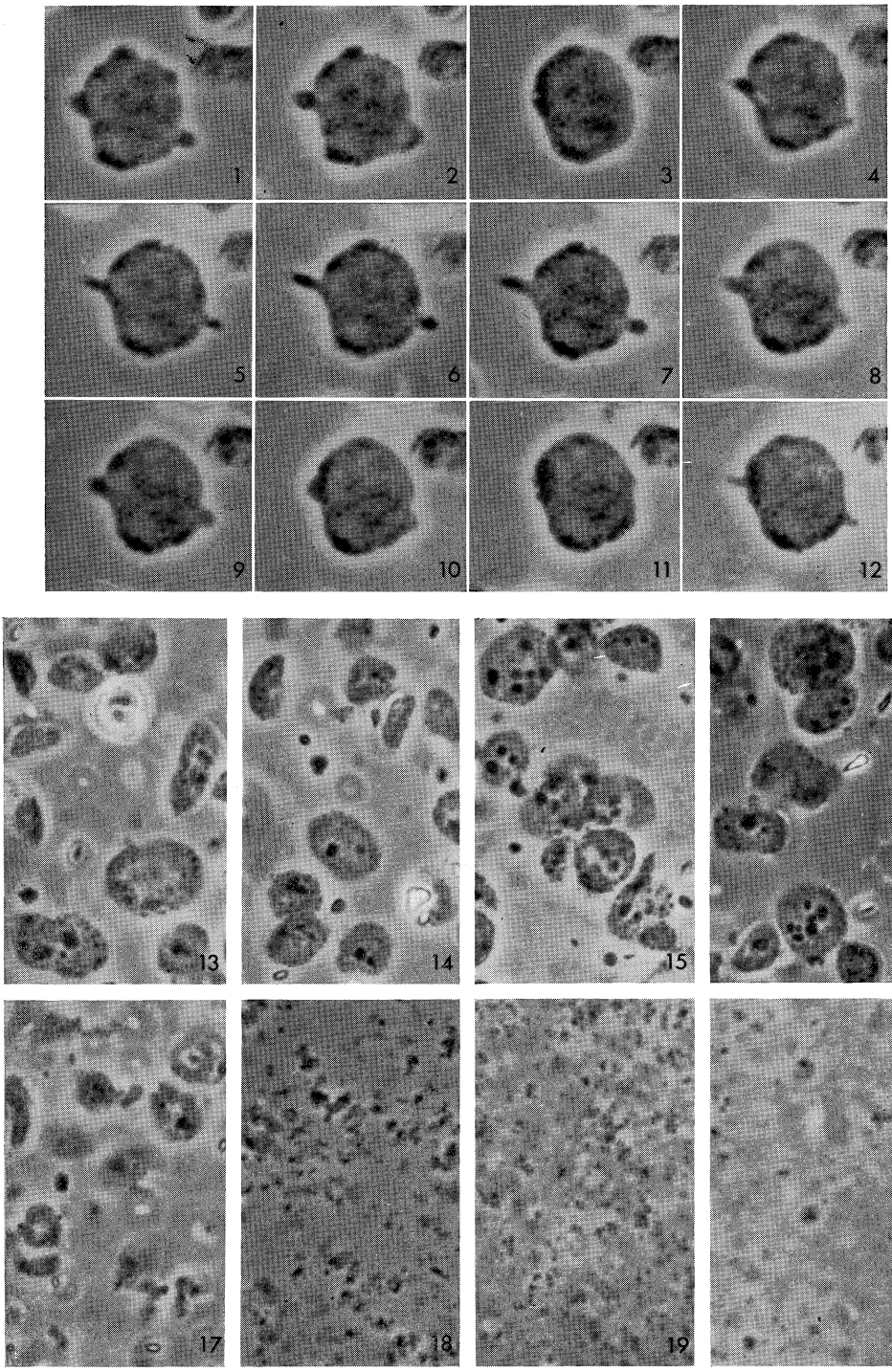

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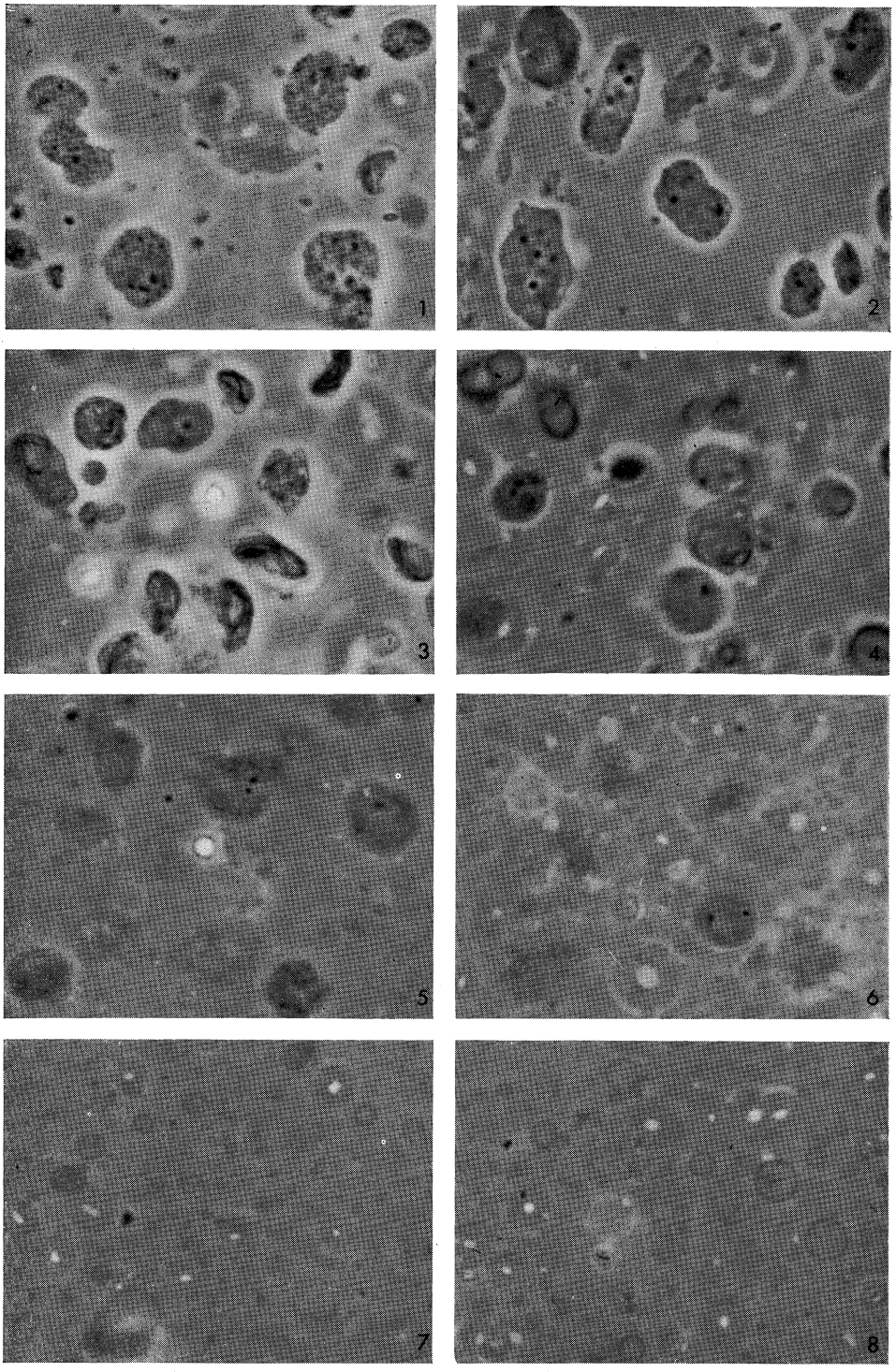

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