BBA 45714

KINETIC STUDIES ON ELECTRON-TRANSPORT COMPONENTS IN ISOLATED CHLOROPLASTS

I. THE EFFECT OF THE POOL OF ELECTRON CARRIERS BETWEEN THE TWO PHOTOSYSTEMS ON P_{700} CHANGES

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(Received January 5th, 1968)
(Revised manuscript received June 17th, 1968)

SUMMARY

Observations are reported on the oxidation–reduction kinetics of P_{700} in isolated chloroplasts. The quantitative effect of the pool of electron carriers between the two photosystems is emphasized. The oxidation and reduction of the pool were correlated by measuring simultaneously the P_{700} and the fluorescence kinetics. The conclusion is that P_{700} reacts with the same pool as measured previously by the fluorescence induction[1,2]. Other kinetic parameters were measured and the results are discussed with relevance to possible reaction schemes.

INTRODUCTION

There are indications[1–8] of the existence of a pool of electron carriers between the two photosystems of photosynthesis, in amounts much greater than those of the reaction centers. Quantitative estimates of the pool size, have given numbers ranging from about 1/20 to 1/50 electron-equiv per chlorophyll[2,4,5]; while the ratio of reaction centers is thought to be about 1/500 to chlorophyll (KOK's[9] P_{700} JOLIOT's[4] E, EMERSON–ARNOLD[10] flash experiments). A detailed kinetic analysis[1–3] of the fluorescence induction showed that this pool consists mainly of two consecutively reacting kinetic components, present in a 1:1 ratio. It was shown that the pool is reversibly reduced and oxidized by Photosystems II and I, respectively[2,6,8].

A previous work of VREDENBERG AND AMESZ[11] showed the existence of induction effects of P_{700} changes in algae, which were attributed by them to the effect of a pool of plastoquinone. This work confirms the existence of marked induction effects in the oxidation of P_{700}. The main purpose was to correlate quantitatively the P_{700} changes with the fluorescence kinetics. A positive correlation would indicate the effect of the same pool on both processes. For such a quantitative study isolated chloroplasts

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seem to be the most suitable system, for under suitable conditions (light intensity and wavelength, absence of a Hill reagent) the pool can be reduced or oxidized completely[2,12]. Also, the induction effects in isolated chloroplasts show simple features allowing a direct quantitative analysis.

METHODS

Spinach chloroplasts were isolated according to a standard procedure[13]. The chloroplasts were diluted for experiment in a medium of NaCl (0.02 M) and 0.05 M Tris–HCl (pH 7.8).

The experiments were carried out in a locally made sensitive difference spectrophotometer, in which the measuring beam passed alternately through the sample and a reference and was detected by a photomultiplier followed by an amplifier and rectifier sensitive only to the frequency of alteration. The samples were contained in rectangular cells of 2-mm optical path, which were held in the horizontal position. The actinic light entered at an angle of about 25° to the horizontal plane of the measured sample. The light intensity of the actinic light was estimated by ferrioxalate actinometry[14] (which gave absolute values for the blue and ultraviolet wavelengths) combined with relative measurements by a thermopile. The wavelength of the measuring beam was set with a monochromator (Bausch and Lomb) at a width of about 1 mμ. The wavelength of the actinic beam was isolated by interference filters. Filters were also placed in front of the photomultiplier to attenuate scattered actinic light. The combination of Schott glasses, RG-5 and BG-36, effectively reduced the scattered far-red (729 mμ) and the short wavelength ($<$640 mμ) actinic lights, while the measurement of P_{700} was made between 690 and 715 mμ.

The fluorescence induction was recorded in the same apparatus, except that the measuring beam was switched off and the photomultiplier was connected directly to an oscilloscope. Both the fluorescence and the P_{700} absorption changes could be recorded alternately with the same sample.

The apparatus, as set for absorption changes, had a relatively slow time response (about 0.5 sec). For faster measurements a time-calibrated camera shutter was placed in the path of the actinic beam to limit illumination to a fraction of a sec. The instrument then recorded the (integrated) change for that period of illumination.

Total absorption of the actinic light by the sample was estimated in a conventional single beam spectrophotometer (Zeiss) by the opal glass method[15]. For illumination by far-red light (729 mμ) the low total absorption (about 1–2 %) was estimated by linear extrapolation, from the observed absorption concentration curves. Due to the different optical path of the actinic light in the experiment, a correction was applied to the absorbance estimation. (The optical path in the apparatus for a 2-mm cell was calculated to be 2.8 mm.) The absorption measurements are estimated to be accurate within 15 %.

RESULTS AND DISCUSSION

Qualitative aspects of P_{700} kinetics

Fig. 1 shows examples of P_{700} reduction by short-wavelength (s.w.) light and oxidation by far-red (f.r.) light. If illumination in short-wavelength light is continued

after complete reduction of P_{700}, the subsequent oxidation by far-red light is slowed down and shows a distinct time lag. This is explained by the pool effect (*cf.* later).

Fig. 2 shows a difference spectrum, defined as the absorbance change after short-wavelength *minus* that after far-red. It has the typical spectrum reported for P_{700} (ref. 9).

In general, the behaviour of P_{700} is very similar to that reported by AVRON AND CHANCE[7] for cytochrome *f* in isolated chloroplasts: short-wavelength light rapidly reduced P_{700}. (There is a rapid oxidation phase at the beginning which is explained by the proximity of P_{700} to pigment system I.) On turning the short-wavelength light off, an immediate further reduction was sometimes observed, which apparently, also reflected the reducing pool between Photosystem II and P_{700} (ref. 7). There was a very slow oxidation in the dark, which was accelerated by turning on the far-red lights (Figs. 1a, b).

The reaction model

The results are interpreted in terms of the following model (Fig. 3). Photosystem II reduces a pool of compounds which consists of two kinetic components Q and P (following the notation of ref. 2; these are presumed equivalent to A_1 and A_2 of JOLIOT[4]). The pool is connected to P_{700}. The higher concentration of pool Q

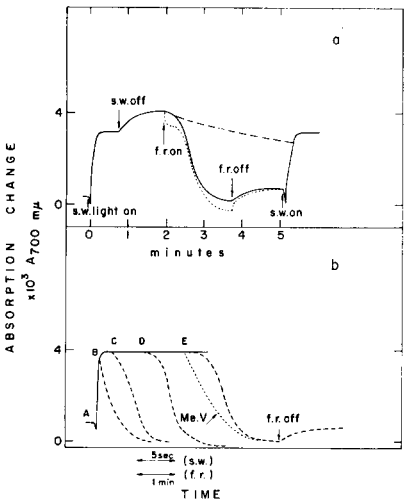

Fig. 1a. Kinetics of P_{700} changes as observed by absorbance changes at 700 mμ. The solid curve follows the following events: short-wavelength (s.w.) light on, short-wavelength light off, far-red (f.r.) light on, far-red light off, short-wavelength light on. The dotted curve is an alternative, found in many experiments for the segment: far-red light on, far-red light off; the jumps are probably artifacts which have nothing to do with P_{700} changes, since their spectrum is flat overall the range from at least 650 to 715 mμ. The broken curve represents a portion of the dark oxidation kinetics after switching off the short-wavelength light. Chlorophyll concentration: 0.1 mM. Amount of light passing through reaction cell: at 617 mμ 1.8, at 729 mμ 1.2 nEinstein/cm²·sec. Absorption: at 617 mμ 51 %, at 729 mμ 3 %.

Fig. 1b. The effect of the time in short-wavelength light on the subsequent oxidation kinetics of P_{700} and the effect of the presence of methyl viologen (Me.V). At a point A short-wavelength light is switched on; at successive points B–E the short-wavelength light is switched off and far-red light is switched on. The time scale is indicated by the arrow and is 5 sec in short-wavelength light and 1 min in far-red light. Other conditions are similar to the experiment in Fig. 1a. The dotted curve represents the oxidation kinetics after saturating pre-illumination in short-wavelength light in presence of methyl viologen (10 μM).

than P_{700} implies a convergence of a number of electron-transport chains to one point (see Fig. 3). Electron carriers such as cytochrome *f*, cytochrome *b* and plastocyanine, are not specified. They may be located between the main pool Q + P and P_{700} or they may be a part of the main pool.

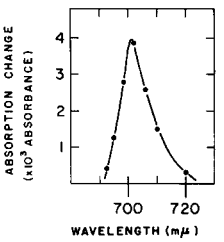

Fig. 2. Spectrum for the change of absorption around 700 mμ, taken as the difference of absorption after switching short-wavelength light off and immediately after switching far-red light off. The same conditions as for Fig. 1.

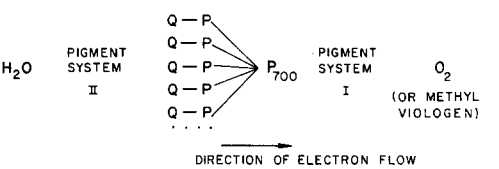

Fig. 3. Schematic diagram for illustrating the model of electron transport involved in P_{700} reduction and oxidation.

Isolated chloroplasts, to which no Hill oxidant is added, perform a photoreduction of O_2 to the level of H_2O_2 with evolution of O_2 from water (MEHLER's reaction[16]). This reaction is saturated at much lower intensities than the Hill reaction and its rate-limiting step is presumed to be between Photosystem I and the O_2 reduction site[2,12]. In strong short-wavelength light one observes mainly the electron transport from H_2O to the pool (the O_2 reduction rate can be neglected) and the pool is finally completely reduced[2,12]. In weak far-red light which is absorbed mainly in Photosystem I the electron transport from the pool to O_2 is observed and the pool is completely oxidized[2].

The pool effect on P_{700} changes

In addition to the further reduction of P_{700} after shutting-off the short-wavelength light, the main effect of the pool is seen by comparing the kinetics of P_{700} oxidation by far-red light for different pre-exposure times of the short-wavelength light. As the pre-exposure to short-wavelength light is increased (Fig. 1b) the time required for complete oxidation of P_{700} increases, up to a limit. In our experiments, the reduction of P_{700} is completed in less than a second, while the delaying effect of short-wavelength light on P_{700} oxidation lasts up to several sec. We assume that P_{700} is the first component to be reduced* by short-wavelength light, and only then are the other electron carriers gradually reduced in the main pool. The more reduced is the pool the longer it takes far-red light to oxidize P_{700}, since P_{700} is kept continually reduced by the pool.

The pool effect can also be seen in the kinetics of P_{700} oxidation, obtained after long pre-illumination in short-wavelength light. Very typically, and especially in very weak far-red light, there is a long lag which may last several sec, before the oxidation begins (Fig. 1a). Presumably, the P_{700} is continuously reduced by the pool, until the latter is appreciably oxidized.

* Compared with our times of measurement, of course. The transient transport of electron from Photosystem II to the pool and from the pool to P_{700} is too fast to be observed, and the measurements are presumably relevant to the equilibrium with respect to the fast dark reactions of the electron transport.

Another effect which depends on the pool is demonstrated by the kinetics of oxidation of P_{700} in presence of an electron acceptor. Addition of methyl viologen*, for example, prior to short-wavelength light removes the pool effect to a large extent (Fig. 1b). Apparently the high steady state of electron transport prevents the pool from being fully reduced by short-wavelength light (at least with our intensities which were a little above the light limiting region of the Hill reaction). This explanation is supported by fluorescence measurements, whereby on addition of an electron acceptor the fluorescence diminishes, denoting the oxidation of at least a considerable part of the pool[2].

Quantitative aspects

Quantitative estimate of the pool size from P_{700} oxidation kinetics

In order to estimate the pool size we need to know the rate of electron transport from P_{700} to the electron acceptor, during the oxidation by far-red light. The rate integrated over the time gives the total amount of electron transport and hence the electron-equiv content of the pool *plus* P_{700}. If one assumes that the rate is not limited on the reducing side of Photosystem I (*cf.* later) and that the light which is absorbed in pigment units, connected to already oxidized P_{700}, is not available for the oxidation of other P_{700} molecules, then the rate would be written:

$$R = \alpha_1 \phi_1 I \frac{P_{700}\text{ reduced}}{P_{700}\text{ total}} = \alpha_1 \phi_1 I p \qquad (1)$$

and the total amount, n, of electron transport from time zero to any time τ' is:

$$n = \alpha_1 \phi_1 I \int_0^{\tau'} p \, d\tau \qquad (2)$$

where α_1 is the fraction of the absorbed light in Photosystem I, ϕ_1 is the quantum yield of the primary photochemical act in Photosystem I, I is the absorbed light intensity and p is defined as the ratio P_{700} reduced to P_{700} total**.

The integral in Eqn. 2 is equivalent to the area described by the P_{700} kinetic curve and the ordinate, normalizing the maximum change to 1 (*cf.* Fig. 4b).

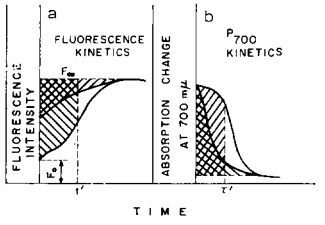

Fig. 4. Illustration of the method for comparing the fluorescence kinetics with the P_{700} kinetics. The comparison is made in two ways: (a) The area described by the P_{700} oxidation curve, obtained after certain time t' in short-wavelength light, with the area described by the "maximal" (after saturation with far-red light) fluorescence curve between times zero and t'. (b) The area described by the "maximal" P_{700} oxidation curve between times zero and τ' and the area described by the fluorescence curve, obtained after τ' in far-red light. In the figure, the areas which are compared are shaded in the same way.

* Methyl viologen is a low potential electron acceptor, which is presumed to act at the low potential side Photosystem I (*cf.* Fig. 1).

** There is one approximation in these relations (1 and 2), namely that the opposing reduction by Photosystem II in far-red light is zero during the whole course of oxidation. This seems quite correct for far-red light.

Eqn. 2 and the considerations which led to it are similar to those which applied in ref. 2 for the case of the fluorescence induction.

Apart for the dependency on the intensity of light which is absorbed in "active" P_{700} (reduced form), the rate of electron transfer must depend also on the state of reduction of the electron acceptor (X) of P_{700}. For example, one may think on the situation that X is in the complete reduced form; in this case the efficiency of electron transport would be zero, even if P_{700} alone is fully active. In support of this consideration it is thought that the rate-limiting step in O_2 reduction is in the reducing side of Photosystem I (refs. 2, 12). Thus Eqns. 1 and 2 cannot be true in all conditions. To overcome this difficulty very weak far-red light is used in order that O_2 reduction is light limited and the primary electron acceptor is assumed to be always oxidized.

According to Eqn. 2 a possible criterion for the use of the area of the P_{700} curve as a measure for n, is that the area be inversely proportional to the light intensity. In checking this experimentally it was found that for low light intensities this was approximately true. At higher intensities the observed area was larger than expected, indicating that the reaction was being limited by other factors in addition to P_{700}. Therefore, for using Eqn. 2, results for the low far-red light intensity region only were taken.

Quantitative estimate of the pool size from fluorescence kinetics

Fig. 5 shows examples of the fluorescence induction obtained in short-wavelength light. The fluorescence change from a lower to a higher value reflects[17] a re-

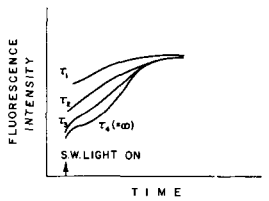

Fig. 5. Fluorescence intensity *vs.* time in strong short-wavelength (s.w.) light. τ_i represent various times (in increasing order) of far-red pre-illumination.

duction of the primary electron acceptor of Photosystem II, Q, $Q \xrightarrow{h\nu II} Q^-$. According to the analysis made in ref. 2 the rate of electron transport to Q, or to the pool, at any time t, is given by:

$$R = \alpha_2 \phi_2 I f \tag{3}$$

where α_2 is the fraction of light absorbed in Photosystem II, ϕ_2 is the quantum yield of the primary act in Photosystem II, f is defined by:

$$f = (F_\infty - F)/(F_\infty - F_0)$$

and is the amount of variable fluorescence, measured from F_∞ and normalized by making its maximum value equal to 1 (*cf.* Fig. 4 for the definition of the fluorescence parameters).

The total amount of reduction, in electron equivalents, which is indicated by

the fluorescence induction, from time zero to any time t', is given by integration of Eqn. 3:

$$n = \alpha_2 \phi_2 I \int_0^{t'} f \, dt \tag{4}$$

The integral in Eqn. 4 is equivalent to the area described above the fluorescence induction curve between the ordinates at $t = 0$ and $t = t'$ (Fig. 4).

Eqns. 3 and 4 for Photosystem II are completely analogous to Eqns. 1 and 2 for Photosystem I.

Comparison of the P_{700} kinetics to the fluorescence kinetics

Procedure of a typical experiment: (a) P_{700} oxidation kinetics: The system was brought to the completely oxidized state by an extensive exposure (about 2 min) to far-red light (729 mμ). This was followed by a brief exposure to short-wavelength light for a known time t, and then the P_{700} oxidation kinetics were recorded in subsequent far-red light illumination (729 mμ). (b) Fluorescence induction: The system was brought to a completely reduced state by exposure (a few sec) to intense short-wavelength light (510–630 mμ), which was followed by exposure to far-red light (729 mμ) for a known time τ, and then the fluorescence induction was recorded in short-wavelength light. The letters t and τ always refer to time in short-wavelength and far-red lights, respectively.

For both fluorescence induction and P_{700} oxidation we obtain series of curves, the characteristics of which are a function of the length of the appropriate pre-illumination (*cf.* Fig. 4). The series of functions f *vs.* t depend on τ. Each of these functions describes an area which measures the amount of reduction, equal to the amount of oxidation in the time τ of pre-illumination. In the same way, the series of P_{700} oxidation curves, obtained after various times, t, of pre-illumination in short-wavelength light, describe areas which measure the amount of reduction caused by the short-wavelength light in the time t.

There are two ways of comparing the fluorescence and P_{700} kinetics: (a) The amount of reduction *vs.* t which is given either directly from the fluorescence curve for $\tau = \infty$ or from the P_{700} oxidation curves for various values of t. (b) The amount of oxidation *vs.* I which is given directly by the complete P_{700} oxidation curve (for $t = \infty$) or by the fluorescence induction curves for various values of τ.

The factors $\alpha_1\phi_1$ and $\alpha_2\phi_2$ can be eliminated by taking the amounts of the total pool as 1. In this case, the amount of oxidation or reduction is given by the relevant area of the kinetic curve divided by the maximum area.

Analytically, we wish to compare (*cf.* Eqns. 2 and 4):

$$\frac{\int_0^{t'} f\,(\text{for } \tau = \infty)\, dt}{\int_0^{\infty} f\,(\text{for } \tau = \infty)\, dt} \text{ with } \frac{\int_0^{\infty} p\,(\text{for } t = t')\, d\tau}{\int_0^{\infty} p\,(\text{for } t = \infty)\, d\tau}$$

which is the amount of reduction done during the time t' by short-wavelength light, measured by the fluorescence or P_{700} kinetics respectively; and

$$\frac{\int_0^{\infty} f\,(\text{for } \tau = \tau')\, dt}{\int_0^{\infty} f\,(\text{for } \tau = \infty)\, dt} \text{ with } \frac{\int_0^{t'} p\,(\text{for } t = \infty)\, d\tau}{\int_0^{\infty} p\,(\text{for } t = \infty)\, d\tau}$$

which is the amount of oxidation done during the time τ' by far-red light, again measured by the fluorescence or P_{700} kinetics, respectively.

If the processes reflected by the fluorescence kinetics and P_{700} kinetics are reduction and oxidation of the same pool, this comparison should result in equal results.

Figs. 6a and 6b show the relative amounts of oxidation and reduction, calculated as explained above. There is a very close agreement between the results of P_{700} and the fluorescence kinetics. Therefore, it is very probable that both types of experiments reflect the same pool.

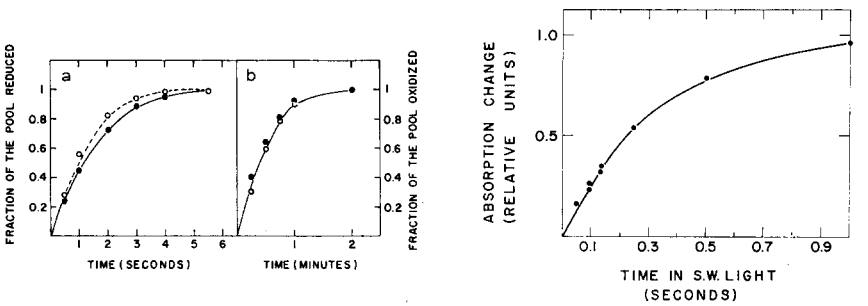

Fig. 6. The relative amount of reduction by short-wavelength light (a) or oxidation by far-red light (b) of the pool. ○, from the fluorescence induction; ●, from P_{700} oxidation kinetics. Chlorophyll concentration: 0.2 mM, short-wavelength light used for the reduction ≡ 510–630 mμ. Far-red light ≡ 729 mμ. Light intensity: for 510–630 mμ 8, for 729 mμ 0.5 nEinstein/sec·cm².

Fig. 7. Kinetics of P_{700} reduction by short-wavelength (s.w.) light. The same conditions as for Fig. 6.

Other kinetic parameters

From the fluorescence induction experiments alone one can deduce the following parameters: $\alpha_2\phi_2$ (= the maximum quantum yield for the reduction of the primary electron acceptor in Photosystem II) for short-wavelength light, and $\alpha_1\phi_1$ (= the maximum quantum yield for the oxidation of the pool) for far-red light (*cf.* ref. 2). In the present experiments we obtained the following typical numbers:

Pool size	= 1/24 of the chlorophyll ($a+b$) amount
$\alpha_2\phi_2$ (for 510–630 mμ)	= 0.25
$\alpha_1\phi_1$ (for 729 mμ)	= 0.8

which agree in general with those obtained previously[2], except that $\alpha_2\phi_2$ is smaller.

The kinetics of P_{700} reduction in strong short-wavelength light could be followed in the following way: A shutter was opened for a specified time and the net change in the reduction state of P_{700} was measured. It was presumed that all the electron carriers had come to an equilibrium state; since P_{700} has the highest oxidation potential of all components between the photosystems it is the first to be reduced. An example for the reduction kinetics done in this way is given in Fig. 7. The initial, maximum, yield, for P_{700} reduction, calculated from the initial slope, using the conventional assumption that the extinction coefficient at the peak is about $10^5\,M^{-1}\cdot cm^{-1}$, was found to be about 0.05, 5 times less than the maximum quantum yield for the pool reduction.

From the extent of absorption change the amount of P_{700} was calculated to be:

P_{700} amount ≈ 1/600 of the chlorophyll ($a+b$) amount

An upper limit to the ratio P_{700}/pool can be obtained by dividing the area of

the P_{700} oxidation curve, obtained for short period of short-wavelength light, just sufficient to reduce P_{700} (*e.g.* point B in Fig. 1b) to the maximal area of the P_{700} oxidation curve, obtained after extensive exposure of short-wavelength light (point E in Fig. 1b). We obtained as a typical number

$$\frac{\text{area, after short short-wavelength light}}{\text{maximum area}} \approx 1/6\text{–}1/5$$

This number is in fact a measure of the total reduction done parallel to the P_{700} reduction. From it and the pool estimate above, it follows:

> Total reduction done during the time needed for reduction of $P_{700} \approx 1/125$ of the chlorophyll $(a + b)$ amount

This result is fully consistent with the low quantum yield of the P_{700} reduction.

A possible explanation for these results is that P_{700} equilibrates, with equilibrium constants close to 1, with other components of the pool, and therefore the total amount of reduction can be much greater than that shown by the initial kinetics of P_{700} reduction. Present experiments, now in progress, show, however, that cytochrome *f* and cytochrome *b* do not make an appreciable contribution to this equilibrium. An alternative is that P_{700} is reduced in parallel to other components, which do not react directly with it (in parallel electron-carrier chains). For example P_{700} may be just one of several components contributing to the P pool. (For this one may assume that P pool, which acts as a single kinetic component, is composed of several, non-identical, electron carriers.) In this assumption P_{700} cannot be the exclusive reaction center of Photosystem I.

The possibility also exists that the extinction value of $10^5\ M^{-1} \cdot cm^{-1}$ assigned for P_{700} is in gross error and must be about 5 times smaller. This will bring the quantum yield of P_{700} reduction to that of the pool reduction, and make the P_{700} amount to be about 1/5 of the pool.

All these possibilities are now being examined, and an attempt is being made to analyze the details of the kinetics of P_{700} changes together with other electron carriers. These will be dealt with in future articles.

ACKNOWLEDGEMENTS

This work was made possible by a personal grant donated by E.M.B.O. to work in the Biophysical Laboratory in Leyden, The Netherlands. Thanks are due to Professor L. N. M. DUYSENS, Dr. J. AMESZ and Dr. T. BEUGELING, as well as the other members of the laboratory for their help.

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