BBA 45624

ANALYSIS OF THE 'WEAK LIGHT EFFECT' ON THE FLUORESCENCE YIELD IN ISOLATED CHLOROPLASTS

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(Received August 14th, 1967)

SUMMARY

1. The effect of light intensity on the fluorescence yield in isolated chloroplasts is analysed. The fluorescence yield changes between two limits, a low yield at low light intensities and high yield at high intensities. The effect is explained by the electron transport induced by O_2 which acts as an electron acceptor. A mathematical analysis is developed to explain the quantitative aspects of this effect, in connection with the electron transport.

2. Comparison of rates, the calculated one from fluorescence experiments and the measured one from O_2 uptake shows that the O_2-mediated electron transport quantitatively accounts for the effect of light intensity on fluorescence.

3. In complete agreement with the above, the fluorescence yield remains maximally high at all intensities under the following conditions: (a) anaerobic conditions; (b) by addition of the strong reductant sodium hydrosulfite; (c) by addition of 3(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of O_2 evolution.

4. There is evidence to show that Photosystem I is involved in the O_2-reduction step.

INTRODUCTION

MURATA, NISHIMURA AND TAKAMIYA[1] have reported that the fluorescence yield of isolated chloroplasts decreases at low light intensities. This effect was explained by assuming a back reaction of the primary photochemical products; a reaction which gains more importance as the light intensity decreases. As a larger fraction of the light excitation energy is used for this reaction at low light intensity, the fluorescence yield consequently decreases.

This explanation and the entire kinetic formulation seem unjustified for the following reasons: (a) It has been stated[1] that in the absence of a Hill oxidant there cannot be any regular non-cyclic electron transport. However, the fact was not considered that in aerobic suspensions O_2 itself may act as an electron acceptor. (b) The kinetic derivations are based on second-order rate equations, which may be applicable to a homogeneous medium, where any molecule can react with any other one. This is not the case in the photosynthetic unit, where a molecule is restricted to reacting with

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; Diquat, 1,1'-ethylene-2,2-dipyridylium dibromide.

a specific neighbor molecule only. If one assumes that the electron transport chains are independent and non-interacting, then one will naturally choose a first-order formulation of the rate equations.

It will be shown in this paper that the effect of light intensity on the fluorescence yield can, in fact, be accounted for by the electron transport induced by O_2. Thus, this effect is just a special case of the fluorescence *vs.* intensity dependence, found a long time ago in many active photosynthesising organisms, and also in isolated chloroplasts which undergo a Hill reaction.

The quantitative analysis used here, of the dependence of the fluorescence yield on the electron transport rate and the light intensity, is in principle analogous to the approach of LUMRY, MAYNE AND SPIKES[2]. The derivations are reformulated in terms of the specific model put forward by DUYSENS AND SWEERS[3]. Analysis of the fluorescence induction in isolated chloroplasts in terms of this model was made by MALKIN AND KOK[4,5]; the terminology and ideas from there[4] form the theoretical basis of the present paper.

THEORY

It is assumed that there exists a compound Q (quencher), which acts both as an excitation trap and an electron acceptor for Photosystem II. The relevant reactions may be written formally as follows:

$$Q \xrightarrow{h\nu_{II}} Q^- + \oplus \qquad \text{(i)}$$

The positive holes react with water to form O_2, in a reaction which is fast enough not to limit the system under normal conditions[4].

$$Q^- + \text{ETC} \longrightarrow Q + \text{ETC}^- \qquad \text{(ii)}$$

where ETC denotes the electron transport chain (which may include Photosystem I).

$$\text{ETC}^- + A \longrightarrow \text{ETC} + A^- \qquad \text{(iii)}$$

where A is any electron acceptor added to the system.

Under aerobic conditions, even when no electron acceptor is added, O_2 may act as A, as indicated *e.g.* by the reaction of MEHLER[6].

$$\text{ETC}^- + \tfrac{1}{2}\, O_2 + H^+ \longrightarrow \tfrac{1}{2}\, H_2O_2 + \text{ETC} \qquad \text{(iv)}$$

In the steady state the redox level of Q is balanced by reactions (i) on the one hand and (ii) on the other. If one assumes that each Q is connected to an independent pigment unit, the following equations are derived (*cf.* ref. 4 for more details).
Rate of reaction (i)

$$= \alpha_2 \phi_2 I \frac{[Q]}{[Q]_0} \qquad (1)$$

Fluorescence yield

$$\equiv F = F_0 \frac{[Q]}{[Q]_0} + F_m \frac{[Q^-]}{[Q]_0} \qquad (2)$$

Where $[Q]_0 = [Q] + [Q^-]$ is the total concentration of Q; $\alpha_2 =$ the fraction of absorbed light which is chanelled to Photosystem II; ϕ_2 = the maximum quantum yield of chemistry in Photosystem II, *i.e.* the quantum yield of the reaction $Q \xrightarrow{h\nu} Q^-$ for a quantum absorbed in unit Q (and not Q^-); I = total light intensity absorbed.

Eqn. 2 expresses a linear relation* between the fluorescence yield and the redox state of Q. F_0 and F_m are the fluorescence yields in the case where Q is completely oxidized or reduced, respectively. In other cases the fluorescence yield may vary between the two extremes F_0 and F_m. Note that here we use the notation F_m rather than F_∞ used in ref. 4. The notation F_∞ indicates the steady level (achieved at $t = \infty$ of the induction period), which, under the conditions of ref. 4, was indeed practically equal to F_m. In the present case, however, we use conditions where the steady level of the fluorescence changes with intensity, and may be quite different from F_m. According to our concepts F_0 and F_m are constants of the system while the steady fluorescence yield, here denoted simply by F, may have any intermediate value, following the reduction state of Q.

Since Q^- does not function as an excitation trap, the fluorescence yield corresponding to Q^- is higher than that corresponding to Q, *i.e.* $F_m > F_0$.

The parameters F_0 and F_m can be obtained experimentally from the fluorescence induction phenomenon, *i.e.* the change of fluorescence yield in the transition from dark to light in short wavelengths ($<$ 680 mμ)[4]. After a long period in the dark, or after appropriate far-red ($>$700 mμ) irradiation, all Q is oxidized[4] and hence the fluorescence yield at $t = 0$ when the short-wavelength light is switched on would be F_0. The subsequent fluorescence rise from F_0 follows reaction (i) (reduction of Q), and is gradually balanced by reaction (ii), aided by (iii) (oxidation of Q). The steady fluorescence yield will have the value F_m in the case when reaction (iii) is inhibited; this is the case under rigorous anaerobic conditions, when no electron acceptor is present, or even under aerobic conditions at relatively high light intensity, as will be shown later.

At the steady state, when the reduction of A is steadily continued, the rate of reaction (i) is equal to the rate of reaction (ii), which is equal to the overall rate of electron transport, R. Therefore, from Eqn. 1:

$$R = \alpha_2\phi_2 I \frac{[Q]}{[Q]_0} \tag{3}$$

By elimination of $[Q]/[Q]_0$ from Eqns. 2 and 3, the fluorescence yield can be related to the electron transport rate and the light intensity.

$$F = F_m - (F_m - F_0)\frac{R}{\alpha_2\phi_2 I} \tag{4}$$

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* Our assumption of linearity between the fluorescence and the redox state of Q is based on the detailed kinetics of the fluorescence induction in isolated chloroplasts, where the experimental curve agrees entirely with the calculated one[4,5]. JOLIOT AND JOLIOT[7], however, presented some evidence against the existence of such a linearity. Their main points come from fluorescence induction at very high intensity and comparison of the O_2 evolved in a short flash to the rate of O_2 evolution in weak light. These experiments involve perhaps a different limiting factor besides Q (perhaps on the oxidizing side of Photosystem II) and so are not conclusive. This question should be investigated more thoroughly. A non-linear relation, such as that suggested by JOLIOT AND JOLIOT[7], does not modify greatly the main conclusions of this paper. Moreover, since the relation between fluorescence and rate is linear, presumably in any case (even if not linear with Q), the quantitative aspects of the paper also remain valid, although the derivation should be modified.

Two extreme situations may be analyzed: (a) Low light intensities. Here one assumes a linear relationship between R and I.

$$R = \Phi \cdot I \tag{5}$$

where Φ is the maximal quantum yield for electron transport. Substituting Eqn. 5 in Eqn. 4 one obtains:

$$F = F_m - (F_m - F_0)\frac{\Phi}{\alpha_2\phi_2} \quad \text{(low light intensities)} \tag{6a}$$

Eqn. 6a contains only the constant parameters of the system. Hence, in the low light region the fluorescence yield is constant and independent of the light intensity. One expects that $\Phi = \alpha_2\phi_2$ if Photosystem II is limiting[8], in this case $F = F_0$ at low intensities. If Photosystem I is limiting, which, as usually thought, is the case for the short-wave ($<$ 680 mμ) region, $\Phi < \alpha_2\phi_2$ (see ref. 8) and therefore F is higher than F_0, but is definitely smaller than F_m, as Eqn. 6a shows. If Photosystem I does not participate at all, $\Phi = \alpha_2\phi_2$ and $F = F_0$.

(b) High light intensities. Here one assumes a saturation of the electron transport rate. Denoting the saturation rate by R_s one obtains:

$$F = F_m - (F_m - F_0)\frac{R_s}{\alpha_2\phi_2 I} \quad \text{(high light intensities)} \tag{6b}$$

As the light intensity increases further, the second term in Eqn. 6b decreases and the fluorescence yield evidently tends toward F_m. The fluorescence yield thus varies between 2 extremes reached at low and high light intensities.

The data of Murata, Nishimura and Takamiya[1] is expressed on the basis of the fluorescence intensity rather than yield. The fluorescence intensity Π is given by F times I. Multiplying Eqns. 6a and 6b by I one obtains:

$$\Pi = \left[F_m - (F_m - F_0)\frac{\Phi}{\alpha_2\phi_2}\right] I \quad \text{(low light intensity)} \tag{7a}$$

and

$$\Pi = F_m I - (F_m - F_0)\frac{R_s}{\alpha_2\phi_2} \quad \text{(high light intensity)} \tag{7b}$$

Eqn. 7b (Π *vs.* I) corresponds to a straight line cutting the I axis at a point I_{0a} (notation by Murata, Nishimura and Takamiya[1]), given by:

$$I_{0a} = \frac{F_m - F_0}{F_m} \times \frac{R_s}{\alpha_2\phi_2} \tag{8}$$

I_{0a} is an experimental parameter which was used by Murata, Nishimura and Takamiya[1] to summarize their results.

From the experimental I_{0a} and use of Eqn. 8, R_s can be calculated. It is seen that Eqns. 7a and b predict that the fluorescence intensity *vs.* I will start as a linear function (7a), will bend to higher slopes and eventually become linear again (7b). This behaviour is indeed found in many photosynthesizing organisms (to quote only a few examples, see refs. 9–11), and the data of Murata, Nishimura and Takamiya[1] seem also to fit into this theoretical prediction.

For intermediate situations between low and high light intensities there is a lack of information about the exact variation of R *vs.* I. As a first approximation we may use a hyperbolic relation like that used for the Hill reaction[12,13]:

$$R = \frac{R_s \Phi I}{R_s + \Phi I} \tag{9}$$

One obtains, by substitution into Eqn. 4:

$$F = F_m - \frac{F_m - F_0}{\alpha_2 \phi_2} \frac{R_s \Phi}{R_s + \Phi I} \tag{10}$$

A practical modification of Eqn. 10 is given by subtracting F_m from both sides and taking reciprocals:

$$\frac{1}{F_m - F} = \frac{\alpha_2 \phi_2}{(F_m - F_0)\Phi} + \frac{\alpha_2 \phi_2}{(F_m - F_0) R_s} I \tag{11}$$

A plot $(F_m - F)^{-1}$ *vs.* I is expected to be linear, allowing the calculation of R_s from its slope. In this calculation the value of the parameters, F_0 and F_m can be estimated from the fluorescence induction (fluorescence *vs.* time) curves, as shown before: F_0 is the initial value (at $t = 0$) and F_m is the steady-state value obtained at high intensities or under anaerobic conditions. $\alpha_2 \phi_2$ is also obtained from the fluorescence induction, as described in ref. 4.

EXPERIMENTAL

Once washed chloroplasts were prepared as described by AVRON[14], but washed in a medium without ascorbate, and diluted in a medium of 0.4 M sucrose, 0.05 M Tris (pH 7.8) and 0.01 M NaCl.

The optical set-up for the fluorescence experiments was similar to that described before[4]. The light source was a high-pressure mercury arc. One beam served as both actinic and exciting light; it was filtered through a broad-band combination of filters, transmitting between 500 and 600 mμ (passing the 546 and 578 mμ Hg lines, as well as some background continuum). The incident light intensity was measured by a combination of actinometric[15] and fluorescence screen (Rhodamine-B (*cf.* ref. 16)) methods. The absorption of the chloroplasts suspension was measured in an integrating (Ulbricht) sphere. The fluorescence was isolated from scattered light by use of cut-off filters ($>$670 mμ) transmitting most of the fluorescence band of chlorophyll *a*. Fluorescence induction curves were recorded on an oscilloscope screen, from which steady-state values were also obtained.

O_2 uptake was measured in a different optical set-up. Here the light source was a 300-W tungsten lamp housed in a projector and the light was filtered through the same filter used in the fluorescence experiments. O_2 was measured polarographically on a commercial apparatus (Oxygraph). The temperature in all experiments was room temperature, 20–22°.

RESULTS AND DISCUSSION

Fig. 1 shows the dependence of the steady-state fluorescence yield, F, on the light intensity. It is seen that F changes between two limits, attained at low and high light intensities. The low limit of F is slightly higher than F_0, which according to Eqn. 6a means that Φ is slightly less than $\alpha_2\phi_2$. This may be explained by the participation of Photosystem I and a limitation by Photosystem I at the region of wavelengths used[8]. F tends to a limit, F_m, at high intensities, although it is not quite reached in our present intensities. The two phenomena, the fluorescence induction (F *vs.* t) and the change of fluorescence yield with the light intensity, are therefore related experimentally by common parameters F_0 and F_m, which is according to our theoretical model.

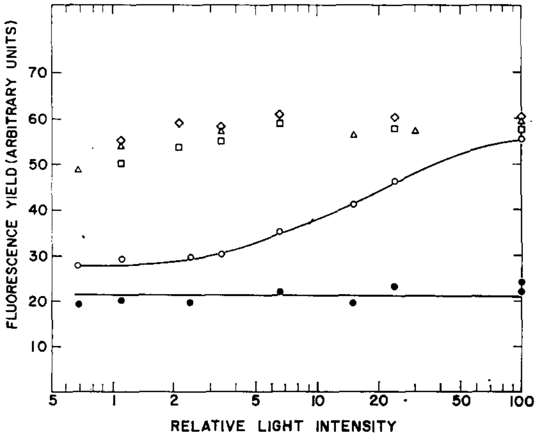


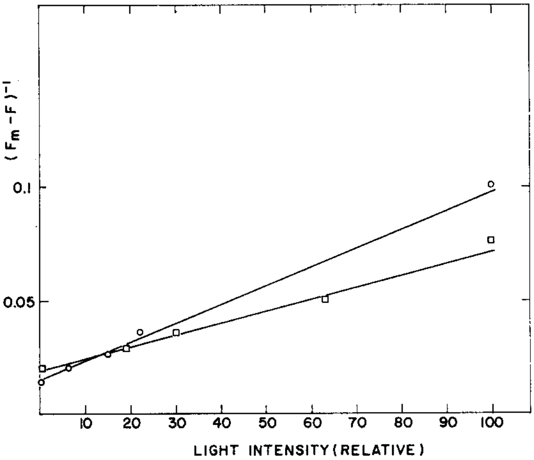
Fig. 1. The dependence of the fluorescence yield on the light intensity. ○, steady-state fluorescence yield (F); ●, initial fluorescence yield, at the beginning of the induction period (F_0); ◇, fluorescence yield in presence of sodium hydrosulfite; △, fluorescence yield under anaerobic conditions; □, fluorescence yield in presence of DCMU. The values for the last three conditions are approximately equal, representing F_m. In this particular experiment there are large variations in F_m. The slight decrease of F_m in anaerobic conditions at low light intensity may be real, since anaerobiosis is not strictly complete. The reaction mixture contained about 20 μM in chlorophyll in a 1.5 ml sucrose–Tris–NaCl medium. Sodium hydrosulfite was added as a solid; DCMU was added to a final concn. of about 10 μM. Anaerobic conditions were established by evacuation and addition of 0.04 M glucose and glucose oxidase. The maximum light intensity (100%) was 10^{-8} Einstein/sec and the absorption of the suspension was estimated to be 11%.

The dependence of the fluorescence yield in the presence of a small amount of the strongly reducing reagent, sodium hydrosulfite, is also shown in Fig. 1. Evidently, in the presence of this reagent, F remains maximally high even at low intensities. The effect of this reagent may be explained either by the anaerobiosis which it induces in the medium, cutting off the supply of O_2 or by a direct chemical reduction $Q \rightarrow Q^-$ by the reagent.

A similar effect is shown by 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of O_2 evolution in the chloroplasts.

The same behaviour is also seen, if anaerobiosis is established by evacuation and maintained by the addition of the enzymatic system glucose/glucose oxidase, which traps any O_2 evolved from the chloroplasts. This shows undoubtly the requirement of O_2 for the effect.

Fig. 2 shows 2 examples of a plot of $(F_m - F)^{-1}$ *vs.* I. This plot is linear, in agreement with Eqn. 11; from its slope R_s was calculated. For this calculation the parameter $\alpha_2\phi_2$ was determined by the method of ref. 4 and found to vary in the range 0.2–0.5. (Ferricyanide was used as an electron acceptor.) The calculated values of R_s for many experiments fall into the range $5–8 \times 10^{-3}$ electron equiv/mole chl·sec.



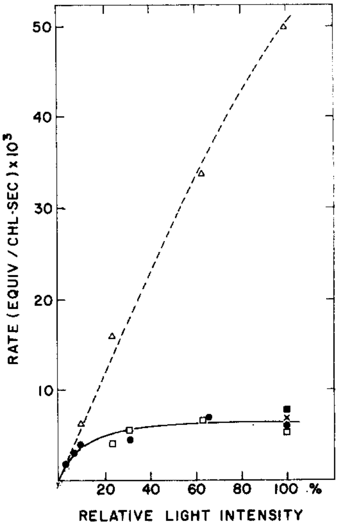


Fig. 2. A plot of $(F_m - F)^{-1}$ *vs.* I for two chosen experiments. In these experiments care was taken to measure F_m as precisely as possible and to discard experiments which showed large variations of F_m. F_m is defined here as the value of F obtained after addition of sodium hydrosulfite. The procedure was to measure F as function of I and then add the hydrosulfite and measure F_m at a range of light intensities, taking an average of several determinations.

Fig. 3. O_2 uptake rate, in equivalents, per mole chlorophyll, per second, for the Mehler reaction (solid line) and the Diquat-catalyzed oxygen uptake (broken line), as a function of the light intensity. □, ●, ■, are points for different samples. The point marked by × was obtained in strong white light and gave essentially the same rate as our standard green-yellow light of 100 % intensity. The maximum rate obtained in white light with Diquat was about 0.15 equiv/mole chl·sec. The reaction mixture contained a dense suspension of chloroplasts (about 0.1 mM in chlorophyll) in the same medium used for the other experiments, with addition of sodium azide (1 μM). 100 % light intensity corresponds to $50 \cdot 10^{-9}$ Einsteins per cm² per second. The Diquat concentration was about 10 μM.

A direct check, that the calculated R_s really corresponds to an electron transport mediated by O_2, was made by measuring Mehler's reaction directly. This was done by recording O_2 uptake in a system which contained also some sodium azide (1 μM), to inhibit the enzyme catalase, which is present in the chloroplasts. In this case, reaction (iv) proceeds continuously with accumulation of H_2O_2. The result is that for each electron transport there is a net 1/4 O_2 uptake. Fig. 3 gives the light intensity dependence of the Mehler reaction. For comparison, the rate of a similar reaction, but with 1,1'-ethylene-2,2-dipyridylium dibromide (Diquat) as an electron acceptor is also given. In the last reaction Diquat acts as an electron acceptor, but is quickly oxidized by O_2 which is then reduced to the level of H_2O_2[17,18]. From the stoichiometry standpoint the Mehler reaction and the Diquat-catalyzed O_2 reduction are similar, but mechanistically the last reaction seems to belong more to the category of a normal Hill reaction. From Fig. 3 one can conclude that while the Mehler's reaction has a low saturation rate, and tends to saturation at low intensities, the Diquat reaction is far from saturation; in fact it only starts to depart from linearity at the highest intensity we achieved. The saturation rate with Diquat was measured separately by using saturating white unfiltered light, and was found to exceed the saturation rate of the Mehler reaction by about 25-fold.

The R_s values as measured by O_2 uptake or calculated from the fluorescence experiments, agree within 25 %, which is quite good, taking into account that the reproducibility in the O_2 uptake experiments is within this factor. Also comparing Figs. 1 and 3 with respect to the light intensity (incident) which corresponds to 50 % of saturation or of change in fluorescence one gets roughly about 3 and 2 $\mathrm{m}\mu$Einsteins$\cdot\mathrm{sec}^{-1}\cdot\mathrm{cm}^{-2}$, respectively. If one takes into account the large absorption of the dense suspension used for O_2 uptake measurements (about 50 % absorption), the average intensity 'seen' by the chloroplasts is about a quarter less and the agreement is quite good. Thus the O_2-induced electron transport quantitatively accounts for the light intensity effect on the fluorescence yield.

An additional point is the effect of far-red light ($> 700\,\mathrm{m}\mu$). If the system is brought to its high fluorescence state (after exposure to strong light of short-wavelength light), then the far-red light accelerates the return to the low fluorescence state[4]. This was interpreted[4] as the oxidation of Q^- by O_2, with the participation of Photosystem I (reactions (ii) and (iii) only). The value reported previously for the saturation rate of this reaction, about $4\cdot 10^{-3}$ equiv/mole chl·sec, is in agreement with the present R_s values, deduced now from steady-state measurements in short-wavelength light. This similarity of rate values is an argument for the possible participation of Photosystem I in the present case.

Values in the literature seem also to agree, at least in order of magnitude. From MEHLER's data[6,19] one can calculate a little higher rate (close to $20\cdot 10^{-3}$ equiv/mole chl·sec). The values reported by FORTI AND JAGENDORF[20] for the O_2-induced electron transport and for the accompanying phosphorylation, are in the range 2–$10\cdot 10^{-3}$ equiv/mole chl·sec. Calculating from the fluorescence data of MURATA, NISHIMURA AND TAKAMIYA[1], by using Eqn. 8 and approximating $(F_m - F_0)/(F_m\cdot\alpha_2\phi_2)$ to unity*, one arrives at figures between $3\cdot 10^{-3}$ and $6\cdot 10^{-3}$, in the same units.

These values of R_s, for the O_2-induced electron transport, seem to be appreciably lower compared to the Hill reaction rate[21] (contrary to the conclusion of MURATA, NISHIMURA AND TAKAMIYA[1]) and the saturation is achieved at rather low intensity, as also illustrated in Fig. 3. This is peculiar to O_2 as an electron acceptor. One may conclude that the rate-limiting step of the O_2-mediated electron transport is in the electron transport chain between Photosystem I and the point of O_2 entry. This is concluded because the part in the electron transport chain, between O_2 evolution and Photosystem I, has a much higher potential rate, as judged by the saturation rate of the Hill reaction itself.

Another evidence for the participation of Photosystem I is that the low fluorescence yield achieved at low light intensities is higher than F_0, meaning that the maximum quantum yield, Φ of electron transport is less than $\alpha_2\phi_2$ (Eqn. 6a). This may be explained by a limitation of the quantum yield by another photochemical system (*viz.* Photosystem I)[8].

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* The values of the parameters $\alpha_2\phi_2$, F_m and F_0 are not reported. Our typical values were: $\alpha_2\phi_2 \approx 0.3-0.5$; $F_0/F_m \approx 0.3-0.5$.

ACKNOWLEDGEMENT

The author wishes to thank Prof. M. Avron for his advice and help in the biochemical work, and for making available the Oxygraph instrument.

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