ACTA CHEMICA SCANDINAVICA 14 (1960) 233–246

Adenosine Triphosphatase Activity in Chloroplasts

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The ATP-ase * activity of isolated spinach chloroplasts has been studied. Two distinct systems with ATP-ase activity are observed in this material. They are different in their pH optima and their response to $MgCl_2$ and chlorpromazine.

At pH 7.5 the ATP-ase activity is rather constant in different chloroplast preparations. It is strongly stimulated by $MgCl_2$ and inhibited by chlorpromazine. The pyrophosphatase activity at pH 7.5 is very low under the conditions of the ATP-ase activity measurements. Thus it can be excluded that the release of phosphate from ATP at pH 7.5 involves a liberation and a subsequent hydrolysis of inorganic pyrophosphate. Dinitrophenol does not stimulate the ATP-ase activity, either in intact or in broken chloroplasts.

At pH 5.5 a variable ATP-ase activity is found. It is unaffected by $MgCl_2$ and chlorpromazine and parallels the pyrophosphatase activity at this pH.

Results of kinetic experiments on the Mg^{++}-stimulated ATP-ase activity of spinach chloroplasts are given.

It is discussed whether the ATP-ase activity of chloroplasts has a functional relationship to the enzymatic mechanism by which phosphorylation of ADP is coupled to electron transport during light-induced phosphorylation.

ATP-ase activity becomes evident in rat liver mitochondria when either DNP is added or their structure is disrupted in some way, *e.g.* by making the mitochondrial suspension hypotonic, by ageing, or by treatment with detergents [1–7]. Addition of Mg^{++}-ions stimulates the ATP-ase activity of disrupted mitochondria. DNP-induced ATP-ase activity is obtained in mito-

* Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; FMN, flavin mononucleotide; FAD, flavinadenine dinucleotide; DNP, 2,4-dinitrophenol; P, inorganic phosphate; PP, inorganic pyrophosphate; Tris, tris(hydroxy-methyl)-aminomethane.

chondria when they are in an "intact" state and disappears when the mitochondria lose their structural integrity. The disappearance of the "intact" mitochondrial structure, involving a loss of the capability to carry out oxidative phosphorylation and of the stimulation of ATP-ase activity with DNP, is concomitant with the development of Mg^{++}-stimulated ATP-ase activity [7].

ATP-ase activity of animal mitochondria is supposed to be a reversal of part of the reaction sequence involved in ATP formation by oxidative phosphorylation [5, 7-15]. The Mg^{++}-activated ATP-ase may involve a one-step transfer of phosphate from ATP to an unknown carrier, followed by a hydrolysis of the compound so formed, whereas the DNP-activated ATP-ase may consist of two consecutive transfer steps, one identical with that involved in the Mg^{++}-activated ATP-ase, and a second yielding a high-energy compound which is subsequently split due to the presence of DNP [7].

Arnon [16] has reported that chloroplasts seem to be rather free of ATP-hydrolyzing enzymes. Avron and Jagendorf [17-18] did not observe any ATP-ase activity in chloroplasts under a large variety of conditions. Baltscheffsky [19], on the other hand, has recently shown that chloroplasts exhibit ATP-ase activity in the presence of added Mg^{++}-ions.

In this paper detailed results of our studies on the ATP-ase activity of spinach chloroplasts are presented. Attention has been directed towards the possibility that this activity is an expression of reversed reactions in light-induced phosphorylation and that information may be obtained about a possible similarity between the mechanisms for generation of ATP in oxidative phosphorylation and in light-induced phosphorylation.

METHODS

Broken spinach chloroplasts were prepared according to the method of Wessels [20]. As no significant difference in ATP-ase activity was found between once washed and four times washed chloroplasts (the activity decreased only 10 % after four washings), they were washed only once. The chlorophyll content of the chloroplast suspension was determined by the method of Arnon [21].

ATP-ase activity was measured by estimating colorimetrically the inorganic phosphate liberated from ATP. The incubation medium, containing ATP and $MgCl_2$ as indicated in the individual experiments, and distilled water to bring the volume up to 1.5 ml, was warmed to 30°. Then 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer, previously warmed rapidly to 30°, was added with stirring. The contents of the tube were incubated in the dark at 30°. The reaction was stopped by the addition of 2 ml of the reaction mixture to 4 ml of ice-cold 9 % trichloroacetic acid. After filtering an aliquot (3 ml) was taken for the estimation of inorganic phosphate by the method of Fiske and Subbarow [22]. The amount of phosphate split from ATP was determined by subtracting (from the values obtained) the phosphate content of a reaction mixture of the same composition, in which the reaction had been quenched directly after the mixture had been made up.

In the pyrophosphatase activity measurements, ATP was replaced by sodium pyrophosphate. Both the ATP and the pyrophosphate solutions were brought to the pH desired in the experiment before being added to the reaction mixture.

ATP, ADP, AMP, FMN and FAD were products of the Sigma Chemical Company.

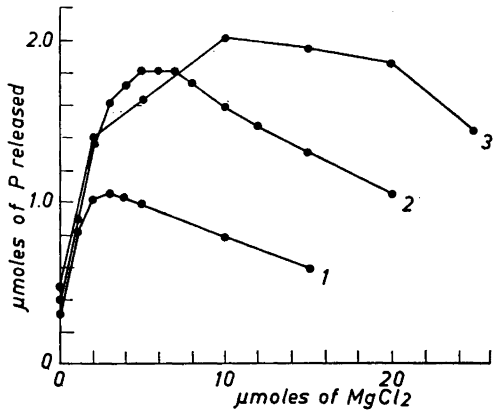

Fig. 1. ATP-ase activity as a function of $MgCl_2$ concentration. The reaction mixture contained, in addition to $MgCl_2$, 5 µmoles of ATP (1), 10 µmoles of ATP (2) or 20 µmoles of ATP (3), and 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer pH 7.5, containing 0.5 mg chlorophyll. Reaction time, 20 min at 30°.

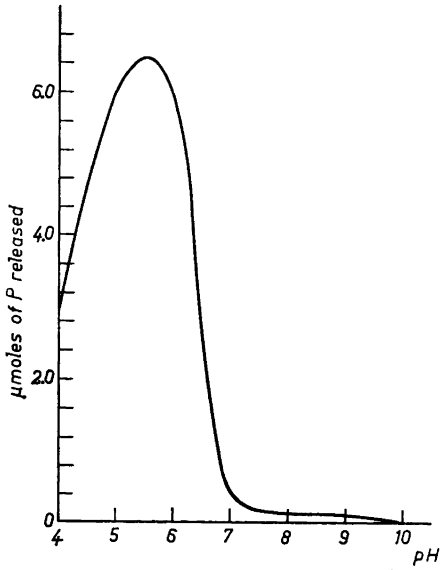

Fig. 2. Effect of pH on ATP-ase activity in the absence of $MgCl_2$. Reaction mixture: 10 µmoles of ATP and 1 ml of a suspension of chloroplasts in 0.1 M buffer, containing 0.5 mg chlorophyll. Reaction time, 15 min at 30°.

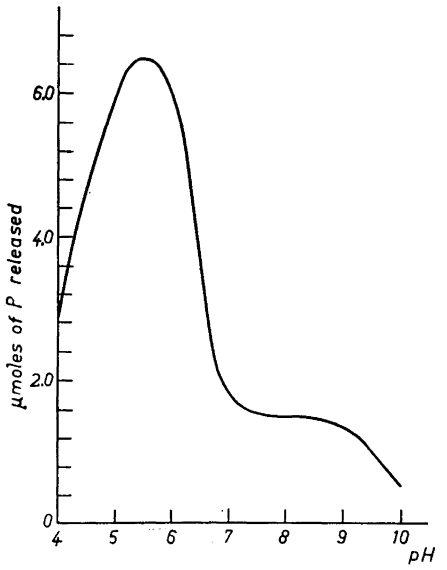

Fig. 3. Effect of pH on ATP-ase activity in the presence of $MgCl_2$. Reaction mixture: 10 µmoles of ATP, 5 µmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 M buffer, containing 0.5 mg chlorophyll. Reaction time, 15 min at 30°.

RESULTS

Fig. 1 confirms the finding of Baltscheffsky [19] that isolated spinach chloroplasts exhibit a Mg^{++}-dependent ATP-ase activity at pH 7.5. The optimal $MgCl_2$ concentration is 0.5—0.7 times the concentration of ATP.

Figs. 2 and 3 show the dependence of ATP-ase activity on pH in the absence and in the presence of added $MgCl_2$. As buffers we used glycine-NaOH (pH 8.5—10), Tris-HCl (pH 7—9), Tris-maleate-NaOH (pH 5.2—8.6) and phthalate-NaOH (pH 4—6). If possible, measurements were performed with two or more different buffers at each pH so as to prevent errors due to the effect of the buffer on the ATP-ase activity.

It must be stressed that the height of the maximum at pH 5.5 was found to be variable, ranging from one to six times the activity at pH 7.5. The amount of ATP split per unit time per unit chlorophyll at pH 7.5, on the other hand, was nearly constant, *viz.* 8—12 μmoles/h/mg chlorophyll. The ATP-ase activity at pH 5.5 was particularly high in chloroplasts from spinach grown in a greenhouse. The maximum at pH 5.5 cannot be ascribed to an impurity of the particular chloroplast preparation, because it did not disappear either after more washings or after digitonin treatment of the chloroplasts. In the latter case the chloroplasts were suspended in a 1 % digitonin solution. The suspension was allowed to stand for 30 min at 0°, and was then centrifuged for 7 min at 1 000 × *g*. The residue was discarded and the supernatant centrifuged for 30 min at 20 000 × *g*. The precipitated chloroplast fragments

Table 1. ATP-ase activity of two chloroplast preparations having extremely high (A) and extremely low (B) activity at pH 5.5 as compared to the ATP-ase activity at pH 7.5.

Chloroplast preparation	Additions (μmoles), unless otherwise indicated	pH	μmoles of P released
A	10 ATP	5.5	8.4
A	10 ATP + 5 $MgCl_2$	5.5	8.6
A	10 ATP + 5 × 10^{-4} M chlorpromazine	5.5	8.4
A	10 ATP + 5 $MgCl_2$ + 5 × 10^{-4} M chlorpromazine	5.5	8.3
B	10 ATP	5.5	1.1
B	10 ATP + 5 $MgCl_2$	5.5	1.6
B	10 ATP + 5 × 10^{-4} M chlorpromazine	5.5	1.1
B	10 ATP + 5 $MgCl_2$ + 5 × 10^{-4} M chlorpromazine	5.5	1.1
A or B	10 ATP	7.5	0.26
A or B	10 ATP + 5 $MgCl_2$	7.5	1.5
A or B	10 ATP + 5 × 10^{-4} M chlorpromazine	7.5	0.11
A or B	10 ATP + 5 $MgCl_2$ + 2 × 10^{-3} M chlorpromazine	7.5	0.20
A or B	10 ATP + 5 $MgCl_2$ + 5 × 10^{-4} M chlorpromazine	7.5	0.44
A or B	10 ATP + 5 $MgCl_2$ + 2 × 10^{-4} M chlorpromazine	7.5	0.89
A or B	10 ATP + 5 $MgCl_2$ + 6 × 10^{-5} M chlorpromazine	7.5	1.2

Chlorophyll concentration, 0.5 mg per sample. Reaction time, 15 min at 30°. The data for "A or B" represent a mean value obtained with a great number of chloroplast preparations of type A or B and of intermediate type. At pH 7.5 no significant difference was found between the mean values for the different types.

Table 2. Effect of inhibitors on ATP-ase activity.

Additions (μmoles), unless otherwise indicated	pH	μmoles of P released
10 ATP + 5 $MgCl_2$	7.5	1.4
10 ATP + 5 $MgCl_2$ + 10 ADP	7.5	1.0
10 ATP + 5 $MgCl_2$ + 10 AMP	7.5	0.88
10 ADP	7.5	0.28
10 ADP + 5 $MgCl_2$	7.5	0.50
10 ADP + 5 $MgCl_2$ + 2×10^{-4} M chlorpromazine	7.5	0.29
10 AMP + 5 $MgCl_2$	7.5	0.02
10 ATP	7.5	0.31
10 ATP + 3×10^{-4} M DNP	7.5	0.30
10 ATP + 5 $MgCl_2$	7.5	1.3
10 ATP + 5 $MgCl_2$ + 6×10^{-5} M DNP	7.5	1.3
10 ATP + 5 $MgCl_2$ + 3×10^{-4} M DNP	7.5	1.3
10 ATP + 5 $MgCl_2$ + 0.01 M KCN	7.5	1.3
10 ATP + 5 $MgCl_2$ + 2 FMN	7.5	1.4
10 ATP + 5 $MgCl_2$ + 10 FMN	7.5	1.4
10 ATP + 5 $MgCl_2$ + 0.01 M NaF	7.5	0.59
10 ATP + 5 $MgCl_2$ + 10^{-4} M *p*-chloromercuribenzoate	7.5	0.49
10 ATP + 5 $MgCl_2$ + 10^{-3} M $Na_2S_2O_4$	7.5	1.9
10 ATP + 5 $MgCl_2$ + 10^{-3} M $Na_2S_2O_4$ + 10^{-3} M KCN	7.5	1.9
10 ATP + 5 $MgCl_2$ + 5×10^{-3} M NaN_3	7.5	0.60
10 ATP + 5 $MgCl_2$ + 10^{-3} M $(NH_4)_2SO_4$	7.5	1.3
10 ATP + 5 $MgCl_2$ + 0.01 M succinate	7.5	1.3
10 ATP + 5 $MgCl_2$ + 5×10^{-3} M atebrin	7.5	0.78
10 ATP + 5 $MgCl_2$ + 0.03 M arsenate	7.5	0.91
10 ATP + 5 $MgCl_2$ + 10 P	7.5	1.1
10 ATP	5.5	5.0
10 ATP + 3×10^{-4} M DNP	5.5	5.0
10 ATP + 5 $MgCl_2$	5.5	5.4
10 ATP + 5 $MgCl_2$ + 3×10^{-4} M DNP	5.5	5.2

Chlorophyll concentration, 0.5 mg per sample. Reaction time, 15 min at 30°.

were resuspended in buffer and diluted to a concentration of 0.5 mg of chlorophyll/ml.

The ATP-ase activities at pH 5.5 and pH 7.5 differed with respect to their response to $MgCl_2$ and chlorpromazine. At pH 5.5 both $MgCl_2$ and chlorpromazine had only a slight effect, especially when the activity was high. At pH 7.5, on the other hand, the ATP-ase activity could be raised 4—10 fold by adding $MgCl_2$ to the reaction mixture. Addition of chlorpromazine resulted in inhibition at pH 7.5, which could not be reversed by FMN or FAD. In Table 1 two extreme types of results obtained with various chloroplast preparations are given:

a) High ATP-ase activity at pH 5.5, showing low response to $MgCl_2$ and chloropromazine as compared to the ATP-ase activity at pH 7.5.

b) Low ATP-ase activity at pH 5.5, showing stimulation by $MgCl_2$ and inhibition by chlorpromazine in the presence of $MgCl_2$.

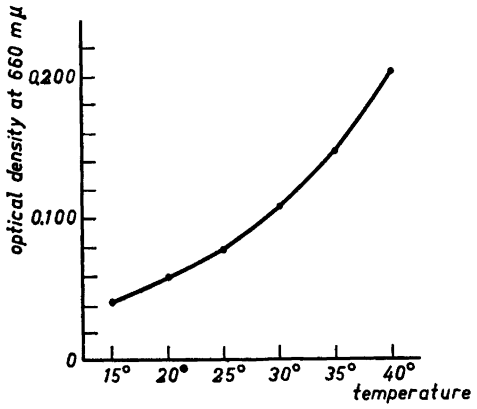

Fig. 4. Effect of temperature on ATP-ase activity (expressed as optical density at 660 mμ). The reaction mixture contained: 10 μmoles of ATP, 5 μmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 Tris buffer pH 7.5, containing 0.5 mg chlorophyll. Reaction time, 20 min.

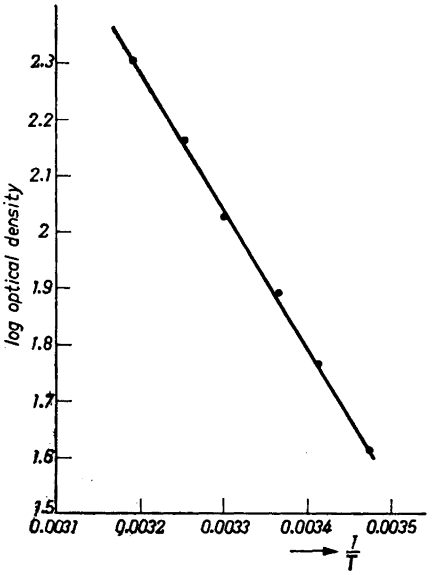

Fig. 5. Arrhenius plot of data of Fig. 4.

Other chloroplast preparations gave results intermediate between those represented in Table 1. The results indicate that chloroplasts contain two enzymes (or enzyme systems) having ATP-ase activity. One enzyme (system) is present in chloroplasts in a rather constant amount, has a broad pH optimum, is strongly stimulated by $MgCl_2$ and inhibited by chlorpromazine. The content of the other enzyme (system) varies in different chloroplast preparations. This enzyme (system) has a pH optimum of 5.5, is not stimulated by $MgCl_2$ and is resistant to chlorpromazine.

As shown in Table 2, ATP-ase activity at pH 7.5 is inhibited by AMP, fluoride, *p*-chloromercuribenzoate, azide, atebrin, arsenate and phosphate. The inhibition by ADP is variable. $(NH_4)_2SO_4$, DNP and KCN had no effect, whereas addition of FMN caused a slight stimulation. A marked stimulation of the ATP-ase activity was observed upon addition of dithionite to the reaction mixture.

Chloroplasts were found to release inorganic phosphate from ADP, but not from AMP. The liberation of inorganic phosphate from ADP at pH 7.5, like the splitting of ATP, was stimulated by $MgCl_2$ and inhibited by chlorpromazine. The release of inorganic phosphate from ADP, the rate of which varied between 20 and 80 % of that of the splitting of ATP, is probably due to the myokinase activity of the chloroplasts. Mazelis [23] has shown that spinach chloroplasts have a high myokinase activity.

It should be noted that with whole chloroplasts similar results were obtained as with broken ones, and that no stimulating effect of DNP could be observed.

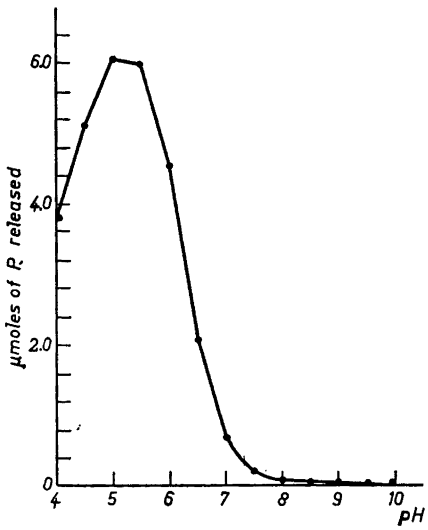

Fig. 6. Effect of pH on pyrophosphatase activity in the absence of $MgCl_2$. Reaction mixture: 10 µmoles of sodium pyrophosphate and 1 ml of a suspension of chloroplasts in 0.1 M buffer, containing 0.5 mg chlorophyll. Reaction time, 15 min at 30°.

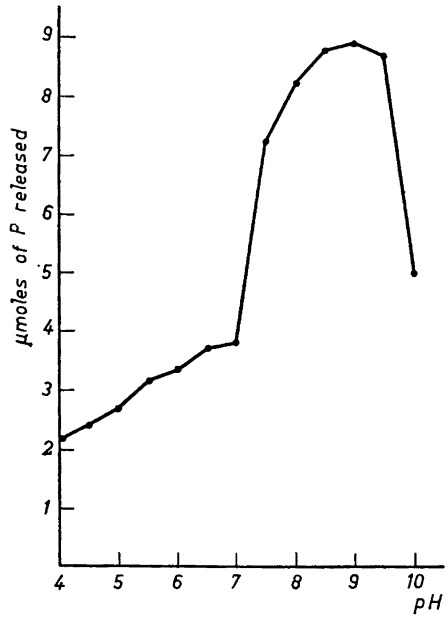

Fig. 7. Effect of pH on pyrophosphatase activity in the presence of $MgCl_2$. Reaction mixture: 10 µmoles of sodium pyrophosphate, 50 µmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 M buffer, containing 0.5 mg chlorophyll. Reaction time, 10 min at 30°.

An increase in temperature resulted in an acceleration of ATP-ase activity (Fig. 4). Fig. 5 shows that a straight line is obtained on plotting the logarithm of the reaction velocity against the reciprocal of the absolute temperature. The value of the activation energy was calculated from the slope and was found to be about 11 500 cal over the temperature range studied.

In order to test whether the cleavage of ATP could be due to apyrase activity, we have investigated the pyrophosphatase activity of spinach chloroplasts at different pH's. Chloroplasts were found to contain two pyrophosphatases which can be distinguished by pH optima and response to $MgCl_2$. One enzyme has a pH optimum of 5.0—5.5 and does not require Mg^{++}. The other enzyme has a pH optimum of 8.5—9.0 and a requirement for high concentrations of added $MgCl_2$ (Figs. 6 and 7; Table 3). These results resemble those of Naganna *et al.*[24] who have reported that green leaves contain both an acid pyrophosphatase and an alkaline pyrophosphatase, the latter being active only in the presence of $MgCl_2$. The pyrophosphatases were found to be insensitive to 5×10^{-4} M chlorpromazine.

The pyrophosphatase activity at pH 5.5 was sufficient to account for the ATP-ase activity of chloroplasts at this pH. This possibility is supported by

Table 3. Pyrophosphatase activity of spinach chloroplasts.

Additions (μmoles), unless otherwise indicated	pH	μmoles of P released
10 ATP	5.5	6.1
10 ATP + 5 $MgCl_2$	5.5	6.0
10 ATP + 30 $MgCl_2$	5.5	4.5
10 PP	5.5	12
10 PP + 5 $MgCl_2$	5.5	11
10 PP + 30 $MgCl_2$	5.5	7.8
10 PP + 5×10^{-4} M chlorpromazine	5.5	12
10 ATP	7.5	0.29
10 ATP + 5 $MgCl_2$	7.5	1.5
10 ATP + 30 $MgCl_2$	7.5	1.2
10 PP	7.5	0.18
10 PP + 5 $MgCl_2$	7.5	0.24
10 PP + 10 $MgCl_2$	7.5	0.81
10 PP + 20 $MgCl_2$	7.5	5.7
10 PP + 30 $MgCl_2$	7.5	8.5
10 PP + 40 $MgCl_2$	7.5	8.9
10 PP + 50 $MgCl_2$	7.5	9.1
10 PP + 30 $MgCl_2$ + 5×10^{-4} M chlorpromazine	7.5	9.0
10 ATP *	5.5	2.9
10 ATP + 10 PP *	5.5	6.6
10 PP *	5.5	6.5

Chlorophyll concentration, 0.5 mg per sample.

our finding that there exists a correlation between pyrophosphatase activity and ATP-ase activity at pH 5.5 in different chloroplast preparations. As a rule the amount of inorganic phosphate released from pyrophosphate was found to be about twice that split from ATP at pH 5.5. As shown in Table 3, addition of ATP did not increase the release of inogranic phosphate from pyrophosphate at pH 5.5.

At pH 7.5, however, the pyrophosphatase activity of spinach chloroplasts in the presence of 5 μmoles of $MgCl_2$ is much lower than the ATP-ase activity. The amount of inorganic phosphate formed by cleavage of pyrophosphate was found to be 10—20 % of the amount split from ATP by the same chloroplast preparation. This indicates that the splitting of ATP at pH 7.5 does not proceed by way of an apyrase, but is caused by a true ATP-ase activity.

KINETICS OF THE ATP-ase ACTIVITY AT pH 7.5

Fig. 8 represents the increase of inorganic phosphate as a function of time in a medium containing 10 μmoles of ATP, 5 μmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer, pH 7.5, (0.5 mg chlorophyll). It

* 0.2 mg of chlorophyll per sample. Reaction time, 15 min at 30°.

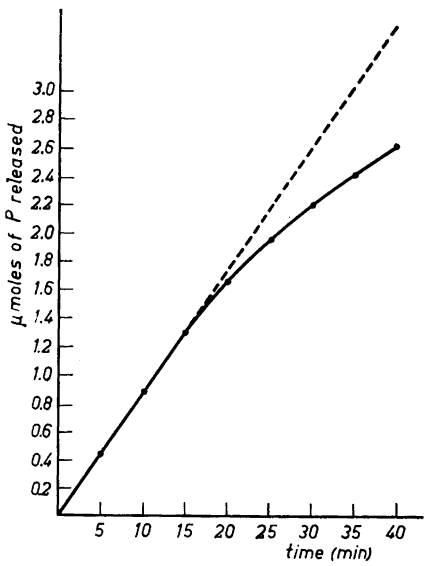

Fig. 8. ATP-ase activity as a function of time. The reaction mixture contained: 10 μmoles of ATP, 5 μmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer pH 7.5, containing 0.5 mg chlorophyll. Temperature 30°.

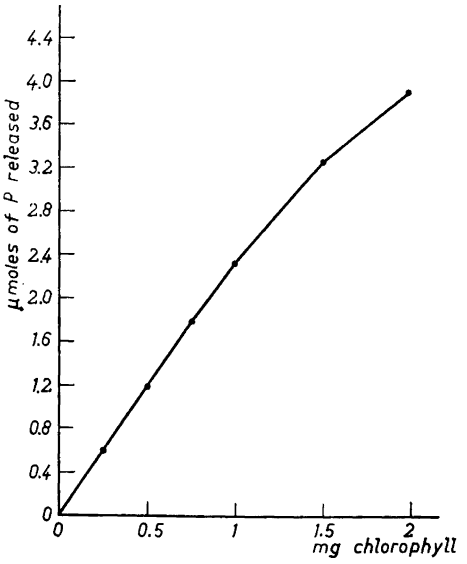

Fig. 9. Effect of varying the amount of chloroplast preparation on the ATP-ase activity. The reaction mixture contained: 10 μmoles of ATP, 5 μmoles of $MgCl_2$ and 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer pH 7.5. Reaction time, 15 min at 30°.

is seen that the reaction rate is constant during the first 15 min of the reaction. In some preparations constancy has been obtained for more than 30 min. As shown in Fig. 9, the rate of ATP cleavage is proportional to the chlorophyll concentration up to 1 mg chlorophyll per sample.

In Fig. 10 the effect of ATP concentration on the velocity of ATP-ase activity is presented, showing the general type of curve obtained with enzyme reactions. For each concentration of ATP the optimal concentration of $MgCl_2$ was added, *viz.* μmoles ATP: μmoles $MgCl_2$ = 2:1. In Fig. 11 the reciprocal of the ATP concentration was plotted against the reciprocal of the rate of production of inorganic phosphate (expressed as optical density at 660 mμ) according to the method of Lineweaver and Burk [25]. The Michaelis constant was calculated from the intercept on the extended 1/S axis and was found to be 1.2×10^{-3} M.

Fig. 12 illustrates the effect of temperature on the maximum velocity (V_{max}) of ATP cleavage, which was calculated from the intercept on the $1/v$ axis of Lineweaver-Burk plots at different temperatures. When log V_{max} is plotted against the reciprocal of the absolute temperature ($1/T$) a straight line is obtained (Fig. 13). The value of the activation energy was calculated from the slope and was found to be 12 800 cal over the temperature range studied. We were unable to study the inhibition of ATP-ase activity by ADP

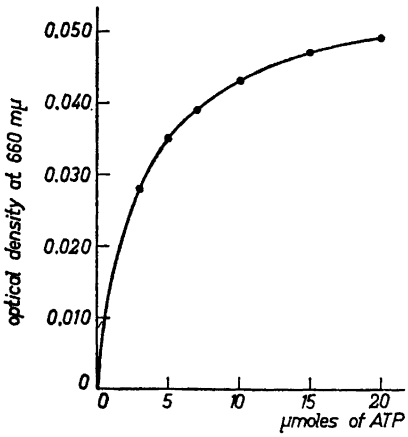

Fig. 10. Effect of ATP concentration on ATP-ase activity (expressed as optical density at 660 mμ). The reaction mixture contained, in addition to ATP, $MgCl_2$ (μmoles $MgCl_2$: μmoles ATP = 1:2) and 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer pH 7.5, containing 0.5 mg chlorophyll. Reaction time, 10 min at 30°.

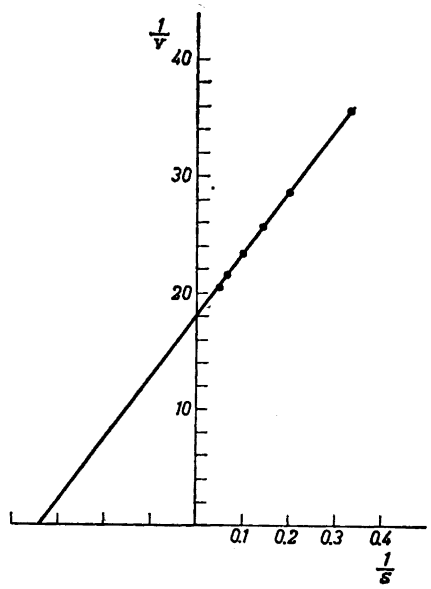

Fig. 11. Lineweaver-Burk plot of the data of Fig. 10. Reciprocal of ATP concentration (1/S) *vs.* reciprocal of optical density at 660 mμ (1/*v*).

kinetically, because of the myokinase activity of the chloroplasts. A kinetic investigation of the inhibition by AMP, on the other hand, was quite possible. Fig. 14 represents the rate of ATP cleavage as a function of ATP concentration in the presence and in the absence of 10 μmoles of AMP. When these data are plotted as 1/*v* against 1/S, as illustrated in Fig. 15, the slope but not the intercept with the 1/*v* axis is found to increase upon addition of AMP. This indicates that AMP inhibits the ATP-ase activity competitively.

DISCUSSION

Lehninger *et al.*[26] have postulated the following reaction scheme for oxidative phosphorylation in mitochondria:

$$\text{Carrier}_{red} + X \longrightarrow \text{Carrier}_{ox} \sim X + 2\ e \quad (1)$$

$$\text{Carrier}_{ox} \sim X + P \rightleftharpoons \text{Carrier}_{ox} + P \sim X \quad (2)$$

$$P \sim X + ADP \rightleftharpoons ATP + X \quad (3)$$

X is an enzyme capable of undergoing reversible phosphorylation. This type of scheme is rather widely accepted, at least as a framework for a general reaction mechanism (recently, however, the necessity of an X-compound between the electron carrier and P has been questioned by Löv *et al.*[27]). The DNP-induced ATP-ase reaction is assumed to involve the splitting of ATP

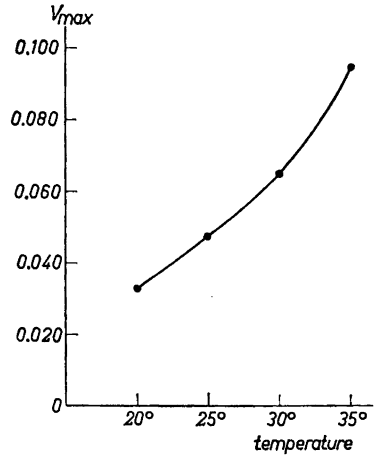

Fig. 12. Effect of temperature on the maximum velocity of ATP cleavage (expressed as optical density at 660 mμ). V_{max} was calculated from Lineweaver-Burk plots at 20, 25, 30 and 35°. The reaction mixture contained, in addition to ATP and $MgCl_2$ in a ratio of 2:1, 1 ml of a suspension of chloroplasts in 0.1 M Tris buffer pH 7.5, containing 0.7 mg chlorophyll. Reaction time, 10 min.

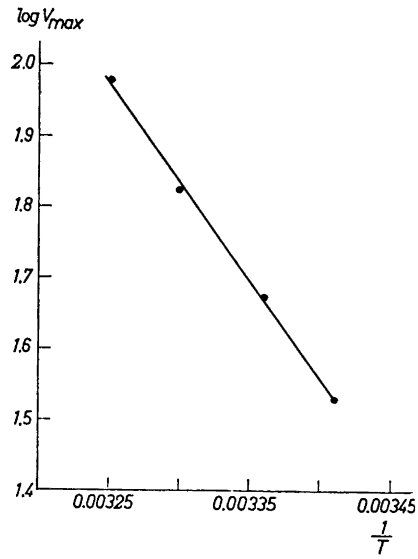

Fig. 13. Arrhenius plot of data of Fig. 12.

by reversal of reactions (3) and (2), followed by a rapid hydrolytic decomposition of $carrier_{ox} \sim X$. Siekevitz *et al.*[7] obtained data supporting the conclusion that the mitochondrial Mg^{++}-activated ATP-ase reaction constitutes a part of the reaction sequence involved in the DNP-induced ATP-ase and is not due to a separate enzyme. The finding by Kielley and Bronk [28] that DNP stimulates ATP-ase activity in phosphorylating fragments of rat liver mitochondria only in the presence of Mg^{++} is in agreement with this view. The Mg^{++}-activated ATP-ase reaction may be a reversal of reaction (3), followed by decomposition of $P \sim X$.

Our results indicate that chloroplasts exhibit two systems with ATP-hydrolyzing activity, one of which is dependent on added Mg^{++}. Contrary to the results obtained with "intact" liver mitochondria, the phosphorylating whole spinach chloroplasts isolated by us consistently show Mg^{++}-stimulated ATP-ase activity. In this respect they resemble phosphorylating heart muscle mitochondria, which are isolated according to Holton *et al.*[29] and are reported to always have a moderately high ATP-ase activity. It is an open question whether the Mg^{++}-stimulated ATP-ase activity should be regarded as an expression of damage in the isolated chloroplasts or as an inherent property of the system. In liver mitochondria the development of Mg^{++}-stimulated ATP-ase activity is paralleled by a loss of the capability to carry out oxidative phosphorylation. In chloroplasts, on the other hand, the Mg^{++}-

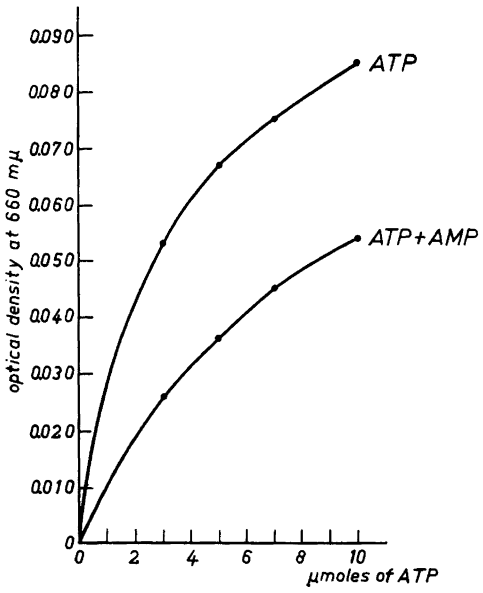

Fig. 14. Inhibition of ATP-ase activity by AMP. Reaction rate (expressed as optical density at 660 mμ) as a function of ATP concentration in the absence and in the presence of 10 μmoles of AMP. The reaction mixture contained, in addition to ATP and $MgCl_2$ in a ratio of 2:1, 1 ml of a suspension of cloroplasts in 0.1 M Tris buffer pH 7.5, containing 0.8 mg chlorophyll. Reaction time, 10 min at 30°.

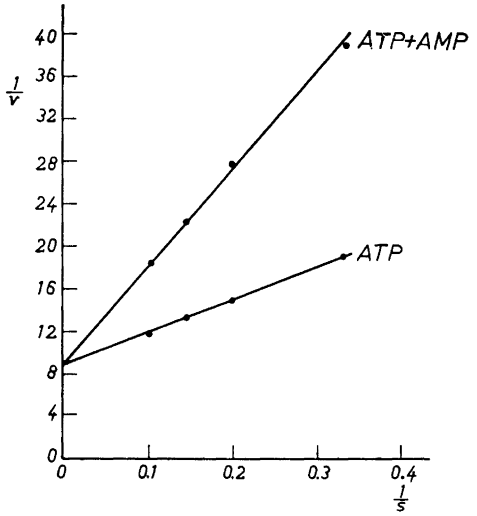

Fig. 15. Lineweaver-Burk plot of the data of Fig. 14, illustrating competitive inhibition of ATP-ase activity by AMP. Reciprocal of ATP concentration (1/S) *vs.* reciprocal of optical density at 660 mμ (1/*v*).

stimulated ATP-ase activity does not increase as light-induced phosphorylation falls off.

Light-induced phosphorylation is not much inhibited by low concentrations of DNP, in contrast to oxidative phosphorylation [30-33]. In fact it was found that DNP can catalyze the generation of ATP by illuminated chloroplasts in the absence of exogenous eletron carriers [34]. The mechanisms of light-induced and respiratory generation of ATP thus seem different in at least one respect. The finding that DNP does not induce ATP-ase activity in spinach chloroplasts may be another reflection of the same difference.

The reported lack of ATP-ase activity and of an exchange reaction between ATP and ^{32}P are according to Avron and Jagendorf [18] confirmatory evidence for the view that the last step in ATP formation in light-induced phosphorylation is an irreversible one. Löw *et al.*[27] have shown that a close relationship seems to exist between the P—ATP exchange and the DNP-induced ATP-ase reactions in isolated mitochondria. Our finding that no DNP-induced ATP-ase reaction could be obtained in chloroplasts may be interpreted to mean, by

comparison, that a P—ATP exchange reaction should not be expected in isolated chloroplasts.

Though the amount of inorganic phosphate released in the ATP-ase reaction of the isolated chloroplasts is rather low (8—12 μmoles/h/mg chlorophyll) as compared to the rate of light-induced phosphorylation in the particular chloroplast preparations (60—80 μmoles ATP formed/h/mg chlorophyll, anaerobic conditions, cofactor 2×10^{-4} M vitamin K_3), there are two reasons to believe that the Mg^{++}-stimulated ATP-ase activity is not due to mitochondrial contamination. First, the chloroplast preparations showed no measurable respiratory activity and second, repeated washings of the chloroplasts did not significantly affect the ATP-ase activity. Arnon's [16] statement that chloroplasts seem to be rather free of ATP-hydrolyzing enzymes has not been supported by any published experiments. The failure of Avron and Jagendorf [17,18] to detect any ATP-ase activity may have been due to the combined effect of short reaction time, low concentration of chloroplasts (*cf.* Ref.[18] Table I), high concentration of $MgCl_2$ and low temperature, as compared to our conditions.

The Mg^{++}-activated ATP-ase activities in spinach chloroplasts and in liver mitochondria were found to be similar in their response to azide, *p*-chloromercuribenzoate, fluoride, AMP, ADP, chlorpromazine and atebrin, which were inhibitory and also to dithionite, which had a stimulating effect (*cf.* Refs.[7,10,35,36]). In spinach chloroplasts KCN had no significant effect, whereas in mitochondria a small but significant stimulation of the Mg^{++}-activated ATP-ase reaction has been reported [35]. $(NH_4)_2SO_4$, which recently was found to be an uncoupler of light-induced phosphorylation in chloroplasts [37], did not influence the ATP-ase activity.

Löw [35,36] has shown that the flavin antagonists atebrin [38-40] and chlorpromazine [41] affect the DNP-induced ATP-ase activity of "intact" liver mitochondria as well as the Mg^{++}-stimulated ATP-ase activity of structurally disorganized mitochondria. He concluded that both of these ATP-ases involve the participation of diaphorase flavin. In chloroplasts, those concentrations of chlorpromazine (3×10^{-4} M) and atebrin (5×10^{-3} M), which inhibited the Mg^{++}-stimulated ATP-ase activity by 50 % also caused a precipitation of chloroplast material. In chloroplasts light-induced phosphorylation in the presence of vitamin K_3 or FMN, as well as the phosphorylation coupled to the reduction of ferricyanide were found to be inhibited by much lower concentrations of chlorpromazine and atebrin (50 % inhibition by 5×10^{-5} M chlorpromazine and 5×10^{-5} M atebrin) than the ATP-ase activity. Thus we are not certain that these agents inhibit chloroplast ATP-ase by a specific action towards flavin.

Summarizing it may be said that the Mg^{++}-stimulated ATP-ase reaction of chloroplasts in several respects is similar to that of disrupted liver mitochondria. Our data do not permit, however, to draw a definite conclusion as to whether the Mg^{++}-stimulated ATP-ase reaction is a reversal of ATP forming reactions in light-induced phosphorylation in chloroplasts.

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Received December 15, 1959.