BBA 45 560

PURIFICATION AND PROPERTIES OF A Mg^{2+}-DEPENDENT ATPase FROM CHLOROPLASTS OF *EUGLENA GRACILIS**

EDGAR F. CARELL** AND JOSEPH S. KAHN

Department of Biochemistry, North Carolina State University, Raleigh, N.C. (U.S.A.)

(Received November 11th, 1966)

SUMMARY

Isolated chloroplasts of *Euglena gracilis* contain highly active ATPases. The ATPase activity in the chloroplasts is usually much higher than the maximal rate of photophosphorylation obtainable and is due to a number of different enzymes.

One of these ATPases, Mg^{2+}-dependent and with a low pH optimum, has been solubilized with the aid of detergents and purified approx. 20-fold. The optimal activity of the enzyme is at pH 5.5, and the optimal Mg^{2+} concentration is $5 \cdot 10^{-3}$ M. The enzyme hydrolyzes dATP more rapidly than ATP, although the K_m for both substrates is the same. Other nucleotide triphosphates are hydrolyzed very slowly while ADP or pyrophosphate are not hydrolyzed at all. Oligomycin, carbonylcyanide *m*-chlorophenylhydrazone, and ADP inhibit the enzyme, the latter competitively.

The level of the enzyme is highest in chloroplasts from rapidly dividing cells, and reaches a minimum in chloroplasts from maturing cultures which have attained their maximal photosynthetic activity. It is postulated that the enzyme has a role in controlling chloroplast development.

INTRODUCTION

Membrane-bound ATPases, both active and latent, have been repeatedly implicated in the mechanism of ATP formation that is coupled to electron transport. In both mitochondria and chloroplasts, the terminal enzymatic reaction leading to ATP formation—called the coupling factor—can act as an active or latent ATPase both *in vivo* and *in vitro*[1–3].

Chloroplasts of *Euglena gracilis* contain a number of active ATPases. One of these, requiring Ca^{2+} for optimal activity and apparently representing the coupling factor, has recently been described in this laboratory[4]. In addition to this coupling factor, Euglena chloroplasts also contain at least two active Mg^{2+}-dependent ATPases—distinguishable by their different pH optima—and have as a rule a much higher rate of ATP hydrolysis than the rate of photophosphorylation observed in these chloro-

* Contribution from the Biochemistry Department, North Carolina Agricultural Experiment Station, Raleigh, N.C., U.S.A. Published with the approval of the Director of Research as Paper No. 2775 of the Journal Series.

** Current address: Department of Biology, University of Pittsburgh, Pittsburgh, Pa., U.S.A.

plasts[5]. The isolation and characteristics of one of these Mg^{2+}-dependent ATPases are described in this report.

MATERIALS AND METHODS

Euglena gracilis, strain z, was grown heterotrophically, and the chloroplasts isolated as previously described[5,6]. Once it was ascertained (see below, Table I) that non-chloroplastic ATPases were essentially absent in the washed chloroplasts, the sucrose density-gradient step was omitted from the chloroplast purification procedure.

Assay of ATPase activity was carried out by incubating the enzyme at 36° for 20 min in a system containing 150 μmoles Tris–malate buffer (pH 5.5), 10 μmoles of ATP, 15 μmoles of $MgCl_2$ and 10–100 μg enzyme (depending on the activity of the preparation) in a final vol. of 3 ml. The reaction was stopped by the addition of 1 ml of of 10% trichloroacetic acid and analyzed for inorganic phosphate by the method of FISKE AND SUBBAROW[7]. Photophosphorylation was measured as previously described[5] using pyocyanin as cofactor.

Protein was determined by the method of LOWRY *et al.*[8] and chlorophyll by the method of ARNON[9].

RESULTS

Purification of the chloroplasts

In order to ascertain that the ATPase activity in the chloroplast preparation was not due to cytoplasmic or mitochondrial contamination, the chloroplasts were repeatedly washed by resuspension in a medium containing 0.5 M mannitol, 0.05 M Tris buffer (pH 8.0) and 0.01 M NaCl followed by centrifugation at 3000 × *g* for 10 min, and finally centrifuged to isopycnic equilibrium in a glycerol density gradient ranging in specific gravity from 1.10 to 1.20. The results, given in Table I, show that after the first washing the ratio of ATPase activity of the chloroplasts, calculated on a protein and chlorophyll basis, approached a constant value and did not change significantly after sucrose density-gradient centrifugation.

TABLE I

EFFECT OF REPEATED WASHING AND OF DENSITY-GRADIENT CENTRIFUGATION ON THE Mg^{2+}-DEPENDENT ATPASE ACTIVITY OF EUGLENA CHLOROPLASTS

For details, see text.

Treatment	*Specific activity*		*Ratio B/A*
	A (*μmoles/mg protein per min*)	*B* (*μmoles/mg chlorophyll per min*)	
Crude chloroplasts	0.324	4.72	14.6
1 × washed	0.408	5.08	12.4
2 × washed	0.364	4.40	12.1
3 × washed	0.348	4.24	12.2
Purified on gradient	0.292	3.24	11.1

Isolation and purification of the enzyme

Twice washed chloroplasts from 100 g of cells were suspended in 50 ml medium containing 0.5 M mannitol, 0.05 M Tris buffer (pH 8.0) and 0.01 M NaCl, sonicated for 30 sec, and centrifuged for 30 min at 100000 × *g*. The supernatant was discarded and the green precipitate layer removed carefully, without resuspending the white precipitate at the bottom (which consisted mainly of paramylum granules and was inactive). The green precipitate was resuspended in 40 ml of 0.05 M Tris buffer (pH 8.0) and reprecipitated by centrifugation. The pellet was then resuspended in 10 ml of 0.05 M Tris buffer (pH 8.0) and Triton X-100 (The Rohm and Haas Co.) added to a final concn. of 1 %. After stirring for 20 min, it was centrifuged for 1 h at 198000 × *g* and the pellet re-extracted with Triton X-100 as described above. The pooled supernatant was fractionated with $(NH_4)_2SO_4$, the fraction precipitating between 25 % and 45 % satn. containing the bulk of the activity.

The $(NH_4)_2SO_4$ precipitate was resuspended in 0.005 M Tris buffer (pH 8.0) containing 10^{-4} M ATP and 10^{-3} M EDTA and was then dialyzed overnight against the same buffer system. It was then layered in 1.5-ml aliquots on a sucrose density gradient ranging in specific gravity from 1.050 to 1.200 made up in 0.05 M Tris buffer (pH 8.0) and centrifuged for 18 h at 90000 × *g* in a Spinco SW 25.1 rotor. After centrifugation the contents of the tubes were fractionated into 33 fractions on a drop-counting fraction collector. Every third tube was assayed for ATPase activity except for the peak region, of which every tube was assayed.

The results of a typical purification procedure are given in Table II and Fig. 1. The increase in total enzymatic activity upon the addition of Triton X-100 was a consistent feature and has been observed before in the case of another chloroplast enzyme[10]. The enzyme purified on a sucrose density gradient was stable for at least a few months when stored frozen or stored in the presence of 10^{-4} M ATP and 10^{-3} M EDTA at 0°.

TABLE II

PURIFICATION OF THE Mg^{2+}-DEPENDENT ATPase FROM EUGLENA CHLOROPLASTS

Chloroplasts were isolated as described in ref. 5 from 212 g of cells. For details, see text.

	Volume (ml)	*Protein (mg/ml)*	*Specific activity (μmoles/mg protein per min)*	*Total activity (μmoles/min)*
Washed chloroplasts	97	15.0	0.204	296.4
Sonicated particles	25	15.8	0.736	290.4
1 % Triton extract	42	10.2	0.792	338.8
25–45 % $(NH_4)_2SO_4$ fraction	20	10.8	1.148	248.4
Peak off sucrose gradient	31	1.74	3.288	176.8

Attempts at further purification of the enzyme by adsorption on DEAE-cellulose, ECTEOLA-cellulose or calcium phosphate gel, or by fractionation with acetone were unsuccessful in that they caused a complete loss of activity.

We could not achieve solubilization of the enzyme with methods not involving detergents. Acetone extraction, prolonged sonication, extraction with 0.6 M KCl or $5 \cdot 10^{-3}$ M EDTA did not solubilize any of this Mg^{2+}-dependent ATPase. Sodium

dodecyl sulfate and deoxycholate solubilized the enzyme, but further purification in their presence could not be achieved without appreciable loss of enzyme activity.

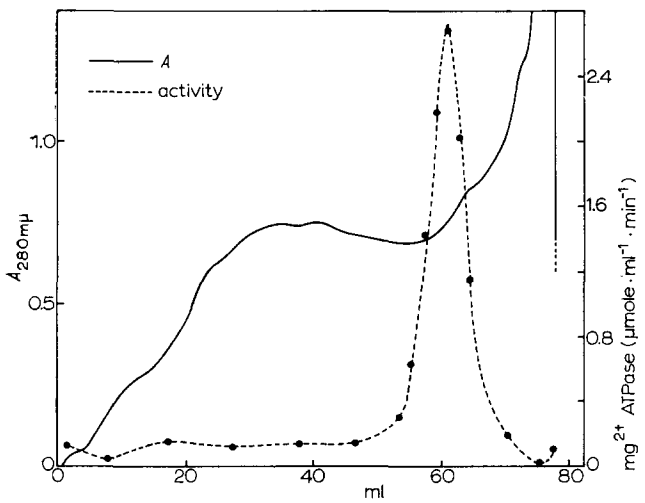

Fig. 1. Purification of the Mg^{2+}-dependent ATPase from Euglena chloroplasts by sucrose density-gradient centrifugation. The absorbance at 280 mμ was monitored continuously, while the activity was determined in every third fraction, except for the peak region where every fraction was assayed. $A_{280\,m\mu}$ represents absorption by pigments and detergent besides protein. For details, see text.

Properties of the Mg^{2+}-dependent ATPase

In initial experiments it was determined that the rate of ATP hydrolysis was proportional to enzyme concentration in the range we used for assay (10–100 μg). The enzyme was also found to be stable for at least 1 h of incubation under conditions of the assay.

The enzyme activity was found to have a sharp optimum at pH 5.5 (Fig. 2). The

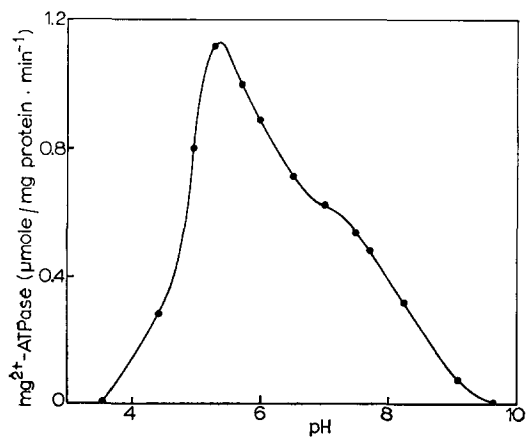

Fig. 2. Activity of the Mg^{2+}-dependent ATPase from Euglena chloroplasts as a function of the pH of the reaction mixture. Tris–HCl and Tris–malate buffers were used, with overlap, to cover the pH range. For details, see text.

small shoulder at pH 7.5 could be due to contamination by the Ca^{2+}-dependent ATPase of Euglena chloroplasts, which has some activity with magnesium and a pH optimum of 8 (ref. 4). Since the Ca^{2+}-dependent ATPase has no activity at pH 5.5, its presence did not interfere with the routine assay of the Mg^{2+} enzyme.

The enzyme has a specific requirement for a divalent cation, with $5 \cdot 10^{-3}$ M Mg^{2+} giving by far the highest activity. Ca^{2+} was inactive and did not, moreover, interfere with the Mg^{2+}-catalyzed activity (Table III). The activity observed with Ca^{2+}, Mn^{2+} and Co^{2+} was the same when their concentration was reduced to $1 \cdot 10^{-3}$ M as it was at $5 \cdot 10^{-3}$ M.

TABLE III

COFACTOR SPECIFICITY OF THE Mg^{2+}-DEPENDENT ATPASE FROM EUGLENA CHLOROPLASTS

For assay conditions, see text.

Cofactor ($5 \cdot 10^{-3}$ *M*)	*Specific activity* (*μmoles/mg protein per min*)	*% of* Mg^{2+} *activity*
None	0.008	< 1
Mg^{2+}	1.220	100
Ca^{2+}	0.068	6
Mn^{2+}	0.288	24
Co^{2+}	0.256	21
Mg^{2+} + Ca^{2+}	1.248	102

Data showing the substrate specificity of the enzyme are presented in Table IV. No activity with ADP or pyrophosphate could be detected in the purified enzyme, although the crude chloroplasts had a highly active pyrophosphatase.

One unusual specificity characteristic is the efficiency of hydrolysis of dATP.

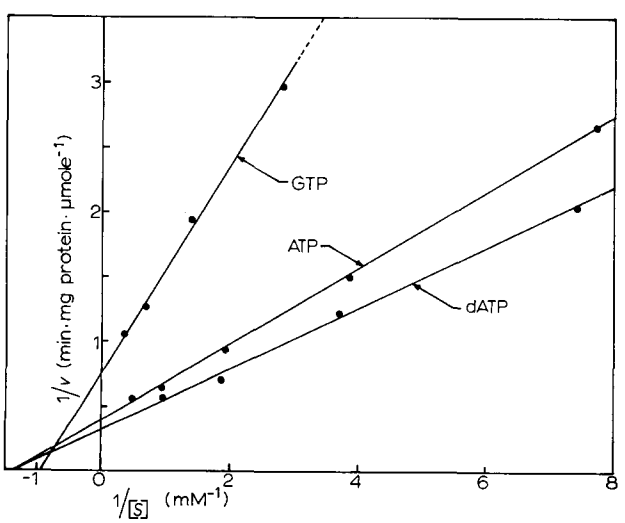

Fig. 3. A reciprocal plot of the rate of nucleotide triphosphate hydrolysis as a function of their concentration. Slopes and intercepts were calculated by least square. For details, see text.

Although the K_m for dATP and ATP hydrolysis are the same, the rate of hydrolysis of the former is consistently higher, and the ratio of the two activities is constant from one enzyme preparation to the other. The kinetics of dATP and ATP hydrolysis, compared to those of GTP and ITP, which are poor substrates, are given in Fig. 3 and Table V.

TABLE IV

SUBSTRATE SPECIFICITY OF THE Mg^{2+}-DEPENDENT ATPASE FROM EUGLENA CHLOROPLASTS

For assay conditions, see text.

Substrate ($3.3 \cdot 10^{-3}$ *M*)	*Specific activity* (*μmoles/mg protein per min*)	% *of ATP*
ATP	1.064	100
dATP	1.256	118
GTP	0.488	46
ITP	0.224	21
UTP	0.132	12
CTP	0.044	4
ADP	0.012	1
Pyrophosphate	0.000	0

TABLE V

KINETICS OF NUCLEOTIDE TRIPHOSPHATE HYDROLYSIS BY THE Mg^{2+}-DEPENDENT ATPASE FROM EUGLENA CHLOROPLASTS

For assay conditions, see text.

Substrate	K_m (*M*)	v_{max} (*μmoles/mg protein per min*)
ATP	$7.6 \cdot 10^{-4}$	2.584
dATP	$7.1 \cdot 10^{-4}$	3.036
GTP	$10.5 \cdot 10^{-4}$	1.308
ITP	$13.5 \cdot 10^{-4}$	1.040

TABLE VI

EFFECT OF UNCOUPLERS OF PHOSPHORYLATION ON THE Mg^{2+}-DEPENDENT ATPASE FROM EUGLENA CHLOROPLASTS

CCCP stands for carbonylcyanide-*m*-chlorophenylhydrazone.

Addition	*Concn.*(*M*)	*Specific activity* (*μmoles/mg protein per min*)	% *inhibition*
None	—	1.016	—
NH_4Cl	$5 \cdot 10^{-3}$	0.960	6
2,4-Dinitrophenol	$1 \cdot 10^{-4}$	0.920	9
Atebrin	$5 \cdot 10^{-5}$	1.072	0
Oligomycin	$5 \cdot 10^{-6}$	0.692	32
	$1 \cdot 10^{-5}$	0.560	45
CCCP	$1 \cdot 10^{-5}$	0.624	38

Oligomycin and carbonylcyanide-*m*-chlorophenylhydrazone partially inhibited the enzyme, while ammonia and atebrin, which inhibit photophosphorylation by Euglena chloroplasts[5] had little effect on the ATPase activity (Table VI). The enzyme was completely inactivated by *p*-chloromercuribenzoate and iodoacetamide, but not by arsenite. ADP inhibited the enzyme competitively, the K_i for ADP being identical to the K_m for ATP ($7.6 \cdot 10^{-4}$ M). A precipitating antibody against a coupling factor for photophosphorylation (CF_1) isolated from spinach chloroplasts did not inhibit the

TABLE VII

EFFECT OF INHIBITORS ON THE Mg^{2+}-DEPENDENT ATPASE FROM EUGLENA CHLOROPLASTS

For assay conditions, see text. Anti CF_1 serum: precipitating antibody prepared against coupling factor from spinach chloroplasts. In the assays with antibody and inhibitors, enzyme and inhibitor were preincubated for the length of time in min, given in brackets; controls were incubated with buffer for the same length of time. PCMB stand for *p*-chloromercuribenzoate.

Addition	*Concn.(M)*	*Specific activity (μmoles/mg protein per min)*	*% inhibition*
None	—	1.056	—
Iodoacetamide	$5 \cdot 10^{-3}$ (20)	0.772	27
	(120)	0.060	94
PCMB	$1 \cdot 10^{-4}$ (20)	0.044	96
Arsenite	$5 \cdot 10^{-3}$ (20)	1.064	0
	(120)	0.876	17
ADP	$1.5 \cdot 10^{-3}$	0.772	27
	$3 \cdot 10^{-3}$	0.552	48
	$6 \cdot 10^{-3}$	0.420	60
Anti CF_1 serum	8:1* (20)	1.012	4

* Ratio of serum to enzyme protein.

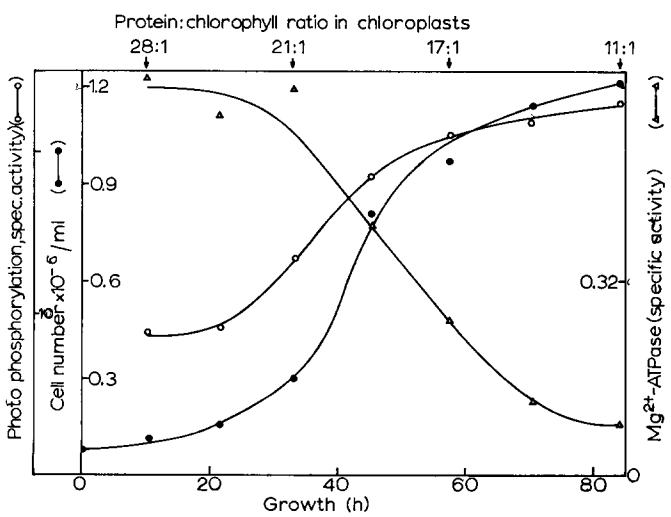

Fig. 4. Relation between the growth rate of a heterotrophic culture of Euglena and the rate of Mg^{2+}-dependent ATPase and photophosphorylation of the isolated chloroplasts. The specific activity of photophosphorylation is expressed as μmoles ATP formed/mg chlorophyll per h, and that of Mg^{2+}-dependent ATPase as μmoles ATP hydrolyzed/mg protein per min.

Mg^{2+}-dependent ATPase (Table VII); an antibody against the coupling factor for oxidative phosphorylation (F_1) from beef-heart mitochondria did not inhibit either.

Changes in the Mg^{2+}-dependent ATPase with cell development

The activity of the enzyme was highest in chloroplasts isolated from young, rapidly-dividing cultures, which had a very low rate of photosynthetic activity. As the cultures matured, the ATPase activity decreased with a concomitant increase in the rate of photophosphorylation (Fig. 4). The enzyme reached its lowest level when maximal photosynthetic activity was reached and before cell number reached its maximum. The changes in the rate of ferricyanide reduction by these chloroplasts corresponded closely to the changes in photophosphorylation.

Effect of the Mg^{2+}-dependent ATPase on photophosphorylation

The presence of residues of Triton X-100 in the purified enzyme preparation makes any evaluation of its effect on photophosphorylation difficult, since Triton is a very potent uncoupler[11]. To date no effect attributable to the ATPase could be found on the rate of phosphorylation by either coupled or uncoupled chloroplasts.

DISCUSSION

Isolated chloroplasts of Euglena differ in many respects from chloroplasts of higher plants. Their photosynthetic capacity is low[5] especially when grown on a heterotrophic medium[12]. Chloroplasts of spinach and swiss chard contain ATPase activity which is low compared to the rate of photophosphorylation obtained by these chloroplasts, and nearly all the Mg^{2+}-dependent activity disappears at pH lower than 6.5 (refs. 3, 13, 14). Spinach chloroplasts contain, however, high ATPase activity with a pH optimum of 5.5, but this activity is absolutely independent of added Mg^{2+} or any other divalent ion[14]. In Euglena chloroplasts, on the other hand, the rate of ATP hydrolysis is usually much higher than the rate of photophosphorylation which we could obtain[5], and the activity at pH 5.5 is moreover, absolutely dependent on the addition of Mg^{2+}.

The ATPase described in this report constitutes a part of the chloroplast structure and could be solubilized only with the aid of detergents. This differs markedly from the Ca^{2+}-dependent ATPase of spinach or Euglena which can be readily and reversibly detached from the chloroplasts and solubilized[2,4,15]. The two enzymes differ also markedly in their sensitivity to uncouplers, the Ca^{2+}-dependent enzyme being unaffected by either oligomycin or carbonylcyanide *m*-chlorophenylhydrazone. Except for dATP, the substrate specificity of both enzymes is similar.

In rapidly-dividing, heterotrophically-growing cells, the photosynthetic activity and chlorophyll formation are suppressed (Fig. 4), while the Mg^{2+}-dependent ATPase is at a maximum. As the culture approaches its maximal growth, the Mg^{2+}-dependent ATPase activity reaches a minimum with a concomitant rapid increase in photosynthetic activity. It is possible that the enzyme has a function in the control of the formation of the photosynthetic apparatus, inhibiting the replication of nucleic acids required for chloroplast development[12]. This would be in agreement with the rapid rate of dATP hydrolysis by this enzyme. Supporting this assumption is the observation

that the level of the enzyme is lowest in chloroplasts from autotrophic cells which have the highest rate of Hill activity.

From experiments to date, this enzyme does not appear to have a role in photophosphorylation, since all experiments on the reconstitution of photophosphorylation in uncoupled chloroplasts with the aid of this enzyme have been negative. No effect of the enzyme on photophosphorylation by coupled chloroplasts could be observed either.

ACKNOWLEDGEMENTS

This investigation was supported in part by U.S. Public Health Service Research Grant GM-09710 from the National Institutes of Health, Division of General Medical Services.

The able technical assistance of Mrs. T. SHOSTAG and Mr. R. THACKER is gratefully acknowledged. The antibody to the spinach chloroplast coupling factor (CF_1) was generously supplied by Dr. R. E. MCCARTY.

REFERENCES

1 H. S. PENEFSKY, M. E. PULLMAN, A. DATTA AND E. J. RACKER, *J. Biol. Chem.*, 235 (1960) 3330.
2 V. K. VAMBUTAS AND E. RACKER, *J. Biol. Chem.*, 240 (1965) 2660.
3 A. BENNUN AND M. AVRON, *Biochim. Biophys. Acta*, 109 (1965) 117.
4 I. C. CHANG AND J. S. KAHN, *Arch. Biochem. Biophys.*, 117 (1966) 282.
5 J. S. KAHN, *Biochem. Biophys. Res. Commun.*, 24 (1966) 329.
6 E. F. CARELL AND J. S. KAHN, *Arch. Biochem. Biophys.*, 108 (1964) 1.
7 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
10 J. S. KAHN, *Biochim. Biophys. Acta*, 79 (1964) 421.
11 J. NEUMANN AND A. T. JAGENDORF, *Biochim. Biophys. Acta*, 109 (1965) 382.
12 A. GNANAM AND J. S. KAHN, *Biochim. Biophys. Acta*, in the press.
13 M. AVRON, *J. Biol. Chem.*, 237 (1962) 2011.
14 J. S. C. WESSELS AND H. BALTSCHEFFSKY, *Acta Chem. Scand.*, 14 (1960) 233.
15 R. E. MCCARTY AND E. RACKER, Federation Proc. Abstr., 25, No. 208 (1966).