THE EFFECT OF CHLORAMPHENICOL AND ACTIDIONE ON CHLOROPHYLL SYNTHESIS[1]

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INTRODUCTION

It has been demonstrated that chlorophyll synthesis is related to protein synthesis and that the inhibition of protein synthesis in leaves results in the inhibition of chlorophyll synthesis[4]. Chloramphenicol and actidione have been reported to inhibit chlorophyll synthesis in previous works[8,11,15].

Chloramphenicol was shown as the specific inhibitor of protein synthesis in bacteria[3], while actidione was reported to inhibit the protein synthesis of algae, fungi, higher plants and animals, but not bacteria.[18]

Studies on the effects of chloramphenicol on a variety of synthetic processes induced by the aging of the red beet have shown that the primary site of action of these compounds is on some other process rather than on protein synthesis in the plant.[5,9,10,13] It has been widely accepted that cytoplasmic ribosomes of plant and animal cells are 80S, while ribosomes from bacteria are 70S. Recently, it has been demonstrated that chloroplasts contain 70S ribosome.[21] It has been suggested in a previous report that chloramphenicol inhibited the function of 70S ribosomes and actidione inhibited the function of 80S ribosomes.[21]

When etiolated seedling leaves are illuminated by light, protochlorophyllide *a* in leaves is converted rapidly by a nonenzymatic photochemical reaction to chlorophyllide *a*; the chlorophyllide *a* so formed is converted enzymatically to chlorophyll *a* without the need of light. The period between the initial conversion of protochlorophyllide to chlorophyllide and the maximal rate of chlorophyll accumulation has been termed the lag phase. The lag phase may last for a few minutes or several hours according to the species and age of plant.[20] The lag phase can be shortened or abolished by treating the plant with a short light period followed by a period of incubation in long darkness.[17] The lag phase in the formation of chlorophyll was suggestive of an enzymatic adaptation. Such an interpretation suggests that brief illumination followed by a period of prolonged darkness eliminated the lag phase by resulting in the synthesis of enzymes necessary for chlorophyll formation.[12]

In this experiment, first, we tried to ascertain whether chloramphenicol and

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actidione could inhibit protein synthesis in the embryo of soybean seeds during germination and to find the proper concentration of the antibiotics that would effect the chlorophyll synthesis in leaves. Secondly, we tried to learn whether chloramphenicol or actidione could inhibit the formation of the light-induced enzymes which are needed for chlorophyll synthesis.

MATERIALS AND METHODS

(1) The analysis of the protein content of the embryo during germination.

Soybean seeds were washed in tap water to wash off any fungal spores. After the seeds were washed, 30 seeds were placed in a 10 cm diameter petri dish, then 15 ml of an antibiotic solution was added to the petri dish. After incubation in the dark at about 30°C for 50 hours, eight germinating embryos were thoroughly ground in a mortar and pestle; 20 ml of distilled water was then added. The homogenate was poured into a large centrifuge tube, and another 10 ml of water was added to the mortar to wash out all the remaining homogenate and this also was poured into the centrifuge tube making a total volume of 30 ml. This was then centrifuged at 700 g for 10 minutes. 5 ml of 10% TCA was used to precipitate the proteins in 5 ml of the supernatent. This was mixed well and left to stand for at least 30 minutes in a refrigerator; then it was centrifuged at 950 g for ten minutes. The protein in the pellet was dissolved in 10 ml 0.1 N NaOH, then analyzed for its protein content by the Lowry method.[12]

(2) The analysis of chlorophyll content of leaves

Soybean plants were grown in the dark for 6 days at about 26°C, and leaves with a cotyledon and a piece of hypocotyl attached were treated with an antibiotic solution[15].

Illumination was performed with white fluorescent tubes at an intensity of 4200 Lux and at about 28°C. Chlorophyll was extracted with 80% acetone and the total chlorophyll content was determined spectrophotometrically by the Arnon method.[1] All manipulation of the plants prior to illumination was performed under a dim safe light.[22]

RESULTS

Chloramphenicol does not inhibit protein synthesis in embryos, whether the treatment is applied at the beginning of germination or after 24 hours of germination in distilled water (Table I). On the contrary actidione (2 μg/ml or 5 μg/ml) inhibits 40% of the protein synthesis after 24 hours of germination in distilled water (Table II). Greater inhibition of protein synthesis in embryos occurs if they are treated with actidione at the beginning of germination.

Chloramphenicol does not affect the germination of soybean seeds, and there is

Table I. The effect of chloramphenicol on protein synthesis of soybean seed embryo in germination for 50 hrs.

Exp.	Treatment	Protein content (μg per embryo)	Inhibition
1.	control (H_2O)	3,840	
	chloramphenicol (0.5 mg/ml)	4,080	−6%
	chloramphenicol (1 mg/ml)	4,080	−6%
2.	control (H_2O)	4,552	
	chloramphenicol (0.5 mg/ml)	4,780	−5%
	chloramphenicol (1 mg/ml)	4,552	

Table II. The effect of actidione on protein synthesis of soybean seed embryo in germination for 50 hrs. Actidione was added after 24 hrs of germination in distilled water.

Exp.	Treatment	Protein content (μg/per embryo)	Inhibition
1.	control (H_2O)	3,555	
	actidione (2 μg/ml)	1,867	47%
	actidione (5 μg/ml)	2,025	43%
2.	control (H_2O)	4,980	
	actidione (2 μg/ml)	2,940	40%
	actidione (5 μg/ml)	2,940	40%

no morphological difference in the germinating embryos between those treated with chloramphenicol and the controls. Actidione completely inhibited the germination of soybean seeds at a concentration of 2 μg/ml or above, provided that the actidione was applied at the beginning of germination; and embryos of soybean seeds germinating in actidione were smaller and more delicate than the controls, if the seeds were first germinated in distilled water for 24 hours and then placed in the actidione solution for 26 hours. So in the following experiment, 2 μg/ml concentration of actidione and 1 mg/ml concentration of chloramphenicol were used to make comparative studies of the inhibition of chlorophyll synthesis.

The leaves of excised etiolated seedlings were treated with antibiotic solutions, and then placed under light. The illuminated leaves were removed at the end 4, 6, and 8 hour intervals for analysis of their chlorophyll content. The results are shown in Table III and IV, a 1 mg/ml solution of chloramphenicol inhibited chlorophyll synthesis about 40%. However the synthesis of chlorophyll in leaves treated with actidione was only slightly inhibited. Plants placed under 4 hours of illumination, showed no inhibition of chlorophyll synthesis after treatment with actidione and plants illuminated for 6 or 8 hours only showed slight inhibition.

Table III. The effect of chloramphenicol (1 mg/ml) on chlorophyll synthesis of soybean leaves. Chloramphenicol was added before illumination.

Illumination 4 hr.	Chlorophyll content (μg/g fresh wt.)	Illumination 8 hr.	Chlorophyll content (μg/g fresh wt.)
control (H_2O)	115	control (H_2O)	316
chloramphenicol	65	chloramphenicol	186

Table IV. The effect of actidione (2 μg/ml) on chlorophyll synthesis of soybean leaves. Actidione was added before illumination.

Exp.	Illumination 4 hr.	Chlorophyll content (μg/g fresh wt.)	Illumination 6 hr.	Chlorophyll content (μg/g fresh wt.)	Illumination 8 hr.	Chlorophyll content (μg/g fresh wt.)
1.	control	125	control	276	control	468
	actidione	125	actidione	256	actidione	419
2.	control	136	control	260	control	483
	actidione	137	actidione	229	actidione	421
3.	control	145	control	291	control	443
	actidione	140	actidione	283	actidione	413
4.	control	126	control	292		
	actidione	118	actidione	283		
5.	control	127				
	actidione	137				

The lag phase for chlorophyll synthesis of etiolated leaves is abolished when a short period of illumination preceeds incubation (Fig. 1). In the above case, if the antibiotic was added at any of the following times, e.g. just before the 10 minutes of short illumination, or just after the 10 minutes of short illumination or 11 hours after the illumination, or at the end of 24 hours of darkness; then following this illuminated again for 6 hours, the following results were obtained and are given in Table V and VI, and these indicate that chloramphenicol will inhibit the formation of the enzymes concerned with the synthesis in the 24-hour dark period.*

The leaves of etiolated seedings were grown under illumination for a total of eight hours, some were only left in distilled water for one hour and then transferred to a solution of chloramphenicol for 7 hours, others were left in distilled water for 2 or 3 or 5 hours before being transferred to the antibiotic solution. The influence of chloramphenicol on chlorophyll synthesis is shown in Fig. 2. The leaves that were treated with chlorampenicol after 1 or 2 hours of incubation in the light showed a significant inhibition; the leaves treated with chloramphenicol after 3 or 5 hours in the water showed much less inhibition of chlorophyll synthesis. No change in chlorophyll content was found if the excised leaves were first treated

* The inhibition was greater when the chloramphenicol was added at the beginning of darkness.

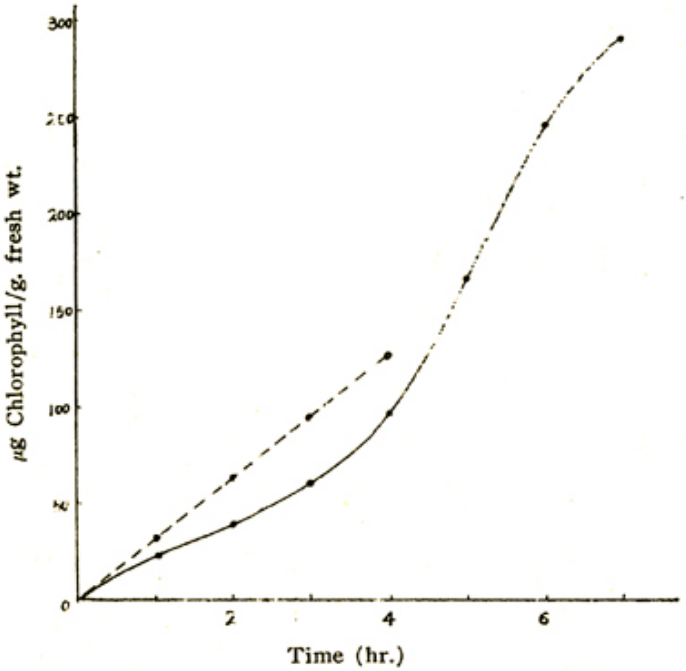

Fig. 1. The effect of chlorophyll synthesis of etiolated leaves by long dark incubation after short period of illumination.
a: No dark incubation is indicated as follows: _____
b: Incubation in 24 hours of darkness after 10 minutes of illumination is indicated as follows: .....

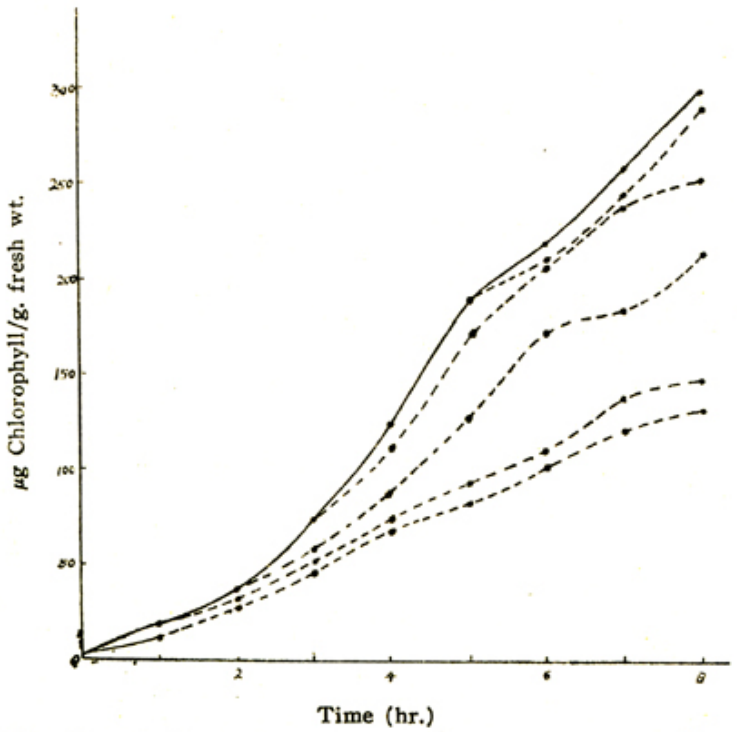

Fig. 2. The effect of chloramphenicol on chlorophyll synthesis during illumination. _____ control, ... chloramphenicol added.

with chloramphenicol and then removed to water after a period of 1, 3 or 5 hours of illumination (Table VIII).

Table V. The effect of time of addition of chloramphenicol (CAM) (1 mg/ml) on the rate of chlorophyll formation. Leaves given a 10 minutes light, incubated for 24 hrs. in the dark. Chlorophyll synthesis was analyzed after the subsequent 6 hrs. of illumination.

Treatment	Chlorophyll content (μg/g fresh wt.)
No addition of CAM illuminated for 6 hr. 10 min. (No dark incubation)	236
Addition of CAM illuminated for 6 hr. 10 min. (No dark incubation)	122
CAM added before 10 minute of illumination	128
CAM added at the end of 10 minute of illumination	128
CAM added 11 hr. after 10 min. of illumination	170
CAM added 24 hr. after 10 min. of illumination	202
No addition of CAM	316

Table VI. The effect of time of addition of actidione (2 μg/ml) on the rate of chlorophyll formation. Leaves were given 10 min. of light and incubated for 24 hr. in the dark. Chlorophyll synthesis was analyzed for the subsequent 6 hrs. illumination.

Treatment	Chlorophyll content (μg/g fresh wt.)
No addition of actidione illuminated for 6 hr. 10 min. (No dark incubation)	251
addition of actidione illuminated for 6 hr. 10 min. (No dark incubation)	230
actidione added before 10 min. of illumination	300
actidione added at the end of 10 min. of illumination	311
actidione added at 11 hr. after 10 min. of illumination	305
actidione added at 24 hr. after 10 min. of illumination	308
No addition of actidione	309

Table VII. The effect of chlorophyll synthesis by leaves transferred from chloramphenicol (1 mg/ml) to distilled water during illumination of 8 hr.

Treatment	Chlorophyll content (μg/g fresh wt.)
Control H_2O	356
Chloramphenicol	196
Leaves transferred to H_2O after 1 hr. incubation of CAM in light	200
Leaves transferred to H_2O after 3 hr. incubation of CAM in light	194
Leaves transferred to H_2O after 5 hr. incubation of CAM in light	193

DISCUSSION

It is commonly held that chloramphenicol has little effect on protein synthesis in higher plant.[6,14] Previous results suggested that the action of chloramphenicol on plant tissue was not the same as on bacterial systems. Ellis reported that chloramphenicol inhibits the development of ion uptake capacity by aging discs.[5] Hanson's experiments suggested that the inhibition of salt accumlation in plant tissue by chloramphenicol could be due to the uncoupling of oxidative phosphorylation rather than to the action of the compound as a specific inhibitor of protein synthesis.[9] Further experiments showed that chloramphenicol increases the rate and extent of mitochondrial swelling which suggests that the compound acts by damaging the mitochondrial membrane rather than by specifically interferring with a phosphorylated intermediate.[10] Margulies has reported that synthesis of chloroplast protein is inhibited by chloramphenicol in excised and intact plants.[16] In contrast, protein synthesis in the cytoplasm of photosynthetic cells appears to be comparatively insensitive to chloramphenicol.[19] Chloramphenicol selectively inhibits enzymes in chloroplasts or the light induced formation of certain photosynthetic enzymes and the light-dependent incorporation of amino acids into the chloroplast fraction, but no such inhibition occurs in the cytoplasm.[18,19,21] Furthermore in contrast to the bacterial system, chloramphenicol does not inhibit protein synthesis of the ribosome system of plant cells in a cell free system.[6,14] Kirk found that the inhibition of leucine incorporation into *Euglena* by actidione was the same in the dark as it was in the light; and suggested that the formation of cell protein, but not chloroplast protein was inhibited.[11]

It was widely accepted that cytoplasmic ribosomes of plant and animal cells are 80S but ribosomes from bacteria are 70S. Recently, it has been demonstrated that chloroplasts contain 70S ribosomes instead of 80S ribosomes. These reports lead to the suggestion that chloramphenicol inhibits the function of the 70S ribosome system. On the contrary, actidione does not affect bacterial growth at all, but inhibits protein synthesis strongly in both plant and animal cells and leads to the suggestion that actidione interferes with the function of the 80S ribosome system.[21] Recent findings of inhibition of mitochondrial protein synthesis by chloramphenicol suggest that mitochondria also possess their own ribosomes with properties closely related to those of bacteria and chloroplasts.[6]

Besides inhibitors of protein synthesis, the inhibitors of nucleic acids also inhibit the formation of chlorophyll in etiolated plant cells.[2]

Chloramphenicol inhibits the formation of protein required for chlorophyll accumulation during illumination of leaves; the formation of protein is necessary for enzyme synthesis.[17] Leaves grown in the dark are capable of accumulating more protochlorophyllide than normal when supplied with δ-aminolevulinic acid (ALA).[20] Gassman and Bogorad have demonstrated that the inhibition of chlorophyll produc-

tion by chloramphenicol during stage III (a period of rapid chlorophyll synthesis which continues until the pigment content approaches that of the normal green leaf) can be partially overcome by the administration of ALA to weakly illuminated leaves.[7] Furthermore, they found that ALA can relieve the chloramphenicol inhibition of protochlorophyllide resynthesis in etiolated bean leaves and they suggested that the limitation of the production of protochlorophyllide by etiolated leaves is due to a lack of a precursor, specifically ALA.[8]

Experiments by Kirk et al[11] showed that the growth of *Euglena* in the light was abolished by actidione at a concentration of 30 μg/ml or above; and chlorophyll *a* synthesis of etiolated *Euglena* organisms incubated for 4 hours in the light is virtually abolished by actidione at concentration of 3 μg/ml or above. They suggested that actidione inhibits the synthesis of a protein, which has protochlorophyllide attached to it and is needed for reduction of protochlorophyllide to chlorophyll, or, for its incorporation of chlorophyll into lamellae. Gassman found that preincubation of leaves with very strong concentration (50 μg/ml) of actidione for 16 hours, in contrast to chloramphenicol, had no effect upon the regeneration of protochlorophyllide in vivo.[8] It is obvious that chloramphenicol and actidione do not affect chlorophyll synthesis of plants in the same way.

This present study indicates that actidione inhibits protein synthesis of the seed embryo during germination. However chloramphenicol exhibits no such inhibition. This supports the theory that chloramphenicol has no effect on the protein synthesis of plants. This experiment showed that chloramphenicol applied during greening, does not stop chlorophyll formation immediately, and confirms the report of Margulies, but disagrees with the report of Gassman.

Chloramphenicol inhibits light-induced enzyme synthesis for the formation of chlorophyll during incubation in the dark, but actidione never inhibits it. The results of this experiment correspond closely with those of previous works,[19,21] and support the theory that chloramphenicol inhibits the function of 70S ribosomes, and actidione interferes with the function of 80S ribosomes. Chlorophyll synthesis inhibited by chloramphenicol may be through the inhibition of the formation of enzymes used for synthesizing of an intermediate or a precursor of protochlophyllide, and this precursor may be ALA. The inhibition of chlorophyll synthesis by actidione may be indirect. The reduction of protochlorophyllide molecules may need some specific proteins synthesized in the cytoplasm. When the protein synthesis of cytoplasm is inhibited by actidione this leads to the shortage of the protein which is required for the reduction of protochlorophyllide molecules, and this causes the inhibition of chlorophyll synthesis.

SUMMARY

Chloramphenicol up to a concentration of 1 mg/ml did not either inhibit seed

germination or protein synthesis in the soybean seed, however, actidione at a concentration of 2 μg/ml inhibited both protein synthesis and seed germination. Chloramphenicol inhibited chlororophyll synthesis via the protein synthesis system in the chloroplast which was insensitive to the inhibitor of 80S protein synthesis, namely actidione. These results, support the earlier findings of various investigators.

The degree of inhibition of chlorophyll synthesis was greater depending on when the chloramphenicol was added. When added one or two hours after illumination, inhibition was decidedly greater than when added after 3 or 5 hours of illumination (fig. 2).

The degree of inhibition of chlorophyll synthesis by chloramphenicol was also different depending on the time of its addition in the dark period. Greater inhibition was observed when it was added at the beginning of dark period. In the case of actidione, such a inhibition was not observed.

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REFERENCES

(1) ARNON, D. I., 1949. Copper enzymes in isolated chloroplasts polyphenol oxidase in Beta vulgaris. Plant Physiol. **24**:1-15.

(2) BERIDIZE, T. G., M. S. ODINTSOVA, N. A. CHERKASHINA, and N. M. SISSAKIAN. 1966. The effect of nucleic acid synthesis inhibitors on the chlorophyll formation by etiolated bean leaves. Biochem. Biophys. Res. Commum. **23**:683-90.

(3) BROCK, T. D., 1961. Chloramphenicol. Bacteriol. Rev. **25**:32-48.

(4) BULL, M. J. and J. LASCELLES. 1963. The association of protein synthesis with the formation of pigments in some photosynthetic bacteria. Biochem. J. **87**:15-28.

(5) ELLIS, R. J., 1963. Chloramphenicol and uptake of salt in plants. Nature. **200**:596-97.

(6) ELLIS, R. J. and I. R. MACDONALD. 1967. Activation of protein synthesis by microsomes from aging Beet disks. plant physiol. **42**:1297-1302.

(7) GASSMAN, M. and L. BOGOARD. 1967. Control of chlorophyll production in rapidly greening bean leaves. Plant Physiol. **42**:774-80.

(8) GASSMAN, M. and L. BOGOARD. 1967. Studies on the regeneration of protochlorophyllide after brief illumination of etioloted bean leaves. Plant Physiol. **42**:781-84.

(9) HANSON, J. B. and T. K. HODGES. 1963. Uncoupling action of chloramphenicol as a basis for the inhibition of ion accumulation. Nature **200**:1009.

(10) HANSON, J. B. and W. A. KRUEGER. 1966. Impairment of oxidative phosphorylation by D-three and L-three-chloramphenicol. Nature **211**:1322.

(11) KIRK, J. T. O. and R. L. ALLEN, 1965. Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. Biochem. Biophys. Res. Commum. **21**:523-30.

(12) LOWRY, OLIVER H., NIRA J. ROSEBROUGH and A. LOWIS FAN. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.

(13) MACDONLD, I. R., J. S. D. BACON, C. VAUGHAN, and R. J. ELLIS. 1966. The relation between ion absorption and protein synthesis in beet disks. J. Exp. Bot. **17**:822-37.

(14) MARCUS, A., J. FEELEY, and T. VOLCANI. 1966. Protein synthesis in imbibed seeds III.

Kinetics of amino acid incorporation, ribosome activation, and polysome formation. Plant Physiol. **41**:1167-72.

(15) MARGULIES, M., 1962. Effect of chloramphenicol on light-dependent development of seedling of *Phaseolus* var. Black Valentine, with particular reference to development of photosynthesis activity. Plant Physiol. **37**:473-80.

(16) MARGULIES, M., 1964. Effect of chloramphenicol on light-dependent synthesis of protein and enzymes of leaves and chloroplasts of *Phaseolus vulgaris*. Plant Physiol. **39**:579-85.

(17) MARGULIES, M., 1967. Effect of chloramphenicol on chlorophyll synthesis of bean leaves. Plant Physiol. **42**:218-20.

(18) MORRIS, I., 1967. The effect of cycloheximide (actidione) on protein and nucleic acid synthesis by *Chlorella* J. Exp. Bot. **18**:54-64.

(19) SCHRADER, L. E., L. BEEVERS, and R. H. HAGEMAN. 1967. Differential effects of chloramphenicol on the induction of nitrate and nitrite reductase in green leaf tissue. Biochem. Biophys. Res. Commum. **26**:14-17.

(20) SISLER, E. C. and W. H. KLEIN. 1963. The effect of age and various chemicals on the lag phase of chlorophyll synthesis in dark grown bean seedlings. Physiol. Plantarum **16**: 315-22.

(21) STUTZ, E. and H. NOLL. 1967. Characterization of cytoplasmic and chloroplast, polysomes in plants: Evidence for three classes of ribosomal RNA in Nature. Proc. Natl. Acad. Sci. **57**:774-781.

(22) WITHROW, R. B. and L. PRICE. 1957. A dark-room safe light for research in plant physiology. Plant Physiol. **32**:244-48.