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NUCLEASE ACTIVITY IN CHLOROPLASTS FROM DODDER FILAMENTS

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Abstract—Stripped chloroplasts prepared from the filaments of three species of *Cuscuta* contain both RNase and DNase activities. Of the total RNase activity in homogenates, 4–7 per cent was recovered from the chloroplasts; the recovery of DNase, highly variable, was somewhat higher. The chloroplast-associated nuclease activity of all three species of parasite was distributed between the stroma and grana fractions, with enrichment of RNase in the stroma fraction. RNase activity in chloroplasts differed from that in the homogenate of the filaments in sensitivity towards alkaline earth metals and sodium and potassium chloride. DNase tended to have a higher pH optimum in chloroplasts than in homogenates. Employing succinic dehydrogenase as marker enzyme, 13 per cent of tissue mitochondria was present as contamination in chloroplasts from *C. reflexa*. Based on DNA determination in the extracts of *C. reflexa* chloroplasts after treatment with Triton X-100, about 17 per cent of the DNA in the chloroplasts fraction originated from intact nuclei. The negligible nuclease activity in the preparation of mitochondria isolated from parasite tissue and the high recovery of nuclease activity in extracts of the stripped chloroplasts with Triton X-100 suggested that both RNase and DNase are integral components of chloroplasts in *Cuscuta*. The nuclease activity of stripped chloroplasts isolated from alfalfa differed in some respects from that of parasite plastids; RNase and DNase in host chloroplasts were essentially insensitive to sodium and potassium chloride and RNase was stimulated by alkaline earth metals, unlike in preparations from parasite. Fractionation of acid-insoluble phosphorus by conventional methods suggested that the chloroplast preparations from the filaments of *C. reflexa* contained both RNA and DNA.

INTRODUCTION

ALTHOUGH chloroplasts have been identified in the filaments of *Cuscuta*[1–3] and a photosynthetic role assigned to chlorophyll in this parasite,[4–6] the plastids have not been isolated and the components delineated. Apart from mitochondria, chloroplasts of higher plants are known to contain RNA[7–10] and DNA[11–13] and also ribosomes.[12,14,15] RNA, but not DNA, has been demonstrated in glyoxysomes.[16] An interesting finding, in animal tissues, is the likely occurrence of DNA in microsomes.[17] Tewari and Wildman[13] demonstrated the presence of DNA-dependent RNA-polymerase activity in tobacco chloroplasts,

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tightly bound to the thylakoids. Whereas there exists no report on the DNase activity in chloroplasts, RNase has been demonstrated, but data as to intraplastid localization are contradictory. According to Biswas and Biswas,[14] the grana fraction had high RNase activity and only low activity was demonstrable in the stroma fraction or in the whole chloroplasts of spinach leaves. On the other hand, the major part of chloroplast RNase activity in wheat leaves was associated with the stroma and the membrane;[10,15] the lamellae fraction had low activity in some samples[10] and high activity in others.[15]

An earlier study from this laboratory related to the nuclease activity in whole homogenates of *Cuscuta*.[18] In the present study, the authors have isolated chloroplasts from aqueous dispersions of the parasite and demonstrated that they contain RNase and DNase as likely integral components. Significant amounts of RNA and DNA were found in the chloroplasts preparations, but an undetermined part of the nucleic acids may have been of extra-chloroplast origin.

RESULTS

1. *Isolation of Chloroplasts by Different Methods from* Cuscuta reflexa

The results of experiments carried out with *C. reflexa* are recorded in Table 1.

TABLE 1. ISOLATION OF CHLOROPLASTS FROM *Cuscuta reflexa*

	Chlorophyll content, mg/40 g tissue equivalent		
	Strained homogenate	Nuclear fraction	Chloroplasts fraction
Isolation according to:			
Arnon	2·020	—	0·362
Chiba and Sugahara	0·901	—	0·0765
Spencer and Whitfeld	1·431	—	0·1002
James and Das	1·543	0·211	0·361
Leech	1·257	0·147	0·290

The apical region of the filaments of *C. reflexa* parasitic on *Lantana camara* was used. The methods of isolation of chloroplasts were as described in text. Different batches of filaments were used in the isolation studies, spread over several days, which would account for the differences in chlorophyll content in the strained homogenates.

On the basis of chlorophyll content of strained homogenates, 7–23 per cent was recovered in the chloroplasts preparation by different methods. By the method of Leech,[19] which was found to be the most suitable and used in the other experiments, 12 per cent of chlorophyll was present as contamination in the 'nuclear fraction' and 23 per cent was associated with the chloroplast fraction. In other experiments, not reported in table, the chloroplast preparation from *C. campestris* contained 20 per cent of the total chlorophyll and that from *C. indecora* 85 per cent.

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2. *Nuclease Activity in Chloroplasts and Intraplastid Localization*

The analytical data for nuclease activity and for chlorophyll and protein content in the stripped chloroplasts fraction and in the separated grana and stroma fractions are recorded in Table 2.

TABLE 2. NUCLEASE ACTIVITY IN STRIPPED CHLOROPLASTS FROM *Cuscuta reflexa* AND *Medicago sativa*

	Chlorophyll content	Protein content	Ribonuclease activity units		Deoxyribonuclease activity, units	
	(mg/g fr. wt. of tissue)		(/g fr. wt.)	(/mg protein)	(/g fr. wt.)	(/mg protein)
				C. reflexa		
Homogenate	0·044	10·7	146·6	13·7	56·0	5·2
Chloroplasts	0·011	0·48	5·0	10·4	11·0	22·9
grana	0·010	0·38	4·2	11·0	4·0	10·1
stroma	nil	0·10	8·3	80·9	1·0	9·7
Supernatant, 1000 *g*	0·031	10·9	125·0	11·5	37·5	3·5
				M. sativa		
Homogenate	1·16	27·03	163·3	6·0	105·0	3·8
Chloroplasts	0·26	0·46	6·7	14·5	6·7	14·5
grana	0·14	0·41	8·9	21·7	6·0	14·1
stroma	0·008	0·16	13·3	83·3	1·3	8·3
Supernatant, 1000 *g*	0·99	21·8	126·7	5·8	88·7	4·1

The chloroplasts were isolated by a modification of the method of Leech from homogenate in medium containing optimal concentration of cysteine and were suspended in cysteine solution. The grana fraction was obtained on osmotic rupture of the chloroplasts. RNase of both parasite and host was assayed at pH 5·0 using acetate buffer. The RNase activity was optimum in parasite chloroplasts at pH 6·0, as found subsequently, but the activity at pH 5·0 (the optimum for homogenate) was only 13 per cent less than at pH 6·0. RNase had the same optimum in host chloroplasts and homogenate (pH 5·0). DNase of parasite was assayed at pH 6·5, using Tris universal buffer and of alfalfa at pH 5·5, acetate buffer. DNase of parasite chloroplasts had the same pH optimum (pH 6·5) as homogenate. DNase of host chloroplasts had optimum at pH 6·5, but the activity at pH 5·5 was only about 10 per cent less. The assays were at 37°, with an incubation period of 1 hr for RNase and 2 hr for DNase.

The chloroplast fraction from *C. reflexa* contained 3·4 per cent of the RNase activity of the homogenate. This order of recovery was reproducible in experiments with different batches of filaments on different days. In the experiment reported, the recovery of DNase was about 20 per cent; the recovery was variable in experiments with different batches of filaments on different occasions, with an average of 10 per cent. Both grana and stroma contained nuclease activity, but the pattern of distribution was different for RNase and DNase. RNase activity recovered in stroma was double that in the grana; also the activity recovered from the isolated fractions was greater than in the original chloroplasts. In contrast, DNase was associated mainly with grana fraction; the recovery from isolated fractions was only about 50 per cent. An 8-fold enrichment of RNase occurred in the stroma fraction, but DNase was not enriched in either fraction.

Less than 5 per cent of the protein in parasite homogenate was recovered in the chloroplast fraction. Of this, about 80 per cent was associated with grana. 25 per cent of chlorophyll in the homogenate was recovered from the chloroplast fraction, the pigment being associated exclusively with the grana fraction. The ratio of protein to chlorophyll (mg/mg) was 44 in the stripped chloroplasts and 38 in the grana fraction.

In experiments (not reported in Table 2) with the filaments of *C. campestris*, about 7 per cent of RNase and 9 per cent of DNase activity of homogenate were recovered in the stripped chloroplasts fraction. The nuclease activity was distributed between the stroma and grana. The protein (mg) to chlorophyll (mg) ratio was 97 in the stripped chloroplasts and 64 in the grana. The chloroplasts isolated from *C. indecora* contained 6 and 8 per cent respectively of the RNase and DNase activity of the homogenate. As with the other species of parasite, the nuclease activity was present both in the grana and stroma. The ratio of protein (mg) to chlorophyll (mg) was 15 in stripped chloroplasts and 5 in grana.

The chloroplast fraction isolated from the shoot of *M. sativa* contained 4 per cent RNase and 6 per cent DNase of whole homogenate. RNase activity was found in both grana and stroma; the recovery from the latter was about 50 per cent higher than from the former. The activity recovered from the isolated fractions exceeded the RNase activity associated with the original preparation. DNase activity was associated essentially with the grana. RNase was enriched in the stroma fraction, but DNase did not show enrichment in either fraction.

Of the protein in homogenate of alfalfa shoot, about 2 per cent was recovered in the stripped chloroplasts, with the major part in the grana fraction. About 22 per cent of chlorophyll of homogenate was in the chloroplasts. The ratio of protein to chlorophyll (mg/mg) was 2 in the chloroplasts and 3 in the grana.

3. *Contamination of Chloroplasts by Intact Nuclei*

The data for phosphorus fractionation in the acid-insoluble, lipid-free, fraction of chloroplasts from *C. reflexa* are recorded in Table 3.

TABLE 3. ACID-INSOLUBLE, LIPID-FREE, PHOSPHATE FRACTIONS IN CHLOROPLASTS FROM *Cuscuta reflexa*

	RNA	DNA	Phosphoprotein
	mg phosphorus/40 g tissue		
Homogenate, strained	4·377	0·955	0·134
Chloroplasts:			
(i) Original preparation	0·134 (3·1)	0·0242 (2·5)	0·015 (11·2)
(ii) Supernatant on treatment with Triton X-100	0·124 [92·5]	0·0199 [82·2]	0·0104 [69·3]

Values in parenthesis represent the percentage content in relation to the total in strained homogenate. Values within square brackets are the recoveries of RNA and DNA in the Triton extracts in relation to the content in the fresh chloroplasts.

Both RNA and DNA were present in the stripped chloroplasts preparation, the former in yields of 3·1 per cent and the latter 2·5 per cent, based on the contents in the strained homogenate. On treatment with Triton X-100, about 93 per cent of RNA and 82 per cent of DNA in the chloroplasts preparation were recovered in the centrifuged extract.

Nuclei are known to contain RNase and DNase.[20] The isolated preparation of chloroplasts had 6·25 units of RNase and 2·1 units of DNase activity per g fresh tissue. With

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the elimination of intact nuclei as sedimentable organelles on treatment with Triton X-100, it was anticipated that the nuclease activity in the extract of the chloroplasts would be a lower value than that of the original chloroplasts fraction. However, analysis showed that the extract of chloroplasts with Triton X-100 contained the same DNase activity and 33 per cent excess RNase activity compared to the fresh preparation of the plastids, suggesting detergent-mediated stimulation both of RNase and DNase.

4. *Contamination of Chloroplasts with Mitochondria*

Of the succinic dehydrogenase activity in homogenate, about 13 per cent was recovered in the chloroplast fraction; this was a measure of contamination due to the mitochondria. Tests for nuclease activity in the mitochondrial fraction isolated from the filaments revealed that whereas RNase activity was barely demonstrable, DNase could not be detected. The inability to demonstrate significant RNase activity in the mitochondrial fraction was in accordance with the findings of Hadziyev *et al.*[10]

5. *Properties of the Nucleases of Chloroplasts*

(a) *pH optimum.* RNase activity in stripped chloroplasts was optimum at pH 5·0 for *C. campestris*, 6·0 for *C. indecora* and 6·0–7·0 for *C. reflexa.* Tested in homogenates, RNase had pH optimum at 5·0 for *Cuscuta* species.[18] DNase of stripped chloroplasts had optimum at pH 7·5 for *C. campestris* and pH 6·5 for *C. reflexa; C. indecora* had two peaks of activity-pH 5·5 and 7·5, with nearly identical activities at the two pH values. Tested in homogenates, DNase from *C. campestris* and *C. indecora* had pH optima at 5·5, but *C. reflexa* had at pH 6·5.[18] In general, the pH optima of the chloroplasts nuclease activity were not sharp.

RNase of *M. sativa* chloroplasts had optimum at pH 5·0 and DNase at 6·5. In whole homogenate of the shoot, RNase and DNase had optimum at pH 5·0 and 5·5 respectively.[18]

(b) *Inhibitors and activators.* Stripped chloroplasts from *C. campestris* and *C. reflexa* and *M. sativa* were suspended in water and tested for the influence of a number of salts and chelating agents used in 1×10^{-2} M concentration on RNase and DNase activities. The basal DNase assay system was devoid of Mg^{2+}. The results obtained are recorded in Tables 4 and 5.

TABLE 4. INFLUENCE OF ACTIVATORS AND INHIBITORS ON RIBONUCLEASE OF TWO PARASITE AND ONE HOST CHLOROPLASTS

Reagents tested	*Cuscuta campestris*	*Cuscuta reflexa*	*Medicago sativa*
$CaCl_2$	−67	−67	+50
$MgSO_4$	−67	−71	+100
EDTA	+67	+19	−50
Sodium citrate	−67	−14	−67
NaCl	−67	−71	0
KCl	−50	−81	0
NaF	−33	−40	0

Chloroplasts were isolated in a medium containing the optimal concentration of cysteine and suspended in water. The enzyme assay was at the optimum pH of 5·0 (acetate) for *C. campestris*, 6·0 (Tris universal) for *C. reflexa* and 5·0 (acetate) for *M. sativa.* The various supplements were tested in final concentration of 1×10^{-2} M. + and − signs indicate respectively activation and inhibition, reported as %.

TABLE 5. INFLUENCE OF ACTIVATORS AND INHIBITORS ON DEOXYRIBONUCLEASE OF TWO PARASITE AND ONE HOST CHLOROPLASTS

Reagents tested	*Cuscuta campestris*	*Cuscuta reflexa*	*Medicago sativa*
$CaCl_2$	+60	+75	+100
$MgSO_4$	+233	+100	+100
EDTA	−66	−50	−79
Sodium citrate	0	0	0
NaCl	−66	−75	+13
KCl	−40	−50	0
NaF	+33	+75	−63

Chloroplasts were isolated in a medium containing the optimal concentration of cysteine and suspended in water. The enzyme assay was at the optimum pH of 7·5 (Tris) for *C. campestris* and 6·5 (Tris universal buffer) for *C. reflexa* and *M. sativa*. The various supplements were tested in final concentration of 1×10^{-2} M. + and − signs indicate respectively activation and inhibition, reported as %.

In parasite chloroplast preparations, NaCl and KCl were common inhibitors of the two nucleases. Mg^{2+} and Ca^{2+} inhibited the RNase activity; EDTA stimulated the activity and citrate inhibited; NaF inhibited. Mg^{2+} and Ca^{2+} stimulated DNase activity and EDTA inhibited. Citrate was without effect. NaF activated the reaction.

In host chloroplast preparation, Mg^{2+} and Ca^{2+} activated not only DNase, but also RNase. EDTA inhibited both activities. NaCl and KCl either did not influence RNase and DNase activities, or elicited only marginal effect (13 per cent activation of DNase by NaCl). NaF did not influence RNase, but inhibited DNase.

DISCUSSION

Chloroplasts of higher plants have the materials necessary for genetic autonomy. They also contain ribosomes which would be necessary to convert the information in chloroplast DNA into proteins.[21] Such a mechanism, if present in the photosynthetically active angiosperm parasites, may be specially important in parasite physiology.

In view of the known low concentration of chlorophyll in *Cuscuta* filaments[22–24] and the high content of starch[25] and occurrence of phenolics,[26] chloroplast isolation was expected to be difficult. The method finally adopted was one patterned after Leech[19] and involved discontinuous density gradient centrifugation, with modifications in the composition of the gradient. A reducing agent such as cysteine was an essential constituent of all isolation media. The ratio of protein to chlorophyll content in the plastid preparations and

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[25] M. SINGH, D. V. SINGH, P. C. MISRA, K. K. TEWARI and P. S. KRISHNAN, *Physiol. Plantarum* **21**, 525 (1968).
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grana fractions from *Cuscuta* was much higher than from *M. sativa*. However, a high protein: chlorophyll ratio need not necessarily indicate extensive mitochondrial contamination.[27]

The stripped chloroplast preparation from all species of parasite contained both RNase and DNase activity. To our knowledge, this is the first report on the presence of DNase activity in a chloroplast preparation from plant tissue. The chloroplast-associated nuclease activity was distributed between the stroma and the grana fraction, with enrichment of RNase in stroma fraction, an observation in agreement with the findings of Hadziyev *et al.*[10] and contradictory to that of Biswas and Biswas.[14] Whether endogenous inhibitors[10] influenced the recovery of nuclease activity in chloroplast fraction and the distribution between grana and stroma was not tested. The assayable RNase activity in tissues is known to be dependent upon a number of unexplained factors.[10,28]

The demonstration of considerable nuclease activity associated with the grana fraction appeared to overrule extensive contamination of chloroplasts with nuclei. That RNase existed in true association with the chloroplasts received support also from the observation that the enzymic activity in chloroplasts differed from that in whole homogenate. Singh and Krishnan[18] reported that RNase activity of *Cuscuta* homogenates was not markedly affected by alkaline earth metals or NaCl and KCl. The isolated chloroplast preparations, on the other hand, were powerfully inhibited under the above conditions. The finding that DNase of *C. campestris* and *C. indecora* chloroplasts had a somewhat higher pH optimum than when tested in the form of homogenates suggested that DNase also was a genuine component of chloroplasts. The finding of nuclease activity in the Triton extract of the chloroplasts, together with the fact that the contribution by mitochondria was negligible, appeared to establish that both RNase and DNase are integral components of the chloroplasts in *Cuscuta*. The presence, if any, of fragmented nuclei in the chloroplasts fraction would result in solubilization of nuclear components on treatment with Triton X-100.[29]

A distinctive feature of nuclease activity of plant tissues is the sensitivity to ions. The rate of nucleic acid degradation may be a critical factor in cell metabolism. Since some of the ions may occur in sufficiently high concentration (10^{-2}M) in the intracellular regions with nuclease activity, they may modulate the hydrolytic degradation of RNA and DNA *in vivo*. The inhibition of parasite chloroplasts RNase and DNase by NaCl and KCl and of RNase by Mg^{2+} and Ca^{2+} and the activation of DNase but inhibition of RNase by fluoride differed from the response by host plastids and suggest differing mechanisms for the regulation of nucleic acid degradation in chloroplasts *in vivo* in parasite and host.

RNA and DNA also appeared to be integrally associated with the chloroplasts. The contribution by intact nuclei was eliminated by carrying out the determination in extracts with Triton X-100; fragmented nuclei, if present, would contribute nuclear components to the extract with detergent. Mitochondrial contamination was only about 13 per cent and could not account for the amount of RNA and DNA found in the chloroplasts fraction. Microsomal contamination was not tested, but probably was not serious in view of the low centrifugal field employed for collecting the chloroplasts. No special measures were adopted to prevent bacterial contamination, nor to determine the bacterial count in the final preparation and effect a suitable correction. During treatment with Triton X-100 bacteria are not extracted, but a higher centrifugal field than used in the present investigation is required to

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sediment the bacteria from the treated preparation.[30] The factors influencing the reliability of chemical determination of RNA and DNA in isolated plastids have been reviewed by Jacobson *et al.*[31]

The presence of RNase in chloroplasts has been shown to result in artifact formation in isolated RNA.[10] In case the data obtained in the present experiments could be interpreted as pointing to the general presence also of DNase in chloroplasts, some of the contradictions on the characteristics of isolated DNA[29,32,33] may be attributed to degradation by DNase action during isolation.

EXPERIMENTAL

Parasite Tissue

The detailed studies on different methods of isolation of chloroplasts were with the abundantly available species *Cuscuta reflexa* Roxb., parasitic on *Lantana camara* L. Ten cm lengths of the filaments were cut and pooled and randomized. The method of isolation found to be most satisfactory for *C. reflexa* was employed in the isolation of chloroplasts also from the whole filaments of *C. campestris* and *C. indecora* growing on *Medicago sativa* L. Nuclease activity determination in the whole preparation and intraplastid localization were carried out on chloroplasts from all three species of *Cuscuta*. The influence of activators and inhibitors was studied with preparations from *C. campestris* and *C. reflexa*. Tests for contamination with intact nuclei and mitochondria were carried out with the preparations from *C. reflexa*. Chloroplasts were isolated from the shoot of *M. sativa* for comparison with parasite plastids.

Isolation of Chloroplasts

The procedure tested for the isolation of chloroplasts from *C. reflexa* were those developed by the earlier workers for tobacco leaves,[34,35] broad bean[19,36] and spinach.[8] The method finally adopted for further studies on *C. reflexa* and for isolation from *C. campestris* and *C. indecora*, described below, was that of Leech,[19] with modifications.

General

All operations were carried out in the cold. The various tissues were used without destarching. Tissue dispersion was effected in Waring blendor operated at low speed for 30 sec; after straining through 4 layers-thick muslin, the suspensions were made to 40% (w/v). The dispersion medium always contained cysteine (freshly neutralized hydrochloride) to prevent enzyme inactivation by phenolics. The concentrations of cysteine found optimum were: 0·1 M for *C. campestris* and *C. indecora*, 0·05 M for *C. reflexa* and 0·04 M for the shoots of *M. sativa*. The final chloroplast pellet was suspended in cysteine solution for nuclease activity determination and for fractionation into grana and stroma; water was used for suspension when the effect of activators and inhibitors was tested. For treatment with Triton X-100, the chloroplasts were suspended in the original dispersion medium.

Detailed Procedure

A homogenate of 40 g tissue in cysteine solution of optimum strength containing 0·4 M sucrose and 0·067 M phosphate buffer, pH 7·3, was centrifuged at 600 *g* for 2 min, the residue discarded and the supernatant centrifuged at 1000 *g* for 12 min. The particles were collected and washed twice with the medium and suspended in 10 ml medium, with the help of a hand-operated glass homogenizer of the Potter–Elvehjem type. The suspension was subjected to density gradient centrifugation employing a top layer of 12 ml 46% (w/v) and a bottom layer of 20 ml 70% (w/v) sucrose in 0·06 M phosphate buffer pH 7·3. The suspension of the chloroplasts settling at the boundary between the two sucrose layers was diluted with medium and sedimented by centrifugation at 1000 *g* for 20 min. Whereas host tissue yielded an apparently homogenous preparation of chloroplasts at the end of above operation, the preparations from parasite tissues had to be resedimented in the discontinuous density gradient to remove additional contaminants. The final preparation was suspended in 10 ml appropriate medium with the aid of the hand-operated glass homogenizer.

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Intraplastid Localization

The stripped chloroplasts from 20 g tissue were suspended in 20 ml cysteine of the same strength as used in grinding medium, the suspension stirred in the cold for 1 hr and centrifuged at 10,000 *g* for 30 min. The supernatant was taken as the 'stroma' fraction; the pellet was the 'grana' fraction and was taken up in 10 ml cysteine solution.

Extraction of Chloroplasts with Triton X-100

Chloroplasts isolated from 40 g filaments of *C. reflexa* and purified twice by density gradient centrifugation were suspended in 20 ml original medium. One half the suspension was set aside for determination of nuclease activity and of RNA and DNA content. The other half was mixed with Triton X-100 in final concentration 1%, the suspension kept stirred with glass rod for 1 hr in the cold and centrifuged at 1000 *g* for 15 min. Nuclease activity and RNA and DNA content were determined in the supernatant fraction.

Isolation of Mitochondria

Mitochondria were isolated from *Cuscuta* according to Beg and Krishnan.[37]

Determination of RNA and DNA in Chloroplasts

The chloroplasts preparation was extracted twice with 10% TCA, the residue treated with ethanol–ether (3:1) to remove phospholipids and the final residue dried at 60°. The acid-insoluble, lipid-free, material was fractionated according to Schmidt and Thannhauser[38] and Schneider.[39] RNA and DNA were determined in the respective fractions by the estimation of phosphorus. Simultaneously, the whole homogenate was analysed for nucleic acids by the same procedure.

Assay of Nucleases

RNase and DNase activities were assayed at 37°, with an incubation period respectively of 1 and 2 hr, as reported earlier.[18]

One unit of RNase or DNase activity was defined as equivalent to an increase of 1·0 in optical density at 260 mμ under assay conditions.

Assay of Succinic Dehydrogenase

Succinic dehydrogenase activity was determined after Slater and Bonner,[40] with $K_3Fe(CN)_6$ as acceptor. The reaction was carried out at pH 7·5 and 37°.

Determination of Protein

Protein was determined with Folin and Ciocalteu reagent according to method standardized in this laboratory.[41]

Determination of Chlorophyll

Chlorophyll was determined spectrophotometrically in 80% acetone extracts according to MacKinney.[42]

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