XXXVII. STUDIES ON CHLOROPLASTS
II. THEIR CHEMICAL COMPOSITION AND THE DISTRIBUTION OF CERTAIN METABOLITES BETWEEN THE CHLOROPLASTS AND THE REMAINDER OF THE LEAF[1]

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THE available information concerning the chemical composition of the chloroplasts and the distribution of elements within the cell has been gained by the use of histochemical methods. The studies reported in this paper were made on the chloroplast substance isolated by the method outlined in the previous paper [1939]. Some information on the composition of the chloroplasts isolated from the leaves of two different species was first obtained. The distribution of N, P, Mg, Fe and Ca in the chloroplasts was next investigated by means of various solvents to obtain information on the nature of the combination of these elements. To determine which elements or compounds were concentrated in the chloroplasts, comparative analyses were made of chloroplast preparations and the corresponding whole leaf tissue; these comprised determinations of Ca, Fe, Cu, P, Mg, K, Na, Mn, SO_4, Cl, NH_4 salts, nitrates, ascorbic acid, catalase and carbonic anhydrase in the chloroplasts and whole leaf tissue of four different species of plants.

EXPERIMENTAL

Analyses were made of the whole leaf tissue and of the chloroplast substance separated from the leaf tissues of *Trifolium pratense* and *Onoclea sensibilis*. The samples were dried *in vacuo* at 60°. The protein content was estimated by determining the N content of the residue remaining after exhaustive extraction of the material with 85% acetone, absolute alcohol, anhydrous ether, 10% trichloroacetic acid and 85% acetone in the order named, and multiplying the value obtained by 6·25. The difference between the total N and the protein-N is represented as the non-protein-N. The ether extract was determined by continuous extraction of the original material in a Soxhlet apparatus with ether only. Starch was determined by the taka-diastase method of Denny [1934] and ammonia by the method of Pucher *et al.* [1935]. In determining nitrates, 0·20 g. of the material was extracted first with ether and then with boiling water; the extracts were combined and freed from ether. The aqueous extract was next made slightly alkaline and evaporated to dryness; the residue was taken up in water and brought to 200 ml. Nitrates were determined in this solution by means of the colorimetric diphenylbenzidine method of Whelan [1930].

Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol according to McHenry & Graham [1935]. The titrations were carried out in triplicate with suspensions of freshly prepared chloroplasts or ground leaf

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tissue, in the preparation of which a few drops of 10% KCN were added to prevent oxidation of ascorbic acid. The suspensions were saturated with H_2S and kept overnight in a closed vessel. The H_2S was displaced by CO_2, the nitroprusside test being employed to determine when all the H_2S had been removed. The titration was carried out in a centrifuge tube with the least possible delay. A few ml. of 2 *M* $CaCl_2$ were added to ensure the flocculation of the suspension, more of the dye solution was added and the suspension centrifuged until the end-point of the titration was reached, which could be easily detected in the clear supernatant liquid. The first titration gives an approximate value; the second and third titrations can be conducted more accurately.

Carotenoid pigments were determined as previously described [1939]. P was determined according to Dyer & Wrenshall [1938]; Fe and Cu by the methods of Parker & Griffin [1939]; Ca by the volumetric micromethod of the A.O.A.C. [1935]; Mg by the hydroxyquinoline method of Greenberg *et al.* [1935]; Na by the method of Salit [1932]; K by the volumetric cobaltinitrite method of Volk & Truog [1934]; Mn by the colorimetric bismuthate method of the A.O.A.C. [1935]; SO_4 by the volumetric benzidine method of Cope [1931] and Cl by the method of Cattle [1935].

The catalase and carbonic anhydrase activities of suspensions of the chloroplast substance and ground whole leaf tissue in distilled water were determined with a differential manometer. It was necessary to use the thermostat at a temperature of 27° because of the high atmospheric temperature prevailing at the time. The amount of gas evolved was plotted against time for suspensions of known dilution and in this way the time was found at which the amount of gas evolved is a measure of the enzymic activity.

In the fractionation of the chloroplasts a 2 g. sample of the moisture-free, finely pulverized chloroplasts was employed in each case. The extractions with anhydrous solvents were made in a Soxhlet extractor, but with the other solvents repeated extractions were carried out in a Büchner funnel fitted with filter paper.

Table I. *Analysis of the chloroplast substance of* Trifolium pratense *and* Onoclea sensibilis

Results expressed as % of the moisture-free substance

	Trifolium pratense	*Onoclea sensibilis*
Total N	9·69	5·90
NH_4-N	0·80	0·12
NO_3-N	0·011	0·006
Non-protein-N	1·66	0·87
Protein-N	8·02	5·05
Total protein	50·11	31·51
Ether extract	21·75	7·21
Starch	Nil	8·43
Ascorbic acid	2·53	0·56
Carotene	0·19	0·10
Xanthophylls	0·24	0·15
Total ash	3·02	4·11
Mg	0·166 : 0·164	0·113 : 0·164
Cu	0·008	0·017
Fe	0·092	0·074
Ca	0·170	0·220
P	0·219 : 0·741	0·326 : 0·254
K	Nil	Nil
Na	0·039	0·939
Mn	Trace	Trace
SO_4	0·894	0·431
Cl	Nil	0·074

Results and discussion

1. *Composition of chloroplasts*

The results are shown in Table I. The high protein and lipoid contents of the chloroplasts, particularly of *Trifolium pratense*, are noteworthy. These results agree as well as could be expected with those of Menke [1938] who records 8·38 % N and 30·9 % ether extract for the chloroplasts of *Spinacia oleracea*. The chloroplasts of *Onoclea sensibilis* are different from those of the other two species; they are considerably larger and appear to store more starch than those of clover, which accounts, in part at least, for the lower lipoid and protein contents. It will also be noted that the P content varied markedly in two preparations from the leaves of the same species cut at different stages of growth.

2. *Fractionation of chloroplasts*

In Table II are shown the scheme of extraction followed and the results obtained. When the initial extraction was made with 85 % acetone more material was dissolved than when it was made with anhydrous ether (Table II B). Furthermore, extraction with 85 % acetone left only a small fraction which was extractable by alcohol and ether, so that most of the lipoid material of the chloroplast was extracted by 85 % acetone. An appreciable amount of P was extracted by anhydrous ether but when the material was first extracted with 85 % acetone the amount of P extracted by ether was considerably reduced. It would appear, therefore, that the chloroplasts contain phospholipins which are appreciably soluble in 85 % acetone. Granick [1937] found that 40 % of the total lipoid-P of the tomato leaf was located in the chloroplasts. Most of the P in clover chloroplasts was extracted by trichloroacetic acid, whereas the P in the chloroplasts of *Onoclea sensibilis* was found to be more difficult to extract.

Nearly all of the Mg contained in the chloroplasts was extracted by trichloroacetic acid, and when compared with this fraction the amount of Mg present in the chlorophyll fraction was negligible. Ca was also nearly all removed by the trichloroacetic acid. This is in accord with the findings of Terlikowski and Sozonski [1937] and Kostychev & Berg [1929], that extraction of leaf tissue with dilute HCl acid effects almost complete removal of the Ca and Mg.

A considerable proportion of the total Fe was extracted by trichloroacetic acid and an appreciable amount was also dissolved by previous extraction with ether, which is to be expected from the known solubility of inorganic salts of Fe in ether. Even after extraction with trichloroacetic acid, Fe was found in a subsequent 85 % acetone extract. The Fe contained in the chloroplasts of *Onoclea sensibilis* seems to be in a form readily soluble in acetone which may account for the comparatively small amount contained in the trichloroacetic acid fraction. The nature of the combination of Fe in the chloroplast is discussed later. The ionic state of the Ca, Mg, P and Fe in the trichloroacetic acid extracts was shown by the fact that the amounts of these elements, as obtained by direct analysis of the trichloroacetic acid solution, were nearly equal to the amounts obtained after digestion with conc. H_2SO_4 plus Na_2SO_4 and HgO.

Most of the N remains in the final residue and is probably all protein-N. Small amounts are extracted by trichloroacetic acid or ether, that dissolved by the latter probably being contributed by lecithin. Granick [1938] found that 80 % of the total N in chloroplasts was protein-N. This agrees with the values reported here.

Table II. *Showing the analysis of various fractions separated from the chloroplasts of* Trifolium pratense *and* Onoclea sensibilis

Results expressed as % of the total amount in the original material

A. Dry, powdered, chloroplast substance extracted with anhydrous ether

Residue
(Extracted with absolute alcohol, followed by asolute acetone)

Residue
(Extracted with 80% acetone, followed by extraction with 10% trichloroacetic acid)

	Ether extract		Acetone extract		Trichloroacetic acid extract	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*	*Trifolium*	*Onoclea*
Dry wt.	21·75	7·21	0·55	0·90	3·35	0·42
N	2·26	0·73	0·18	0·93	67·97	14·12
P	3·10	1·13	*	0·015	90·85	78·76
Mg	*	9·74	*	*	66·96	54·21
Fe	6·11	*	3·49	6·52	92·12	103·64

B. Dry, powdered, chloroplast substance extracted with 85% acetone

Soluble in 85% acetone
(Residue extracted with light petroleum)

Residue
(Extracted with absolute alcohol and anhydrous ether)

	Extract		Extract	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*
Dry wt.	25·14	12·24	3·75	1·53
N	5·86	1·37	0·32	0·32
P	1·42	6·90	0·23	0·64
Mg	*	*	*	*
Fe	*	*	1·53	2·44

Residue
(Extracted with 10% trichloroacetic acid)

Residue
(Extracted with 80% acetone)

	Extract		Extract (bound chlorophyll)		Residue	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*	*Trifolium*	*Onoclea*
N	3·18	0·32	3·03	0·63	82·73	85·51
P	55·43	19·31	3·88	11·57	—	—
Mg	87·80	61·95	*	*	—	—
Fe	47·76	26·49	2·29	7·74	—	—
Ca	92·12	83·62	—	—	—	—

* If present, the amount found was too small to be determined by the methods employed.

3. *Comparative analysis of chloroplasts and whole leaf tissue*

The results of the analysis of the whole leaf tissue of different species of plants and of the chloroplast substance separated from the same leaf tissue are shown in Tables III, IV and VII. When reported as % of the total ash

Table III. *Showing the comparative compositions of the ash of chloroplasts and whole leaf tissue*

Species	Total ash (% dry wt.)	% of the total ash									
		Fe	Ca	Mg	P	Mn	K	Na	Cu	SO_4	Cl
Trifolium pratense:											
Chloroplast substance	8·04	0·44	6·15	1·99	9·20	—	*	0·48	0·101	11·11	*
Whole leaf	9·91	0·27	20·87	3·08	3·84	—	20·01	3·21	0·042	7·31	1·11
Onoclea sensibilis:											
Chloroplast substance	5·23	1·08	6·33	3·04	4·90	—	*	0·74	0·332	8·23	2·12
Whole leaf	9·75	0·44	13·31	5·12	3·80	0·06	21·01	2·14	0·077	10·58	5·63
Elodea canadensis:											
Chloroplast substance	9·74	2·56	—	2·07	4·01	0·24	*	2·57	0·165	6·89	—
Whole leaf	12·89	1·14	—	3·41	3·56	0·52	25·28	5·16	0·068	6·21	—
Arctium minus:											
Chloroplast substance	5·32	1·18	9·62	3·00	6·39	—	*	0·39	0·160	8·09	0·74
Whole leaf	11·75	0·59	14·02	4·86	2·64	—	21·68	3·35	0·031	5·21	3·54

* Presence doubtful—not detectable by the methods used.

Table IV. *Showing the distribution of ammonia, nitrates, sulphates and ascorbic acid in leaf tissue*

Results are expressed as % of the moisture-free substance

Species	Ammonia (% NH_4-N)			Nitrates (% NO_3-N)		
	Chloroplasts	Whole leaf	Non-chloroplast fraction	Chloroplasts	Whole leaf	Non-chloroplast fraction
Trifolium pratense	0·794	0·335	0·165	0·0110	0·0062	0·0045
Onoclea sensibilis	0·125	0·088	0·066	0·0060	0·0034	0·0051
Elodea canadensis	0·234	0·110	0·033	0·0006	0·0044	0·0073
Arctium minus	0·217	0·172	0·149	0·0032	0·0156	0·0208
	Sulphates			Ascorbic acid		
	Chloroplasts	Whole leaf	Non-chloroplast fraction	Chloroplasts	Whole leaf	Non-chloroplast fraction
Trifolium pratense	0·89	0·66	0·57	2·529	2·557	2·566
Onoclea sensibilis	0·43	1·03	1·38	0·566	0·285	0·126
Elodea canadensis	0·67	0·80	0·88	0·653	0·433	0·325
Arctium minus	0·43	0·61	0·70	0·497	0·677	0·770

(Table III), or as % of the total amount in the whole leaf which is contained in the chloroplast fraction (Table V), the values show which elements or compounds are concentrated in the chloroplasts relative to the other fractions. The whole leaf material is always higher in total ash content than the chloroplast substance. From these results the following observations may be made.

A. *Fe and Cu* are concentrated in the chloroplasts, Cu showing an even greater tendency to collect in the chloroplasts than Fe. The fact that Fe and Cu show the same localization supports the view that some oxidative reactions are centred in the chloroplasts which particularly require their catalytic action.

Table V. *Showing the distribution of elements between the chloroplasts and the remainder of the leaf tissue*

Results are expressed as % of the moisture-free substance

		Trifolium pratense	*Onoclea sensibilis*	*Elodea canadensis*	*Arctium minus*
% of chloroplasts in the leaf tissue		27	37	40	33
Fe:	Whole leaf	0·025	0·043	0·147	0·070
	Chloroplast substance	0·035	0·057	0·249	0·063
	Non-chloroplast fraction	0·022	0·035	0·092	0·077
Ca:	Whole leaf	1·91	1·43	—	2·45
	Chloroplast substance	0·17	0·22	—	0·72
	Non-chloroplast fraction	2·55	2·14	—	3·29
Mg:	Whole leaf	0·28	0·50	0·44	0·57
	Chloroplast substance	0·16	0·16	0·20	0·12
	Non-chloroplast fraction	0·33	0·70	0·60	0·78
P:	Whole leaf	0·35	0·37	0·46	0·31
	Chloroplast substance	0·74	0·26	0·39	0·34
	Non-chloroplast fraction	0·21	0·44	0·50	0·29
K:	Whole leaf	1·82	2·05	3·26	2·54
	Chloroplast substance	*	*	*	*
	Non-chloroplast tissue	2·49	3·25	5·43	3·79
Na:	Whole leaf	0·29	0·21	0·67	0·39
	Chloroplast substance	0·04	0·04	0·25	0·02
	Non-chloroplast tissue	0·38	0·32	0·95	0·07
Cu:	Whole leaf	0·0038	0·0025	0·0087	0·0038
	Chloroplast substance	0·0081	0·0174	0·0160	0·0085
	Non-chloroplast fraction	0·0022	0·0017	0·0038	0·0009
Cl:	Whole leaf	0·10	0·61	—	0·62
	Chloroplast substance	*	0·07	—	0·05
	Non-chloroplast fraction	0·14	0·94	—	0·90
Mn:	Whole leaf	Trace	0·01	0·07	Trace
	Chloroplast substance	Trace	Trace	0·02	Trace
	Non-chloroplast fraction	—	—	0·10	—

* Presence doubtful—not detected by methods used.

Determinations of inorganic Fe and Cu showed that whilst most of the Fe in the chloroplasts is present in the inorganic form there is an appreciable amount which may be in organic combination, and that Cu is present in the chloroplasts largely in organic combination (Table VI).

Table VI. *Nature of the combination of Fe and Cu in the chloroplasts and whole leaf tissue of* Trifolium pratense

Fe (mg. per g. dry tissue):	Chloroplasts	Whole leaf
Total	0·772	0·479
In 10% acetic acid extract	0·572	0·292
Inorganic Fe (% of total Fe)	74·1	61·0

% of the Fe in the whole leaf which is contained in the chloroplast fraction: (*a*) inorganic, 51·7%; (*b*) organic, 57·5%.

Cu (mg. per g. dry tissue):		
Total	0·081	0·038
In 10% acetic acid extract	Nil	0·004
Inorganic Cu (% of total Cu)	—	10·5

% of the total Cu in the whole leaf which is contained in the chloroplast fraction: 74·6%.

B. *Ca and Mg* show a similar localization, being found mainly in the cytoplasm, although the chloroplasts do contain appreciable amounts of these elements. This distribution is interesting from the viewpoint of Laville [1933] who believes that Mg antagonizes Fe by its antioxidant activity; the two elements show opposite localizations in the leaf thus allowing each other greater freedom of action.

C. *P*, relative to the other constituents of the ash, is concentrated in the chloroplasts; the degree of concentration, however, is variable. Granick [1937] has recorded that 20 % of the total P and 40 % of the lipoid P in tomato leaves is contained in the chloroplasts. This does not show an accumulation of P in the chloroplasts unless the chloroplast content of this leaf is very small. That the P content of the chloroplasts of *Trifolium pratense* may vary widely is shown from the two results given in Table I, which represent the P contents of the chloroplasts of the young growing plant and of the flowering plant respectively. According to Seissl [1909] the P content of the leaves is at a maximum during the period of greatest CO_2 assimilation.

D. *Na and K* are concentrated outside of the chloroplast; K is probably absent from the chloroplast. These observations are in agreement with the findings of Weevers [1913] and Lloyd [1925], by histochemical tests, that K was localized in the vacuoles and of Funcoka [1921] that Na, by similar tests, is localized in the cell plasma.

E. *Mn* appears to be present in both the chloroplasts and non-chloroplast material of the leaf and particularly in the latter. In some cases there is only a trace of Mn present.

F. *SO_4 and Cl* show a variable distribution in the leaves of different species. SO_4 in particular shows no evidence of a definite localization, whereas Cl appears to be concentrated outside the chloroplast, presumably as a result of the high concentration of potassium in the cell sap (see Table IV).

G. *NH_4 salts* appear to be more highly concentrated in the chloroplasts, whereas nitrates, like sulphates, do not show any definite plan of localization.

H. *Ascorbic acid* appears to be present in appreciable quantities both in the chloroplasts and in the other parts of the cell. This is in accord with the findings, by histochemical tests of Giroud *et al.* [1934; 1935] and of Dischendorfer [1936] who conclude further, that there is a positive correlation between the amount of chlorophyll and ascorbic acid present and that the latter is bound in the chloroplasts.

I. *Catalase and carbonic anhydrase* are found in the chloroplasts, the former being highly concentrated in the chloroplasts, in all of the species. Carbonic anhydrase is also present in the other parts of the leaf in amounts varying with the species. The values for the whole leaf tissue are a little too low from the fact that it was difficult to rupture all the cells by grinding. This explains why the values in Table VII show a higher localization of catalase in the chloroplasts than is theoretically possible. However, at least 80–90 % of the cells should be ruptured since 10 g. of the material were ground as thoroughly as possible in a large porcelain mortar. Von Euler and his associates [1929; 1930] have found a relationship between the chlorophyll content and catalase activity, and between the number of chloroplasts present and catalase activity, which appeared to be hereditary.

It is interesting to note that Fe, Cu and catalase, which are concentrated in the chloroplasts, are oxidative catalysts. This would indicate that active respiratory processes occur in the chloroplasts.

Table VII. *Showing the distribution of carbonic anhydrase and catalase in leaf tissue*

	Trifolium pratense		*Arctium minus*		*Onoclea sensibilis*	
	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase
Activity of chloroplast* substance	13·89	7·06	57·90	12·12	50·04	13·63
Activity of whole leaf*	15·37	1·78	24·84	3·83	38·22	4·78
Activity of whole leaf due to chloroplasts (calculated)	3·75	1·90	19·30	4·30	18·51	5·04
% of total activity of leaf which is due to chloroplasts	24·39	106·74	77·69	112·27	48·43	105·43

* Carbonic anhydrase activities are represented in μl. CO_2 released per mg. of dry tissue in 30 sec. at 27°.

Catalase activities are represented in μl. O_2 released from 0·01 *M* H_2O_2 per mg. of dry tissue in 2 min. at 27°.

Summary

Chloroplasts consist chiefly of protein and lipins. They contain a relatively high percentage of lipins as compared with the rest of the cell. Nearly all the lipin fraction may be extracted with 85 % acetone.

Cu, Fe, P and NH_4 salts are concentrated to a certain extent in the chloroplasts. Ca, Mg, Mn, Na, K and Cl show an opposite localization in the cell. SO_4 and NO_3 do not follow any general rule.

The Cu in chloroplasts appears to exist chiefly in organic combination. Part of the Fe and P is also combined organically but Ca and Mg are present chiefly in the inorganic state.

Most of the catalase in the leaf cells is found in the chloroplasts. Carbonic anhydrase and ascorbic acid are found in appreciable quantities both in the chloroplasts and in other parts of the cells.

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