XXXII. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

I. PREPARATION AND GENERAL CHARACTERS.

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IN a previous paper [Chibnall and Grover, 1926], there has been described the preparation in bulk of the cytoplasm from green leaves; in that paper it was shown that the cytoplasm consists chiefly of proteins and substances soluble in fat solvents and the methods of preparation and the properties of these proteins were discussed. As part of this cytoplasmic protein appeared to be in some state of physical or chemical combination with substances of fatty nature, it was deemed wise to attempt to study the nature of the ether-soluble substances of cytoplasm before embarking on more advanced studies of the proteins themselves.

A review of the existing literature at once discloses the rather surprising fact that although numerous references to the isolation of individual substances of fatty nature abound, no thorough investigation of the fatty materials present in the leaf has been attempted. This is probably due to the fact that the extraction of the fatty material from fresh leaves—in which both the cell wall and cytoplasm are heavily imbibed with water—is attended with considerable difficulties. In such work on the plant as has been reported, the seed or young seedlings appear to have been studied chiefly, and the method usually adopted has been to extract with boiling alcohol, evaporate the extract to dryness and to treat the residue obtained with ether. This method cannot be satisfactory when applied to fresh leaf material in bulk. In the first place, the extraction of say 20 kg. of fresh leaves would require a prohibitive amount of alcohol; secondly, the dilution of the alcohol by the water in the leaf is sufficient to cause the solution of a certain amount of the sugars, water-soluble nitrogenous substances and inorganic salts which have to be subsequently removed; thirdly, substances insoluble in alcohol but soluble in ether may be present, and this entails subsequent exhaustive extraction of the bulky leaf material with ether.

Alternatively the leaf material has been dried and ground to a very fine

powder. Although this process results in a much less bulky product and one which can be directly treated with fat solvents, there are objections to it, for the drying of large quantities of leaf material is necessarily slow, with the resulting danger of considerable alteration of the substances present due to enzyme action to an extent which we have no means of judging. Further the continual grinding, which must be carried out if the material is to be reduced to that very fine state of division which is so necessary for successful extraction, may result in a considerable degree of oxidation.

Even if the difficulties of dealing with moderately large quantities of material are overcome, there still remains the fact that the yield of fatty substances from green leaves is, in general, very small in terms of the weight of the fresh leaf taken, even though the fat constitutes no inconsiderable proportion of the total leaf solids. Thus during the investigation reported in this paper, upwards of 200 kg. of fresh leaves have been used, and yet as will be seen later, we have been limited throughout the work for want of sufficient fatty material. These considerations are probably sufficient to explain the paucity of information available on the chemistry of the leaf fats.

The problem of obtaining sufficient material is thus one of devising means whereby very large quantities of leaf material can be reduced rapidly to small bulk in a form which can be directly extracted with fat solvents without the necessity of preliminary drying.

GENERAL EXPERIMENTAL METHODS.

The present paper deals not with the ether-soluble substances of the total leaf but only with those which appear to be part of the colloidal complex which constitutes the cytoplasm of the leaf cells. In previous papers methods of obtaining this cytoplasm free from cell-wall material, and the preparation of proteins from it have been described [Chibnall and Grover, 1926]. In those papers it was considered justifiable to give to these proteins the generic name of cytoplasmic proteins. This terminology neglects the nucleus. However, in the mature leaf-cell the mass of the nucleus is very small compared to that of the cytoplasm. Therefore it may be well if we emphasise here the fact that the proteins were obtained in such quantity as to make it quite definite that they must be derived in bulk from the cytoplasm itself and not from the nucleus. Similarly in the present work we have found no single ether-soluble substance which can definitely be assigned to the nucleus, for the methods available for the isolation of the individual constituents of any complex fatty material are such that unless a very large quantity can be used it is a matter of considerable difficulty to obtain them in a pure state unless they are present to the extent of 5 % or more. We have thus been compelled to confine our attention to those constituents which are present in amounts too large for them to be present in the nucleus only, and therefore just as in the work on the proteins, we may regard the ether-soluble substances with which we are dealing as forming part of the cytoplasm.

The aim in this work has been to study as quantitatively as possible the ether-soluble extractives of the cytoplasm of one leaf only in order that the knowledge so obtained may be of possible use later in a similar study with small preparations from different species of leaves. With small quantities of material, little can be done save to determine the usual fat constants, and the results of the investigation into a larger quantity of similar fat will help to make the interpretation of those constants more of a probability and less of a gamble. The green leaves of headed cabbage—*Brassica oleracea*—were chosen for this work because they can be obtained throughout the year. Further, in this initial study, it has been desired to enquire in greater detail into the nature of the phosphorus-containing substances soluble in ether, and as preliminary investigation into the fats of a number of species of leaves seemed to show that the cabbage leaf was richer in ether-soluble phosphorus than the leaves of the other species which were used, that leaf was finally chosen.

Details of preparation.

After the separation of the leaves and the removal of their thick mid-ribs, the material was coarsely minced, mixed with an equal volume of distilled water, then finely minced and finally squeezed through fine silk. The residue of cellular material, after being treated with water, was again minced and squeezed out as before. Previous experimental work has shown that this deep green solution contains most of the vacuole constituents and part of the cytoplasm, the latter being dispersed in colloidal form, and that it does not contain any cell-wall material. The green juice is then heated to 70° when the cytoplasmic material is coagulated, and on cooling as rapidly as possible it settles as a green amorphous mass, leaving a clear brown fluid which is easily filtered through folded filters. The coagulated cytoplasm was enveloped in stout filter cloth, placed in a Buchner press and submitted to a pressure which was slowly increased to 400 kg. per sq. cm. and maintained until no more liquid was expressed. The product thus obtained was a dark green hard mass which could be readily powdered in a mortar in spite of the fact that analysis showed that it still contained about 40 % water. Such material is in a form which can be directly extracted with ether.

Prepared by this method the crude mass of cytoplasmic material will contain also those substances present in the vacuole fluid which are precipitable by heat. Previous experimental work [Chibnall, 1923] has shown that these are present in relatively small amounts, and consist of protein and inorganic material. Consequently, as the ether-soluble substances of the cytoplasm and not the proteins are now under investigation, these latter may be disregarded. Further, water-soluble substances from the vacuole will be present in the water still retained by the preparation, but, since the final solvent used for extraction of the fat is anhydrous ether, the presence of these substances will not affect the composition of the material obtained. A more

refined method of preparing cytoplasm has been already described [Chibnall and Grover, 1926], but on account of its more lengthy nature it could not be used in the present research. The essential point was to obtain the ether-soluble substances of the cytoplasm in large quantities, and the presence of vacuole protein and salts will not affect the question.

The material was extracted with ether in a Soxhlet apparatus with frequent changing of the receiving flasks, until the final extract was virtually colourless.

It was considered wise to make a study of the rate at which the fat was removed by ether, and the following table shows the weights of fat obtained during the various stages of a typical extraction.

Table I. *Showing the rate of extraction of the ether-soluble substances from 149 g. of cabbage leaf cytoplasm.*

Batch F, 9.5 kg. of leaves picked May 20, 1926.

Number of extract	Duration of extraction hours	Weight of extract g.
1	16	17.0
2	24	0.6
3	24	0.15
After regrinding of the powder:		
4	24	0.23
5	24	0.02

Schulze and Steiger [1889] reported that the phospholipins can only be completely removed from seed material if the ether extraction is followed by successive extractions with alcohol at 60°. It became of interest to see whether this finding applied also to the cytoplasmic phospholipins of the leaf. Accordingly after the exhaustive extraction with ether tabulated above, the residue was treated three times with large volumes of alcohol maintained at 60° and renewed every half hour. The combined alcohol extracts were evaporated to dryness at low pressure, and the residue treated with anhydrous ether. The total weight of this extract was 0.88 g. Finally, to ensure that there was no appreciable amount of fatty material left in the residual powder, the whole of the latter was hydrolysed for 3 hours with *N* HCl on a boiling water-bath. The mass was filtered and the residue washed several times with alcohol and then with ether. The alcohol-ether washings were combined with the ether extract of the filtrate and after thorough washing with water to remove alcohol, were evaporated to dryness. The weight of fatty acid so obtained was 0.69 g.

It will be seen that the extraction by ether is virtually completed at the end of 40 hours, when an extract weighing 17.6 g. has been obtained. Subsequent treatment of the residue with alcohol yielded 0.88 g. and hydrolysis 0.69 g. We therefore adopted ether extraction for 40 hours as our routine method, and the relatively small amount of material which can be obtained by subsequent treatment with alcohol and by hydrolysis has been ignored in this paper. It may be mentioned in passing that the alcohol extract does

not yield any quantity of phospholipin as Schulze showed to be the case in seed material.

The ether extracts contained a little water which had been removed by the ether from the coagulum. It has been our experience that the presence of water in such a solvent as ether will allow a small but definite amount of inorganic material and amino-compounds to go into solution. Since subsequent work on the extract was carried out with a view to determining the nature of the phosphorus-containing substances present, and since the percentage of nitrogen in those bodies is very low, it was considered essential to evaporate every crude extract to dryness *in vacuo* and make an anhydrous ether extract from it¹. There always remained behind a small amount of material undissolved by that solvent, and this material was rejected.

Preliminary analysis of the extracted cytoplasm.

Tables II and III give details of the various samples of cytoplasm prepared. The method employed for the preparation of the cytoplasm in bulk is such that much of the cytoplasm is left behind in the cell residues. The amount actually obtained is dependent on the thoroughness with which the leaves were ground: only those cells which have been torn open will have their cytoplasm dispersed into colloidal solution. Some information may be obtained regarding the percentage of cytoplasm extracted by a determination of the protein content of the original leaf and of the cytoplasmic coagulum respectively. If such a rough method of calculation be adopted, it is only with the knowledge that it may involve an error of 5 %.

Table II. *Details of cytoplasm prepared from green leaves of cabbage.*

(Batch E, 20 kg. of leaves picked April, 1926.)		
Total leaf solids		2550 g.
„ N		67 g.
„ water-soluble N		23.9 g.
„ protein-N		43.1 g.
„ protein (N $\times$ 6.25)		269.3 g.
„ cytoplasm (calculated)		405.1 g.
Percentage of total cytoplasm extracted		47 %
Extracted cytoplasm		
	g.	% total leaf solids
Protein (N $\times$ 6.25)	129	5.73
Ether-soluble substances	39	1.73
Ash	12	0.53
Undetermined	14	0.62
	194	8.61

From Table III it will be seen that the percentages of protein and ether-soluble substances in the various preparations are very constant for biological material. Further the protein : ether-soluble substances ratio is always about 3 : 1. These concordant figures are good evidence that the samples of material with which we are dealing are truly representative of the cytoplasm as a

¹ In this and subsequent papers the expression anhydrous ether means “0.720 ether” dehydrated by sodium.

whole. Thus, to consider extreme cases, in batches D and H, where the percentage of cytoplasm extracted was 41 and 28 respectively, the ratio is the same. Further evidence as to the uniformity of the different samples will be obtained by a study of the figures in Table IV which deal with the composition of the ether-soluble substances.

Table III. *Analyses of cytoplasm prepared from several different batches of green leaves of cabbage.*

	Autumn sown picked April and May				Spring sown picked August	
	D 20	E 20	F* 9.5	G 9.0	H 32	M 50
Weight of fresh leaves, kg.						
	g.	g.	g.	g.	g.	g.
Weight of cytoplasm extracted	167.6	194.4	79.6	70.6	194.0	389.2
Protein ($N \times 6.25$)	110.3	129.0	50.9	44.1	122.0	223.6
Ether-soluble substances	35.4	39.0	18.0	14.2	40.0	75.4
Ash	9.5	12.4	2.7	3.8	14.2	40.7
Undetermined	12.4	14.0	8.0	8.5	17.8	49.5
Ratio protein/ether-soluble substances	3.1	3.3	2.83	3.1	3.1	3.0
Composition of cytoplasm:	%	%	%	%	%	%
Protein ($N \times 6.25$)	65.8	66.4	63.9	62.4	62.9	57.2
Ether-soluble substances	21.8	20.1	22.6	20.1	20.6	19.3
Ash	5.7	6.4	3.4	5.4	7.3	10.5
Undetermined	7.4	7.1	10.1	10.1	9.2	13.0
Percentage of total cytoplasm extracted	41	47	39	36	28	32

* Exhaustive ether extraction.

A point worthy of notice is that the ether-soluble substances constitute one-fifth by weight of the total cytoplasmic solids. The ether extract on evaporation to dryness yielded a solid fat deeply pigmented with chlorophyll. We should have preferred to have removed the chlorophyll from the coagulum before the extraction of the fat. Since it was desired to obtain the fat in a state as little altered as possible from that in which it occurred in the leaf, the use of chemical methods of separating the chlorophyll was rendered impossible. Though many attempts were made to extract the pigment by the use of solvents, these proved fruitless, because all those solvents which will remove chlorophyll will dissolve some part of the fat. Therefore, in order to keep the work as quantitative as possible, the chlorophyll and fat were weighed together. The presence of this chlorophyll has necessarily modified the constants which would have been obtained had the fat been chlorophyll-free. Evidence will be adduced in a later paper, however, to show that the difficulties caused by the presence of the pigment can be overcome, and an approximate estimate of its amount and its effect on various determinations made.

The figures given in Table IV define the general characteristics of the ether-soluble substances. The percentage of material given under I was calculated by weighing the precipitate obtained when a solution of these substances in

dry ether was treated with 4 volumes of acetone. This high percentage figure for acetone-insoluble material does not represent phospholipins, as might be expected. It will be shown subsequently that not more than half of the weight of this fraction is due to substances containing phosphorus, and that these substances are nitrogen-free and therefore not phospholipins in the strict chemical sense. The other components of this fraction, which do not contain phosphorus, consist of one or more hydrocarbons and alcohols. The acetone treatment was found to remove all the phosphorus-containing substances and the figures given for the percentage of phosphorus-free material in II are obtained by difference.

The filtrate from I was evaporated to dryness *in vacuo*; on this material determinations of iodine value (Wijs) and saponification value were carried out. Attention is drawn to the very high iodine value and to the fact that the low saponification value is due not to the presence of fatty acids of very high molecular weight, but to the large percentage of unsaponifiable matter.

Table IV. *Showing the general characters of the ether-soluble substances of the cytoplasm.*

(All weights given are percentages of total ether extractives.)

	D	E	F	K
I. Weight of material precipitated by acetone	41.5	42.0	41.0	44.0
II. Weight of phosphorus-free fatty material	48.5	58.0	59.0	56.0
Iodine value (Wijs)	141	147	152	154
Saponification value	151	158	152	149
III. Weight of unsaponifiable matter	16.9	16.5	17.0	24.1
Iodine value (Wijs)	113	117	119	105
Weight of sterol	3.63	2.65	3.70	3.80
IV. Weight of fatty acids	23.2	23.1	23.0	15.0
Iodine value (Wijs)	173.5	203.5	201	206
Neutralisation value	209	206	198	197

The unsaponifiable matter was determined by the method already described [Drummond, Channon and Coward, 1925], the second saponification being carried out with sodium ethylate in alcohol, which is particularly necessary in dealing with plant materials containing definite quantities of the esters of the higher alcohols. The sterol content was determined by the digitonin method [Windaus, 1910].

The fatty acids were prepared after the removal of the unsaponifiable matter and were rendered free from chlorophyll degradation products.

The figures given in Table IV indicate how very constant the various values obtained from different batches of materials are. This confirms the results recorded in the previous tables and suggests that the fraction of cytoplasm obtainable is quite representative of the whole.

SUMMARY.

The difficulties of preparing the ether-soluble substances of leaves are discussed. A method is given for preparing in bulk the ether-soluble substances of leaf cytoplasm, and the fat constants are given for several preparations made from cabbage leaves. The general uniformity of the results obtained is taken as evidence that the portion of cytoplasm extracted for analysis is representative of the whole.

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