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

VI. SUMMARY AND GENERAL CONCLUSIONS.

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In a series of papers [Chibnall and Channon, 1927, 1, 2, 3; Channon and Chibnall, 1927], we have recorded certain results which we have obtained in the analysis of the ether extract of the leaf-cell cytoplasm of the cabbage. The object of the present paper is to discuss these results so far as is at present possible from their chemical and physiological aspects, and, in passing, to refer to our investigations of the pigments and of the unsaturated portion of the unsaponifiable fraction of which no account has yet been given.

For simplicity in presentation, we propose to confine all further remarks on this investigation to batch *R*, consisting of 220 g. of ether extract prepared from 220 kg. of cabbage leaves. By methods already described, this extract was separated into the following fractions.

A. The fraction precipitated by acetone, which was further subdivided into
   - I. The fraction insoluble in hot acetone, wt. 57·8 g.
   - II. The fraction soluble in hot acetone, ,, 35·0 g.

B. The fraction soluble in acetone-ether, ,, 119·9 g.

   Saponification of an aliquot part showed that this fraction contained
   - III. Fatty acids soluble in light petroleum, wt. 38·3 g.
   - IV. Unsaponifiable matter ,, 48·6 g.
   - V. Acidic products insoluble in light petroleum, consisting of chlorophyll derivatives and possibly hydroxy-acids.

As a preliminary to the further study of fractions (IV) and (V) it was necessary to determine the chlorophyll content of the original ether extract.

Determination of chlorophyll and carotenoids.

Extensive investigations have been made by Willstätter and his colleagues [Willstätter and Stoll, 1913] into the chlorophyll content of leaves; we have therefore thought it sufficient to determine the total chlorophyll content only, and not the α- and β-chlorophyll separately. Willstätter's method is to shake the ether extract with methyl alcoholic potash and to compare the green colour of the resulting phyllins with that given by a specimen of pure chlorophyll submitted to similar treatment. This method was applied to the

ether extract of the cabbage but the green tint of the resulting phyllins did not match that given by a specimen of pure chlorophyll. This suggested that during the coagulation of the cytoplasm by heat part of the magnesium had been removed from the molecule. Accordingly the ethereal solutions of the cabbage extract and of pure chlorophyll were first shaken with an aqueous solution of oxalic acid to remove the magnesium and so convert the chlorophyll to phaeophytin. The resulting solutions of phaeophytin were then submitted to Willstätter's treatment with methyl alcoholic potash and an aqueous solution of the complex potassium salts was prepared. The green tints of these were easily comparable and subsequent calculation showed that the chlorophyll content of the acetone-ether fraction B of batch *R* was 9·3 %.

The carotenoids were determined at the same time by Willstätter's method. The acetone-ether fraction B contained 0·86 % carotene and 1·39 % xanthophyll, giving a ratio $\frac{\text{carotene}}{\text{xanthophyll}} = 0{\cdot}62$, which is of the same order as that found by Willstätter for the pigments of the whole leaf. On the other hand, the ratio $\frac{\text{chlorophyll}}{\text{carotene} + \text{xanthophyll}}$ is 7·7, which is about twice that found by Willstätter for whole leaves. Two explanations of this high value suggest themselves: (*a*) these pigments are not located solely in the leaf cell cytoplasm; (*b*) the colours of the pigments had faded somewhat before they were quantitatively determined.

Unsaturated unsaponifiable matter (Fraction IV).

106·2 g. of the acetone-ether filtrate B yielded 42·5 g. of unsaponifiable matter, a brown gummy mass having iodine value 105 and acetyl value 168, and containing 21·8 % of sterol precipitated by digitonin. Present in this fraction also are the lipochrome pigments, *i.e.* 0·92 g. carotene and 1·48 g. xanthophyll, together with 6·13 g. phytol derived from the chlorophyll and present in the extract. The fraction contained in addition that portion of the hydrocarbon fraction which remained in solution when the original ether extract was precipitated with acetone. It is clear that the effective fractionation of a mixture of substances in the presence of higher alcohols and sterols having various similar solubility properties is no easy matter. Repeated attempts to obtain pure fractions by crystallisation from a variety of solvents left us convinced that we were obtaining products contaminated by the hydrocarbon, which we were unable to remove by this method. It was decided, therefore, to submit the material to fractional distillation *in vacuo*; a liquid fraction B.P. 175° to 185°, and a small fraction B.P. 222° to 240° at 2 mm. pressure were removed in this way. The first fraction by careful purification was shown to consist mainly of phytol, whilst the second was crude hydrocarbon (M.P. 63°–65°). Attempts to distil the remainder, however, resulted in marked decomposition, as was shown by the nature of the products obtained and by the fact that the residue failed to give any precipitate with digitonin. From this distillate there were isolated white crystalline products

of various melting points, the highest observed being 129°. This latter fraction was shown by the digitonin method to contain only 82 % of sterol. In view of these difficulties and the lack of sufficient material for a large scale fractionation, we were reluctantly compelled to leave over the question of the nature of these higher alcohols and sterols until a larger amount of material was available.

General summary.

We now propose to discuss what we consider the essential results of these researches in both their chemical and physiological aspects.

The following table shows the results which we have obtained in the analysis of a typical sample of cabbage-leaf cell cytoplasm. It is admittedly incomplete in some respects and must remain so until material can be obtained in still larger bulk. During the course of this work some 10 cwts. of cabbage have been used and a description of some of the difficulties and uncertainties which we have encountered in what appears to be the first attempt to study such an ether extract is given in the footnotes to the table for the guidance of any who may wish to extend these results.

The methods used in the separation of the various fractions have already been discussed.

Table I. *Summary of the ether-soluble substances of cabbage-leaf cytoplasm.*

(Batch *R*, consisting of 220 g. of material.)

	Wt. g.	% of total material
Pigments		
Chlorophyll (α and β)	20·5	9·3
Carotene	1·1	0·5
Xanthophyll	1·7	0·8
Substances containing phosphorus		
Calcium phosphatidate	40·6	18·4
Unidentified calcium salts, possibly of fatty acids and phosphoric acid	11·3	5·0
Unidentified iron compound	6·7	3·0
Glycerides and waxes		
Containing palmitic, stearic, linolic and linolenic acids	38·3	17·5
Glycerol	2·8	1·3
Unsaponifiable matter		
Saturated fraction, chiefly nonacosane and di-*n*-tetradecyl ketone	27·0	12·3
Unsaturated fraction		
Sterols (by digitonin)	9·8	4·5
Unidentified products, probably alcohols and hydrocarbons	29·2	13·3
	189·0	85·9

Note 1. *Cause of losses.* It will be observed that the percentage total of the fractions is only 85·9 and that there has been an apparent loss of 14·1 %. In considering these losses it may be pointed out, firstly, that about one-third of this loss is entailed by the use of methods necessary for purifying the hydrocarbon fraction (36 g. of crude product gave only 23 g. of purified product), and, secondly, the large number of manipulations in obtaining the various fractions has of necessity caused considerable mechanical losses, which cannot be avoided when working with fatty materials. Even so, there

remains the probability that about 5 % of the total material is unaccounted for, and a similar loss has been encountered in every batch. As to the reasons for it, we have little information. It always occurs as the result of the separation of the acetone-ether-soluble fraction into fatty acids, unsaponifiable matter and chlorophyll degradation products, and the material remains in the aqueous phase after extraction of the fatty acids. This fact suggests that there may be present an ether-soluble substance which is destroyed on treatment with alcoholic alkali, or alternatively that acidification of the soap solution after the removal of the unsaponifiable fraction has resulted in the precipitation not only of fatty acids and acidic chlorophyll products, but also of other acids sparingly soluble in ether. Although the acidified solution was extracted many times with ether, it was found necessary that the aqueous solution should be very dilute in order to facilitate extraction, and hence any substance, soluble to a limited extent only in ether and water, would remain to some extent in the aqueous phase. Such substances might be hydroxy-acids.

Note 2. *Unidentified iron compound.* As we mentioned in an earlier paper, only about 1 g. of this curious substance was isolated. (Found C = 52·4 %, H = 8·1 %, P = 5·3 %, Fe = 8·9 %. Nitrogen is absent.) We are not yet in a position to give any further information. In the table the gross amount of this substance has been computed from the iron content of the calcium phosphatidate. This is greatly in excess of the amount actually isolated, but it must be remembered that we treated this material as an incidental impurity and removed it whenever possible when purifying the calcium phosphatidate.

Note 3. *Residual ether-soluble phosphorus.* The ether-soluble phosphorus present as calcium phosphatidate represents about 60 % of the whole. In a previous paper [Chibnall and Channon, 1927, 2] it was suggested that the remainder was present as the calcium salt of an easily hydrolysable monoglyceridephosphoric acid, because on shaking the ether-soluble phosphorus compounds in ether solution with mineral acid part of the phosphorus passed into the aqueous layer and fatty acids appeared in the ether solution. Our subsequent investigations have tended to weaken this view for two reasons. Firstly, the iron compound which contains 5 % of phosphorus and no calcium represents about one-tenth of the ether-soluble phosphorus fraction. Since the ratio of calcium to phosphorus in this fraction was originally unity, it is clear that there must have been present some calcium which was not in combination with phosphorus and that the unity ratio was merely fortuitous. Secondly, shaking the original ether-soluble phosphorus solution with mineral acid removed part of the phosphorus as phosphoric acid and not, as we had thought, as glycerophosphoric acid. Further, determinations of glycerol on the phosphatide fraction have shown that the amount of glycerol in the fraction is no more than is required by the calcium phosphatidate present. Hence the fatty acids which appeared in the ether layer cannot have originated as the result of the hydrolysis of a monoglyceridephosphoric acid. We have

obtained no further information as to the chemical nature of the compounds giving rise to the phosphoric acid and fatty acids on shaking with mineral acid; and this question remains therefore undecided at present. We incline to the view that the sources of these substances are calcium soaps and calcium phosphate, and that only 60 % of the ether-soluble phosphorus represents phosphatides.

Note 4. *Glycerides and waxes.* Estimations of glycerol by the methods of Zeisel and Fanto [1903] on the acetone-ether-soluble material of batch *R* gave a ratio of $\frac{\text{fatty acids}}{\text{glycerol}} = 7{\cdot}8$, slightly higher than that recorded for batches *G* and *M* in a previous paper. This method in our hands has given a theoretical yield of glycerol from barium glycerophosphate, but we have recently found that it gives very low yields from distearin and tristearin. The method of Fanto [1904] was found to give a theoretical yield with tristearin, and applied to batch *R* gave a ratio $\frac{\text{fatty acids}}{\text{glycerol}} = 13$. The original method, which has the advantage of requiring only 0·2 g. of material, is clearly inapplicable to these complex plant extracts, possibly on account of the unidentified substances mentioned under note 1 above. Tristearin gives a ratio of $\frac{\text{fatty acids}}{\text{glycerol}} = 9{\cdot}3$, so that about one-third of the fatty acids present in the acetone-ether-soluble material of batch *R* was present as substances other than glycerides, probably as esters of higher alcohols.

General discussion.

The yield of the chief constituents.

By methods which were referred to in the first paper of this series [Chibnall and Channon, 1927, 1] we calculate that the total amount of ether-soluble material in cabbage-leaf cytoplasm is about 3·5 % of the total leaf solids. This figure may not represent the total amount of these substances present in the leaf, because our preparations of coagulated cytoplasm were free from cell-wall material which, in consequence, has not been ether-extracted. The variations which we have found in the amounts of the chief constituents are given in Table II.

Table II. *Ether-soluble substances. Variation in chief constituents.*

	% of total ether-soluble material	Estimated % of total leaf solids
Total ether-soluble substances	—	3·5
Calcium phosphatidate	9–18	0·3–0·6
Fatty acids in glycerides and waxes	19–27	0·6–0·9
Saturated hydrocarbons and ketones	8–13	0·3–0·5
Other unsaponifiable material	16–25	0·6–0·9

As the presence of fat-soluble substances containing calcium and iron has not been demonstrated hitherto, it appeared to us to be of interest to calculate roughly what proportions of calcium, phosphorus and iron of the cabbage

leaf are present in the form of calcium phosphatidate and the iron compound. The results of our analyses of the cabbage leaf for these inorganic materials are given in Table III.

Table III. *Distribution of Ca, Fe and P in the cabbage leaf,* (% *of total leaf ash*).

	Whole leaf	Water-soluble	Water-insoluble	Ether-soluble
CaO	12·6	8·0	4·6	0·45
Fe_2O_3	12·9	—	2·9	0·15
P_2O_5	16·0	11·8	4·2	0·73

Water-soluble iron was too small to be determined gravimetrically as Fe_2O_3. Hoagland and Davies [1923] in a discussion of the cell sap of higher plants emphasise the fact that for a proper understanding of the metabolism of such plants it is necessary to gain some insight into the forms in which inorganic elements are held by the plant. They have analysed different plants (species not given) at various stages of growth for water-soluble inorganic elements, and have found that in practically every case all of the potassium is soluble in water as well as the major portion of the other elements, with a few exceptions in the case of calcium. We determined total ash and ether-soluble ash on samples of 300 g. of fresh leaves. As the ether-soluble calcium, iron and phosphorus are computed from figures obtained from the analysis of 220 kg. of fresh leaves, it is obvious that the two sets of figures given in the table are not strictly comparable. They show, however, that about one-twentieth of the total leaf calcium, phosphorus and iron is ether-soluble, and that about one-tenth of the water-insoluble calcium can be ascribed to calcium phosphatidate.

Phosphatides.

One of the most interesting results of these researches has been the discovery of the existence in the cytoplasm of calcium phosphatidate, and experiments are clearly necessary to determine its relation to the glycerides. Thus in a plant which is being starved, does utilisation of this phosphatide and of the glycerides take place? This question of the utilisation of fat by a starving plant is one which has suffered severe neglect because the ratio of carbohydrate to fat was thought to be so high as to make it appear that a study of fat from the tissue was a matter of minor importance. It will be of interest, therefore, to see whether utilisation of the fatty acids, either of the glycerides or of the phosphatide, occurs, and if there is a decrease in the fatty acids it will be necessary to determine whether the fatty acids utilised are those of the phosphatide or of the glycerides. One is naturally tempted to compare these substances with those present in animal tissue, and in this connection the high degree of unsaturation, not only of the phosphatide but also of the glycerides, is interesting. It will be remembered that the fat of adipose tissue is saturated relatively to that of such organs as the liver and that during

starvation this adipose fat is utilised, while that of the liver persists even if death from inanition occurs. The conclusion drawn from these results is that the liver fat, consisting of unsaturated phosphatide, cannot be utilised without causing destruction of the cells of which it is an integral part. Hence, if calcium phosphatidate is fulfilling in the leaf the rôle which lecithin and the other phosphatides play in the animal, we should expect that on starvation the plant would utilise the glycerides only and that the calcium phosphatidate would remain constant in amount. Experiments on this subject are in progress; it is clear that, if calcium phosphatidate is as vital a constituent of the leaf as lecithin is of the animal, it should be present in the leaves of all plants. This question has not been systematically investigated by us yet, but some experiments with spinach suggest that there is a definitely smaller percentage of calcium phosphatidate in spinach leaves than in those of the cabbage.

The literature on plant phosphatides, although extensive, is very unsatisfactory on account of the great difficulties encountered in freeing phosphatidic substances from sugars, amino-acids, etc., and it gives the impression that very little definite information as to the nature of plant phosphatides is available. However, the results of Levene on soya-bean meal show that lecithin is present in that material. This opens up the question as to the physiological relationship existing between the phosphatides of the seed and of the leaf, and it will be interesting to investigate whether such a plant as soya bean, having lecithin and cephalin in its seed, has the calcium or some other salt of phosphatidic acid in its leaves, and, if so, the reasons why storage products should differ from those of physiologically active tissue. Such a finding as the occurrence of different phosphatides in the leaves and seeds would be totally unexpected if we regard the phosphatide as an essential constituent of the cell. On the other hand, such a finding might be understood if the rôles of the two types of phosphatide were different either in their effects on permeability or as agents in the transport of fat. Any view which we can express on these subjects at the present time must be speculative, but the fat-solubility of the calcium salt of phosphatidic acid and the water-solubility of its sodium salt suggests that these phosphatides may be in part responsible for the alteration in cell permeability which can be brought about by changes in the proportion of sodium and calcium ions.

The fatty acids.

A point of interest regarding the fatty acids is that the iodine value of the glyceride fatty acids is about 200 compared with 137 of the fatty acids present in the phosphatide. This higher degree of unsaturation of the glyceride fatty acids is the reverse of the findings in the fats of animal tissues, for in the latter the fatty acids of the phosphatides are considerably more unsaturated than those present in the neutral fat.

As to the question of unsaturation, the suggestion of Leathes and Raper [1925] that the temperature at which fats are formed in plants is one of the

features which determine the degree of unsaturation of the fatty acids is of interest. Recently Terroine *et al.* [1927] and Pearson and Raper [1927] have shown that the degree of unsaturation of the fatty acids present in *Aspergillus niger* grown at temperatures varying from 17° to 35° decreases with a rise of temperature. If such a finding can be applied to a higher plant, it would be expected that there would be a variation in the degree of unsaturation of the fatty acids of autumn- and spring-sown cabbage. The former are picked in April-May and will be referred to as "winter-grown," while the spring-sown plants which are gathered in July-August will be referred to as "summer-grown." Actually, we have found no significant change in the degree of unsaturation of the fatty acids of the winter- and summer-grown cabbages, the extreme values being 195 and 206, the value for batch *D*, as shown in Table I of a previous paper of this series [Chibnall and Channon, 1927, 1], being invalidated by the fact that in the early preparations we had been unable to free the fatty acids completely from chlorophyll degradation products.

Table IV. *Comparison of glyceride fatty acids and crystalline crude hydrocarbon* (% *of total ether-soluble material*).

	Autumn-sown (picked April–May)		Spring-sown (picked July–August)		
	E	F	H	M	P
Wt. of crude hydrocarbon (*a*)	11·0	10·9	19·0	17·6	16·0
Wt. of glyceride fatty acids (*b*)	23·1	23·0	15·0	16·3	16·6
Iodine value of fatty acids	203	201	206	—	195
Calculated iodine value of (*a*) and (*b*) mixed	137	136	91	—	99

This finding leads us to wonder whether the hypothesis of Leathes and Raper regarding the variation of the iodine value of fatty acids with temperature may not need extension to include not only fatty acids but other fat-soluble substances present. It is to be remembered that the unsaponifiable matter of a leaf fat constitutes a large proportion (up to 40 %) of the whole fat and if variation in the liquidity with temperature is to be expected it seems possible to us that a variation of the degree of unsaturation of the unsaponifiable matter as well as of the fatty acids may be anticipated. Our reason for this is that the amounts of crude saturated hydrocarbon, M.P. 63°, present in our extracts have shown considerable variation with the season of picking, as is shown by Table IV, in which it is seen that 11 % of hydrocarbon is present in winter-grown cabbage, whereas between 16 % and 19 % is found in the summer-grown plants. At the same time, the percentage of fatty acid fell from 23 % to 16 % with no alteration in the iodine value. We admit that the unsaturated portion of the unsaponifiable matter may also require consideration in this connection, but we tend to regard the hydrocarbon as being concerned in the metabolism of fatty acids and being in its physical properties closely akin to the saturated acids. If this hypothesis be

accepted, namely, that the hydrocarbon fraction may be regarded from the point of view of liquidity as saturated fatty acid, calculation shows that the iodine value of the hydrocarbon-fatty acid mixture in the winter-grown cabbage fat is 136 as against 91 for the summer-grown cabbage. Whether this variation in the fat derived from cabbages grown in winter and summer respectively is significant must await further investigation.

We will conclude this paper by referring to a matter to which Leathes and Raper have already drawn attention, namely, the use of the word "fat" in biological chemistry. They have pointed out that much time and labour have been lost in researches in which attempts have been made to follow the metabolism of "fat" by the determination of the amount of ether-soluble material which can be extracted from tissues under various conditions, and they have emphasised the necessity of the determination of the amount of the fatty acids present in the ether extract rather than of the amount of the ether extract itself. Our experiences in this work on the ether extract of cabbage-leaf cell cytoplasm illustrate this point vividly, for the fatty acids make up only about 27 % of the material soluble in ether, and there are present not only calcium phosphatidate and an iron compound containing phosphorus, but also a higher paraffin and ketone, together with unidentified alcohols; in addition, the ether-soluble pigments are present in the extract. In biochemistry, it is frequently necessary, after drying a tissue suitably, to extract it with ether or some fat solvent in order to obtain one particular fat-soluble substance. Such an ether extract as in the present case contains so many and various materials, that it seems desirable to us that some word be adopted merely to denote such an ether extract irrespective of what it may contain. This seems to us more advantageous than the adoption of the word "lipide" which is used by Bloor [1925] to denote a variety of substances soluble in fat solvents and related to fatty acids as esters or potential esters, for nonacosane and di-*n*-tetradecyl ketone may be as intimately concerned in the metabolism of fatty acids as the higher alcohols which are included in such a classification.

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