XXXVII. ON THE EVIDENCE FOR PHOSPHATIDES IN THE EXTERNAL SURFACE OF THE PLANT PROTOPLAST.

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(Received January 6th, 1928.)

INTRODUCTION.

DURING recent years Hansteen Cranner [1910, 1914, 1919, 1922, 1925, 1926], and subsequently Grafe and his co-workers [1925, 1926, 1927], have published results of the examination of the substances leached into aqueous liquids from living parenchymatous plant tissues. On the assumption that such substances must come from the external surface of the protoplast, or from the cell wall, as the semipermeability of the protoplast is still maintained, these experiments have been widely quoted as evidence for the presence of phosphatides in the external protoplasmic layer or "plasma membrane." This conclusion does not necessarily follow and will be critically examined elsewhere. The present paper is confined to a brief record of a somewhat extensive series of such leaching experiments in which it has proved impossible to substantiate the presence of appreciable quantities of phosphatides in the aqueous extracts.

LEACHING EXPERIMENTS ON STORAGE TISSUES.

The substances obtained by leaching cut slices of Scotch seed potatoes, variety Sharpe's Express, were first examined. The detailed description of the procedure and examination of products will be omitted as it is substantially the same as that to be described later. As the result of the examination of the accumulated basic lead acetate precipitate (100 g.), and also the filtrates evaporated and freed from lead, derived from leaching 6-8 kg. of fresh, washed slices of tissue, a turbid aqueous extract was obtained which contained calcium, magnesium and potassium, and the anions chloride, phosphate, and carbonate, and only reduced Fehling's solution slightly, even after inversion with mineral acid. On warming, some coagulation occurred, and the coagulum contained both nitrogen and phosphorus, and gave biuret and xanthoproteic colour reactions. The turbid solution, enclosed in a parchment dialyser and dialysed against distilled water, yielded inside the dialyser a gelatinous solid which also contained nitrogen and phosphorus and was probably a globulin.

The total amount of crude ether-soluble fatty acid which could be obtained by sulphuric acid hydrolysis of 100 g. of the lead acetate precipitate was 0.5 g. By repeated crystallisation this yielded a small amount of a white, solid, fatty acid of low melting point, which was, however, inadequate for further investigation. No ether-soluble unsaponifiable material was obtained, but a small amount of ether-insoluble acid similar to that isolated by Rhodes [1925] from potato cork was noticed. In spite of a careful examination for nitrogen bases (other than the more complex amino-acids which might be obtained from protein hydrolysis) amongst the hydrolytic products of the lead acetate precipitate, no evidence could be obtained even of traces of choline or betaine. Similarly glycerol was not detected.

The evaporated, aqueous filtrates from the lead acetate precipitation were examined with the following results.

1. Very little phosphorus was present.
2. Negligible traces of fatty acids and no glycerol were found.
3. Choline was detected only microchemically as the crystalline periodide. Having due regard to the sensitiveness of this reagent and the large volume of extract from which this amount of choline had been obtained, this choline provides no evidence of the presence of a phosphatide but probably diffused as such from the cell sap.
4. The evaporated aqueous residues consisted of a thick syrup, which contained sugar, was very deliquescent, and, though readily soluble in water and moist alcohol, was insoluble in all dry organic solvents. This was obtained in a relatively large amount, and does not appear to be mentioned by Cranner or Grafe and Horvat [1925].

It is quite clear that the results obtained by leaching potato tissue do not confirm those of Cranner. The experiments of Grafe, however, were carried out with sugar beet tissue. In order to enable a strict comparison between our results and those of Grafe and Horvat [1925], leaching extracts from sugar beets were examined. Two methods of leaching were adopted. It was obvious that in the earlier experiments much of the turbidity of the solutions was due to bacterial activity, plate cultures of turbid extracts always revealing extensive contamination. Moreover, sterile leaching experiments on a small scale indicated that at room temperature the leaching liquid remained clear indefinitely, provided the conditions were aseptic. Hence the following method of leaching was adopted to supplement that of Cranner, and Grafe and Horvat.

The tissue, arranged in layers, was separated by "grids" of bent glass rod which fitted over the legs of an inverted glass tripod, contained in the leaching vessel, which consisted of an inverted bell jar with a gas-tight lid. Tubulures in the bell jar and lid were fitted with ground glass stoppers and tops. Air entering the apparatus did so through cotton wool plugs. Connections were made so that communication could be made at will with three storage vessels containing (1) boiled distilled water cooled out of contact with air, (2) alcoholic mercuric chloride solution, and (3) 0.25 % formaldehyde

solution. The apparatus was sterilised by being completely filled with alcoholic mercuric chloride, washed with boiled distilled water, and partially filled with 0.25 % formaldehyde solution. The tissue was quickly inserted "en bloc," the apparatus then being completely filled with 0.25 % formaldehyde, and emptied by siphoning back into the storage vessel by means of the water-pump. After thorough washing with boiled distilled water, leaching was allowed to proceed with daily changes of distilled water. This procedure reduced bacterial growth to a minimum, especially over the short periods (4 or 5 days) during which one sample of tissue was in use. It is to be noted, however, that the temperature of leaching was about 18° and since all air was excluded by recently boiled water the conditions were approximately anaerobic. Extracts were also collected by the method of Cranmer [1922] and Grafe and Horvat [1925], namely, immersion of well washed slices of tissue in single layers in distilled water contained in shallow trays at 20° for 24 hours. After this period the tissue and water were changed. The shallow tray method assures more efficient aeration, but involves greater bacterial contamination.

Utilising this leaching technique, extracts were collected from both red and sugar beetroot slices. These are entirely confirmatory and only the results with sugar beet will be described. A further check experiment, in which 150 g. of the dried lead acetate precipitate were obtained by leaching in a large glass tank, also corroborated the data given below. The method of lead acetate precipitation was adopted for critical purposes only, for it is not one which is usually employed in the isolation of phosphatides. The results obtained were verified by extraction of the aqueous leaching extract with ether. Using the same concentration of normal lead acetate adopted by Grafe and Horvat, the precipitation was effected directly without the preliminary concentration at 28° practised by these authors. In all cases both the lead acetate precipitate and filtrates were examined for the decomposition products of phosphatides.

Examination of products for phosphatides.

The hydrolytic agent applied to the washed, dried lead acetate precipitate was 5 % sulphuric acid, allowance being made for the lead content of the precipitate. It is clear that the system of fractionation utilised by Cranmer [1925] had not justified itself and it was not adopted either in these experiments or those of Grafe. The hydrolysis was effected by boiling under a reflux condenser for 6 hours. During this period carbon dioxide was evolved and the supernatant liquid became dark coloured. The residual lead sulphate, after being dried and extracted in a Soxhlet apparatus with alcohol and ether for fatty acids, gave an estimate of the lead content of the precipitate correct to about 1 %. The contents of the alcohol and ether extracts of the lead sulphate were united to the ether extract of the acid filtrate, and the saponifiable and unsaponifiable contents determined approximately by the usual

methods. Bases were precipitated from the ether-extracted filtrate, after hydrolysis, by means of phosphotungstic acid, also in 5 % sulphuric acid. Standard methods were utilised in the hydrolysis and fractionation of the phosphotungstates obtained [see Barger, 1914; Guggenheim, 1924]. These removed bases other than those of the simple choline or betaine type. Figures typical of several sets obtained are given in Table I, and for comparison those calculated from the formula of Grafe and Horvat for the lead acetate precipitate are cited.

Table I.

Leaching method	Weight of ppt. g.	PbSO ₄ g.	% Pb.	Fatty acid		Weight of phosphotungstates g.	% Choline	% Glycerophosphoric acid
				Weight in g.	%			
Approx. "sterile"	55	61	75.2	0.50	1	0.37	*	*
"Shallow" tray	20	21	71.2	0.12	0.6	1.0	*	*
Grafe and Horvat	—	—	36.10	—	47.0	—	5.3	15

* None detected.

The complete failure to detect choline and glycerophosphoric acid or glycerol, as hydrolytic products of the lead acetate precipitate, and the remarkably small amount of total fatty acids obtained as compared with that obtained by Grafe and Horvat, is a confirmation of the previous results on potato tissue.

The following analyses given in Table II seem to suggest that the bulk of the lead acetate precipitate is inorganic in nature.

Table II.

Leaching method	% N	% CO ₂	% Pb	% P
"Approx. sterile"	0.115	8.26	75.2	0.42
"Shallow tray"	0.47	9.3	71.2	—

Nitrogen estimated by Kjeldahl method; carbon dioxide in a modified Schrötter flask; phosphorus by the Pemberton-Neumann method.

Since lead and CO₂ account for 86 % of the total precipitate, not more than 10–13 % can be organic. Moreover, of the phosphorus content a considerable percentage is inorganically combined. The liquid obtained by leaching 40 g. of beetroot, in the form of discs, in 100 cc. of distilled water was analysed for phosphorus by the method of Stewart and Archibald [1925]. It was found that 25 cc. contained 0.000232 g. of phosphorus of which 0.000105 g. (or about 45 %) was inorganically combined. This would appear as lead phosphate in the precipitate obtained with normal lead acetate.

It would appear then, that the precipitate obtained by adding normal lead acetate to the aqueous extracts of living tissue collected by the two methods described does not contain appreciable amounts of phosphatide. This is in harmony with our previous results on potato tissue and contrary to Grafe and Horvat, whose procedure has been duplicated in the shallow tray method at 20°. The results given indicate that the molecular formula

derived by Grafe and Horvat cannot represent even an appreciable fraction of the precipitate examined. Moreover, the widespread use of lead acetate, as a general precipitant for various plant products, would indicate that the isolation of a pure chemical compound by its application to a liquid, which probably represents a very dilute solution of the more diffusible cell contents, is extremely improbable. The obscurity in the attachment of the four lead atoms also detracts from the value of this formula. Whilst the substance is described as a single chemical compound of constant composition, the lead atoms (amounting to 36 % of its weight) are stated to be "physically combined" [Grafe and Horvat, 1925, p. 464]. It is true that the preliminary concentration at 28° utilised by Grafe and Horvat may have decreased somewhat the carbon dioxide content of the solutions precipitated, but in view of the low temperature of evaporation, which is little higher than the leaching temperature and lower than that adopted by Cranner (30°), this will not materially effect the criticism that in our experiments the precipitate examined did not contain phosphatides, but consisted mainly of inorganic salts, with a small percentage of fatty acids and amino-acids and probably protein decomposition products. It is probable that a small organic residue, which is not fatty in nature, remains undescribed.

Examination of the filtrates from lead acetate precipitation.

Since no evidence of phosphatides could be detected in the precipitate it became of importance to examine the filtrate for phosphatides or their decomposition products.

The accumulated, concentrated filtrates from lead acetate precipitation of leaching extracts from sugar-beet were examined for fatty acids, bases and glycerol. Excess of lead was removed by sulphuric acid and the slightly acid, filtered liquid extracted with ether. Only negligible traces of ether-soluble material were obtained. Bases were precipitated in 5 % sulphuric acid by phosphotungstic acid by the usual method, and the bases regenerated and fractionated by the Schultze method for separation of choline and betaine. Since betaine is a diffusible constituent of the cell sap, it was obtained in appreciable quantity. Of the crystalline hydrochloride (M.P. 227° with decomp.) 0.2 g. was obtained in a state of purity and the crystalline gold salt (M.P. 240°) and picrate (M.P. 180°) prepared. The derivatives agreed in general properties with the respective betaine derivatives. The choline fraction was comparatively small. An alcoholic solution containing a hydrochloride, which yielded a crystalline gold salt, not due to betaine, and also gave the Florence crystal reaction attributed to choline, was obtained. As only microchemical reactions could be applied, it was not possible to state with certainty that choline was present, although this is probable.

After the separation of bases as phosphotungstates, excess of sulphuric and phosphotungstic acids were removed by baryta solution and excess of barium as barium carbonate. The concentrated filtrates were tested unsuccess-

fully for glycerol, but revealed the presence in relatively large amount of the deliquescent syrup, previously noticed in the experiments on potato tissue. In bulk, this appears to be the principal constituent of such leaching extracts, and, whilst some sugar is undoubtedly present, a fraction could be obtained, by repeated precipitation from dilute alcohol by acetone, in a form almost free from nitrogen, phosphorus and reducing sugar, being very soluble in water or moist alcohol, insoluble in all dry solvents and not yielding any crystalline or volatile derivative. No conclusion as to its nature can be drawn, but a somewhat similar product is described by Szent-Györgyi [1925] and has also been examined by Platt and Wormall [1926]. The previous investigators of leaching extracts do not describe this material.

The examination of the filtrates from lead acetate precipitation gives no indication that the leaching extract contained phosphatides, or even fatty acids, which are not precipitated by lead acetate.

In order to test the possibility that Grafe and Horvat might have obtained their results from tissue whose semi-permeability had been impaired, 19 g. of dried lead acetate precipitate, obtained by precipitating the solution containing the products leached from beetroot into water containing chloroform, were hydrolysed. Whilst this showed a closer approximation to Grafe's figures, the total amount of fatty materials was still much below that required by Grafe's data (see Table III and compare Tables I and II).

Table III.

Weight of precipitate hydrolysed	= 19 g.
Total material soluble in alcohol or ether	= 2.5 g. or 13.1 %
Ether-soluble acid after saponification	= 1.1 g. or 5.2 %
Unsaponifiable material soluble in ether	Nil
Ether- and water-insoluble acid after saponification	= 0.33 g. or 1.7 %
Lead sulphate	= 11.4 g. or 40 % Pb.
Phosphorus	= 0.87 %

An attempt to isolate phosphatides from potato tissue by extraction methods will now be described. The procedure was as follows.

1. Whole tubers, free from the cork layer of the skin, were finely minced.
2. Juice was pressed out in a hand press, the residual material being grey-white and in the form of a hard cake.
3. The solid residue was air-dried and finally ground to a fine powder in a coffee mill and sieved through a fine mesh wire gauze. 700 g. were then obtained for extraction in a large Soxhlet apparatus.
4. Direct ether extraction of the aqueous liquid (3 litres) was not practicable owing to its opalescent, colloidal nature, so that the reaction was adjusted electrometrically with hydrochloric acid to $p_H = 4.4$. After standing, centrifuging and decanting, a yellow-brown solution and a coagulum, which could be examined separately, were obtained. The coagulum was dried in a vacuum desiccator and ground in a mortar and was thus suitable for direct extraction with fat solvents. The products obtained from the dry potato

residue, the protein coagulum and the clear aqueous liquid are described below.

The residual liquid was treated as follows:

The only fractions which, according to normal technique, would be regarded as crude phosphatide are the acetone-insoluble but ether-soluble ones, amounting in all, from 4 kg. of potato tissue, to 0.16 g. It is also significant that 0.14 g. of this was obtained from the dry residue and not from the aqueous suspension. The materials in question gave qualitative phosphorus reactions, but were not light-sensitive. Whether or not they really contained phosphatides it was impossible to decide with the available material.

In the light of these results obtained by extraction of potato tissue with fat solvents, the following previous observations are of interest.

1. Lippmann [1887] extracted sugar beet with alcohol and ether and was able to isolate lecithin, hydrolyse it and identify the decomposition

products—oleic acid, glycerol, phosphoric acid and betaine. A subsequent experiment gave choline and no betaine.

2. Gallagher [1923], who thought that lecithin could function as an autoxidisable substance in connection with the potato oxidase system, extracted 3 kg. of finely minced tubers with 5 litres of 90 % alcohol. The evaporated solution yielded 1.6 g. of substance soluble in ether, dehydrated by sodium sulphate, and insoluble in acetone. This contained phosphorus, but not choline, and no mention is made of any isolation of fatty acids or glycerol. This also indicates that the amount of lecithin is at any rate small, although the figure for ether-soluble, acetone-insoluble material is somewhat higher than that already given. The effects of variety may be important in this respect, and also the time of extraction relative to the rest period. The tubers used in the author's experiments had been stored during the winter, and were analysed in June.

3. Chibnall and Channon [1927] were unable to isolate lecithin in experiments involving the extraction of some 200 kg. of cabbage leaves. The substance isolated, of a phospholipin character, proved to be in the form of the insoluble calcium salts of glyceridephosphoric acids—in effect lecithin with choline replaced by calcium—and the possibility of true lecithin being present was negatived.

SUMMARY.

Experiments with various parenchymatous tissues lead to the conclusion that leaching experiments do not yield evidence that phosphatides are present in the surface layer of the protoplasm, and diffuse from living tissues into distilled water.

The author desires to acknowledge his indebtedness to Professor J. H. Priestley, in whose laboratory this work was carried out, for his continued help and advice, and also to the Department of Scientific and Industrial Research for a grant of maintenance.

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