LXVIII. THE CHEMICAL NATURE OF THE MEMBRANE OF POTATO CORK.

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INTRODUCTION.

IN all the vascular plants a very important part is played by cell walls the physical properties of which have been altered by deposition of fatty substances in and upon them. As these bodies modify considerably the penetrability of the wall to water and aqueous solutions it is very essential to have information as to the nature of the fatty deposit, its continuity, permanence and physical properties. Such information is gradually being accumulated in this laboratory, but a much fuller knowledge is required of the chemical nature of the fatty bodies involved than is at present available and the present paper is a first contribution to that end in the case of cork.

In the shoot of the flowering plant there are two very different types of fat-impregnated membranes. From its earliest appearance the shoot is clothed with a cuticle, a fatty layer which is continuous over the whole surface of the shoot, as a deposit upon the outer walls of the outermost layer of cells. Frequently in older stems this layer is replaced in later life by a layer of cork which has quite a different origin. In this case a layer of cells, always lying below the actual epidermal cell, enters into a stage of active division and growth. As a result, a series of cells are cut off to the outside of this meristematic cell of which the oldest is the outermost (Fig. 1 A). Within each of these cells there now forms an inner layer which possesses fat-staining properties and which is called the suberin lamella. Similar fatty substances impregnate the thin wall medianly placed between two adjacent cells and known as the middle lamella, but the continuous suberin lamella within the cell is laid down simultaneously in all parts of the cell and undoubtedly is formed from the contents of the cell itself which have accumulated at the surface of the protoplast (Fig. 1).

Within this suberin lamella is present a layer of cellulose impregnated to some extent with fat but quite recognisable when all fatty bodies are removed. The fatty substances which are under investigation therefore in cork are not in a continuous sheet, as in the cuticle, but mainly concentrated within a series of isolated lamellae lining individual cells, the suberin lamellae, and also to some extent diffused through a carbohydrate basis of cellulose or of pectates in the middle lamellae.

A brief historical account of our chemical knowledge of suberin has been given by Priestley [1921]. The basis of the assumption that it is fatty in nature is its behaviour towards fat stains such as Sudan III and Scharlach R. On the other hand, neutral fat solvents only remove a small proportion of these substances [Gilson, 1890] of which only a small fraction has been shown to be glycerides of fatty acids [Kügler, 1884]. The rest of the material of the suberin lamella is removed by boiling in alcoholic potassium hydroxide [Van Wisselingh, 1886-1895], and this extract is usually regarded as consisting in the main of salts of various organic (suberogenic) acids [Gilson, 1890]. The relation of these acids to fatty acids is not clear, but, in view of the fact that they all lose water at higher temperatures with consequent changes such as a rise in melting point and loss of solubility in fat solvents, they would appear to be of the nature of hydroxy-fatty acids.

Fig. 1.

- A. Series of cork cells formed from meristem, 1 oldest and outermost cell, 6 youngest.
 Details of wall shown in 1.
- B. Formation of a new cork layer below cut surface of potato tuber.

In the present work, as a result of the experience gained with these somewhat intractable substances, the experimental methods of Gilson have been abandoned for methods similar to those used in the paint and varnish industries, where processes are involved in which linoxyn-like products are formed from the fats and oils derived from plants. It is hoped to show that such processes have considerable bearing upon the deposition of the suberin lamellae within the serried ranks of the cork cells.

EXPERIMENTAL METHOD.

The material chosen for examination was the skin of washed potatoes because it could be readily obtained in large amounts. It was washed in running water, dried fairly quickly some few feet from a fire in a well-ventilated room and then ground fairly finely.

The actual method of treatment, as now presented, is felt to be capable of yielding consistent semi-quantitative results for the various types of fatty substances involved and consists of three successive operations:

- (1) Removal of chloroform-soluble material in a Soxhlet apparatus.
- (2) Saponification of residual material with 3 % alcoholic sodium hydroxide.

(3) Treatment of alcoholic solution so obtained:

- (a) Removal of unsaponifiable material.
- (b) Removal of normal fatty acids.
- (c) Removal of hydroxy-fatty acids.

(1) *Removal of chloroform-soluble material.*

The extraction apparatus used was made of metal and had a capacity of about 2 litres; it belongs to Messrs Rowntree & Co. of York, to whom grateful acknowledgments are due. This made it possible to free from fat, in one operation, quite a large bulk of tissue. The chloroform extract was distilled to small bulk, carefully washed into a tared dish, taken nearly to dryness on a water-bath and the last traces of liquid removed by repeated evacuation—while slightly warm—in a desiccator. With samples of the same tissue, results correct to less than 1 % can easily be obtained.

(2) *Saponification with alcoholic sodium hydroxide*¹.

The extracted tissue, after removal of chloroform by exposure to air was boiled for 6 hours with about 12 times its weight of alcoholic sodium hydroxide. The alcoholic liquid was filtered off and replaced by absolute alcohol in which the tissue was boiled for 1 hour before again filtering and adding a fresh supply of alcohol. This boiling with successive lots of alcohol was continued until the filtered extract ceased to show colour. The combined alcoholic extracts were taken to dryness on the water-bath.

The residual solid matter was quite free from suberin and dissolved completely in cellulose solvents.

(3 a) *Removal of unsaponifiable material.*

The mass obtained after removal of alcohol was taken up in water. Usually a large volume of water was required, as some of the materials present seem to have relatively insoluble sodium and potassium soaps. The aqueous solution was repeatedly extracted with ether² in a separating funnel until the ethereal layer was no longer coloured and a test sample gave no stain on a watch glass. The combined extracts were concentrated, carefully washed into a tared vessel and the remaining solvent removed in a vacuum desiccator. Evacuation was repeated until constant weight was reached. The practice of repeated evacuation was adopted in preference to the standard practice for unsaponifiable fractions, namely, heating to 110° for some hours, because the presence

¹ At first it was found impossible to obtain agreement between the amounts of material in subsequent stages 3 a, b and c. This was found to be due in large measure to the fact that the saponifying agent was made up from rectified spirit. When it was prepared by dissolving NaOH in its own volume of distilled water and making up to the required volume with absolute alcohol, the results for subsequent stages could be consistently repeated.

² The ether used was purified by re-distilling after standing over stick potash for several days. Unless so treated, consistent values could not be obtained, as the ethereal extracts were found to contain varying amounts of resinous bodies.

of relatively volatile bodies was early noted and it was thought that their preservation might be useful. The solution of sodium soaps was then made acid with dilute HCl, giving a suspension of free acids.

(3 b and c) *Removal of normal acids and "hydroxy"-acids.*

It was early noted that in this mixture of acids were bodies which, although soluble in ether, could not be removed by light petroleum (B.P. 40–60°) and were similar to those described by Lewkowitsch [1909] and Fahrion [1911] and called hydroxy-acids. The method for the separation of normal and hydroxy-fatty acids described by Lewkowitsch [1909] seemed to be the only one available and proved to be suitable. The suspension was repeatedly shaken with light petroleum (B.P. 40–60°) and the solvent layer separated until a test sample gave no stain on a watch glass. The combined petroleum extracts were treated in the same manner as for chloroform-soluble fat and the amount of normal acids thus obtained.

The aqueous suspension, freed from normal acids and consisting of hydroxy-acids, was filtered and the weight of hydroxy-acids determined after drying to constant weight in a vacuum desiccator.

The filtrate freed from fatty bodies was tested for glycerol.

EXPERIMENTAL.

Sharpe's Express potatoes were used in the following experiments. It was noted that, having removed the cork layer, leaving the potato in air for a few days resulted in the formation of a second, "regenerated," cork layer, and as this involved further suberisation and might provide useful data, these regenerated layers were also taken for examination each time within one week after removal.

With samples of the same cork layer semi-quantitative numbers could be obtained. The results of duplicate experiments are given in Table I.

Potatoes of gradually increasing age up to maturity were examined, the samples being taken from the same crop at different periods. Regenerated cork layers on these potatoes were also examined. Details are given below.

Normally formed Cork Layers.

Date of collection 1924	Cork layer (code letter)	Details of potato
July 14	A	From potatoes of size slightly larger than a pea nut
	B	Larger than A. Maximum length about 1"
	C	Larger than B. Maximum length about 1½"
August 26	D	Larger than C. Maximum length about 2"
	E	Larger than D. Maximum length about 3"
	F	Larger than E. Not quite mature
October 20	G	Larger than F. Mature

Regenerated Cork Layers.

Period of regeneration	Code letter of cork layer	Details of layer
Three weeks	H	Formed on A, B and C taken together
Three weeks	I	Formed on D
	J	Formed on E
	K	Formed on F
Four weeks	L	Formed on G

Table I.

	Weight of tissue taken g.	% on original dry weight of tissue			
		CHCl ₃ -soluble fat	unsap. material	normal acids	hydroxy-acids
Exp. 1	620	0.95	0.055	0.18	2.05
Exp. 2	663	0.95	0.058	0.17	2.17

*Results of Examination of Skins A-L.*Table II. *Normally formed Cork Layers.*

	Cork layer	Dry weight % CHCl ₃ -soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxy-acids	Dry weight % normal plus hydroxy-acids
July 14	A	2.43	0.37	0.58	7.97	8.55
	B	2.74	1.86	3.12	6.28	9.40
	C	2.71	0.19	4.98	4.36	9.34
Aug. 26	D	3.30	1.53	3.23	11.23	14.46
	E	3.23	3.80	2.21	8.91	11.12
	F	3.41	4.83	5.93	6.61	12.54
Oct. 20	G	1.65	0.36	3.0	2.40	5.40

Table III. *Regenerated Cork Layers.*

Cork layer	Dry weight % CHCl ₃ -soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxyacids	Dry weight % normal plus hydroxy-acids
H	1.57	0.76	3.45	2.86	6.31
I	1.74	0.90	1.59	6.47	8.06
J	1.50	0.49	0.80	5.15	5.95
K	1.57	1.02	1.66	6.16	7.82
L	1.53	0.511	2.28	5.11	7.39

EXAMINATION OF PRODUCTS.

(1) *Chloroform-soluble fats.*

These bodies were all dark coloured, quite solid and possessed a rather sweet smell not unlike chocolate. The iodine numbers for the series A-L are given.

Table IV.

Normal cork layer		Regenerated cork	
Code letter	Iodine no.	Code letter	Iodine no.
B	70	H	74.6
C	60	I	76.0
D	68	J	74.0
F	64	K	63.0
G	56.7	L	62.4

The iodine numbers for other normal mature skins not included above, were all in the region 60–66, whilst for other regenerated cork extractions they varied from 70 to 75. Such tissues, if left in air for some weeks before extraction, yielded fats whose iodine numbers were considerably lower. A regenerated cork layer yielded a fat with an iodine number of 71, whilst after extraction 23 weeks later the iodine number was 53.

Fats from mature corky tissues had saponification values of 186–190 and 0.2–0.5 % ash in which sodium and potassium were detected. No marked solubility change was produced by exposure to sunlight for 40 days or powerful electric light for 70 days.

The amounts of constituent types of fatty acids from the fats of normal and regenerated cork were compared by saponifying them with 3 % alcoholic sodium hydroxide and following out the treatment as if for a skin.

	% unsap. material	% normal acids	% hydroxy- acids	% total acids
Fat from mature normal cork	15.3	50.0	33.6	83.6
Fat from regenerated cork	18.0	38.9	24.6	63.5

The aqueous residues after saponification were tested for glycerol, using the acrolein test. The fat from mature cork apparently contained only a trace, that from the regenerated cork evidently much more.

The normal acids thus obtained reduced alkaline permanganate slightly. They contained a little yellow colouring matter which was very difficult to remove. By repeated deposition from a mixture of light petroleum, ether and ethyl acetate, a pure white sample of an acid was obtained melting at 71–72°. Assuming mono-basidity, its molecular weight by the silver salt method was 283. The acid is assumed to be stearic acid. It had no iodine number.

The unsaponifiable material from both fats was heated to 70° on a hot plate for some hours. That from the young fat contained 23 % volatile material as against 0.2 % in the old fat. Tests of the non-volatile portions for phytosterol by heating with acetic anhydride according to usual practice were negative.

(3 a) *Unsaponifiable materials from cork.*

These bodies had invariably a strong penetrating odour and a yellow to brown colour. Attempts to detect phytosterol always failed. Heating to 70° removed in many cases considerable amounts of volatile material. Samples from regenerated cork contained in some cases as much as 70 % volatile bodies.

(3 b) *Normal acids.*

The invariable accompaniment of coloured extraneous matter, difficult to remove, has hampered progress. In order to remove it, it has been found best repeatedly to salt out the sodium salts with NaCl, finally acidifying and washing free from chloride. By this means a small amount of acid has been obtained which when moist is quite colourless but dries to a slate-grey colour. Its melting point is again 70–71°.

(3 c) *Hydroxy-acids.*

Although originally dark coloured, repeated salting out of the sodium salts gave a solid, quite white when moist, which again dried to a very pronounced slate colour, even *in vacuo* in the dark.

Gilson found great reduction of solubility with the suberogenic acids when heated in sealed tubes to high temperatures. Some hydroxy-acids from mature skins were heated in air for 27 days at 50°. They then showed a decrease in solubility of 50 %, indicating behaviour similar as regards solubility to the suberogenic acids. Gilson's procedure for obtaining the individual pure acids was followed without success.

DISCUSSION.

In considering the data obtained it must be remembered that the fatty products obtained are derived not only from the suberin lamella itself but also from the fatty impregnations of other lamellae. Microscopic examination has revealed in normal cork layers the presence of an occasional epidermal cell outside the cork, indicating that a trace of the original cuticle may be present. In the case of regenerated cork layers, much of the extracted material will arise from the deposit—also termed suberin—which occurs in and along the wall of cells at the exposed surface (Fig. 1 B).

The chloroform extract of normally formed cork contains very little neutral fat. The bulk appears to consist of fatty acids with only traces of sodium and potassium soaps.

The values in Tables II and III are expressed as percentages of original dry weight of tissue. Obviously, comparisons must be drawn with caution, as on different occasions different amounts of extraneous tissue may be removed with the cork and be taken for examination. On these grounds such comparisons are not made in spite of what at first sight would appear to be interesting differences in chloroform extract percentages. If the total amounts of fatty acids released on saponification be compared with the amount of chloroform-soluble material, it will be observed that the ratio

$$\frac{\text{Hydroxy-acids + normal acids}}{\text{Chloroform-sol. bodies}}$$

is approximately constant. With the exception of D, in which the yield of acids on saponification is high, the ratio for *normal cork* layers only varies from 3.3–3.67 (Table V).

Table V.

Normally formed cork		Regenerated cork	
Tissue letter	Hydroxy-acids + normal acids Chloroform-sol. fat	Tissue letter	Hydroxy-acids + normal acids Chloroform-sol. fat
A	3.52	H	4.02
B	3.42	I	4.6
C	3.45	J	3.96
D	4.38	K	4.9
E	3.44	L	4.8
F	3.67		
G	3.3		

When the chloroform extract is small the acids released on saponification are low also and in proportion. The modifying factor may be the amount of extraneous tissue accompanying the pure cork layers. We may accept as a first conclusion that, in the normal cork layer at all stages of development, the proportion of substance soluble in fat solvent to fatty substances extractable after saponification is approximately constant. As the series contains tissues with increasing numbers of mature cork cells, the numbers increasing from 3 or 4 to 10 or 15 without any appreciable change in the above ratio, we may conclude that there remains in the suberin lamella (Fig. 1 A) a definite proportion of material soluble in fat solvents. This result is also indicated by other investigators working on bottle cork which consists of still older cork cells, but in which Kgler [1884] found some 9 % of substances soluble in chloroform (excluding cerin). Experiments with chloroform-extracted tissues show that the chloroform-soluble material is responsible almost entirely for the staining reactions of the suberin lamella with Sudan III.

In the regenerated cork layers a larger and variable proportion of the more resistant bodies is present (Table V). Here, however, it must be remembered that we are dealing not only with internal suberin lamellae but with an irregular though continuous layer formed in and on the cellulose walls near the exposed surface, from which one would expect an excess of resistant bodies, owing to a more complete "drying" of this more exposed layer.

It can be definitely stated that the chloroform-soluble material itself contains both normal and hydroxy-acids, and Kgler's statement that stearic acid is present in a chloroform extract of *Quercus suber* adds support to the tentative identification of stearic acid in the corresponding extract of potato cork. No fatty acids have been obtained which are liquid at normal temperature, and the solid nature of all fatty bodies isolated in this work, coupled with the fact that saponification values would agree with the presence of acids of the stearic type as major constituents, suggests that only small amounts of unsaturated acids are present. The iodine value may be due to these small amounts of unsaturated acids, together with any unsaturated colouring matter or unsaponifiable body, associated with the main bulk of saturated material in the extract. It will be seen that the saponification of such an extract of normal cork yielded some 99 % of products as non-saponifiable, normal and hydroxy-fatty acids, the normal acids being mainly stearic, and showing only slight reaction to alkaline permanganate. The unsaponifiable material present in the fats is characterised by its greater content of volatile bodies in the case of regenerated cork.

After the removal of the normal and hydroxy-fatty acids from the corky tissue microscopical examination showed the complete absence of fatty materials in the residues. The walls disappeared entirely under the action of cellulose solvents, a result obtained previously by Payen [1868] with potato cork. Payen, however, before extraction with alcoholic potash had soaked the tissue for days, first in 4 % HCl and then in 20 % acetic acid. Whilst, however, the

ratio of the sum of normal and hydroxy-acids to chloroform-soluble material remained constant, the amounts of each constituent were very variable (Tables II and III), this variability holding also in the regenerated cork layers. Coupling these facts, it is obvious that either the method of extraction is not reliable and the separation of hydroxy- and normal acids not efficient, or else there is genetic connection between the hydroxy- and normal acids, the one replacing the other and the whole remaining approximately constant. The consistent results in Table I negate the first alternative so that one is led to assume that the hydroxy-acids are formed at the expense of the normal acids. In the suberin lamella itself these hydroxy-acids are insoluble in fat solvents, and are only released on saponification, thus indicating some form of combination or condensation. Stearic acid is almost certainly present in the fat, and the normal acids from the products of tissue saponification seem to be very similar in all respects. The hydroxy-acids obtained by tissue saponification and by saponification of chloroform-extracted material behave similarly. These bodies plus the unsaponifiable material constitute all that is present in the walls of the cork cells. There can be little doubt that the suberin lamella owes some of its properties to the presence of normal and hydroxy-acids in a state of combination or condensation, and that the hydroxy-acids are derived from the normal acids by some process of oxidation, and if the bulk of these should consist of stearic acid, as is suspected, then perhaps saturated acids will have to be considered as a possible starting point.

Little can be said regarding the unsaponifiable material. It certainly contains no sterols. In the chloroform-extracted material glycerol is present in the normal cork in traces only but in appreciably larger amount in the regenerated cork. No glycerol has ever been detected in the alcoholic sodium hydroxide extracts. These facts are not in disagreement with the suggestion of Schmidt [1910] that the fatty acids first accumulate as glycerides, later being split up and the glycerol oxidised to CO_2 and water. The large amounts of volatile bodies present in the unsaponifiable material from regenerated cork whether from chloroform or alcoholic sodium hydroxide extracts may be by-products from the oxidative changes discussed. That these volatile bodies should be less in amount in older normal cork layers is to be expected. Dr P. W. Clutterbuck, working on the oxidation of stearic acid, has noted the appearance, as by-products, of bodies with similar appearance and properties.

SUMMARY.

A method is described by which the fatty constituents of the cork layer of the potato skin may be entirely removed, leaving the walls completely soluble in cellulose solvents. The removed bodies can be separated into chloroform-soluble material, unsaponifiable material, normal and hydroxy-acids and semi-quantitatively estimated.

It is concluded that the suberin lamella of the cork cell arises by changes

taking place in the fatty material resulting in the appearance of bodies no longer soluble in fat solvents.

A relatively constant proportion of the soluble fatty substances never undergoes this change and this soluble component of the suberin lamella is mainly responsible for its staining properties.

In the main the suberin lamella consists of relatively insoluble normal and hydroxy-fatty acid complexes which can be released by prolonged saponification as soluble soaps.

The non-saponifiable substances of the regenerated cork layers contain considerable amounts of volatile bodies.

Glycerol is found only in the chloroform extracts and then only in traces except in the case of regenerated cork layers.

The bearing of these facts on the formation of the suberin lamellae in cork cells is discussed.

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