XLVIII. WAX METABOLISM IN THE LEAVES OF BRUSSELS SPROUT[1].

By PREM NATH SAHAI and
ALBERT CHARLES CHIBNALL.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received February 26th, 1932.*)

In connection with an investigation which is being made in this laboratory into the metabolism of paraffins in the higher plants it was thought that further light might be thrown on the chemistry of paraffin synthesis in the leaf if the composition of a leaf-wax known to contain large amounts of paraffin could be determined at various stages throughout the life history of the leaf. The wax of cabbage leaves (*Brassica oleracea* v. *capitata*), which was shown by Channon and Chibnall [1929] to consist chiefly of the paraffin *n*-nonacosane and the closely allied ketone 15-nonacosanone would have been a suitable one for such an experiment, but unfortunately the headed cabbage, which is a bud with its leaves densely compacted together and in different degrees of maturity, is unsuitable for this purpose. The Brussels sprout (*B. oleracea* v. *gemmifera*) on the contrary appeared to be quite suitable for this purpose and as the botanical difference between the two plants is a varietal one only it seemed to us probable that the leaf-waxes would be similar. Accordingly we first determined the composition of the Brussels sprout leaf-wax and having found that it consisted chiefly of *n*-nonacosane, 15-nonacosanone and 15-nonacosanol we investigated the change in composition throughout the life history of the leaf.

In the experiments detailed below it is to be clearly understood that the term Brussels sprout leaf refers to the leaves growing on the main axis of the plant, and is in no way concerned with the bud-like shoots commonly referred to as "sprouts."

Analysis of the wax.

Several samples of wax ("crude hydrocarbon") had been obtained in a previous research from the mature leaves of Brussels sprout by Chibnall and Sahai [1931]. These were collected and saponified and the unsaponifiable material (22 g.) was fractionated by the phthalate method of Chibnall *et al.* [1931].

Primary alcohols. Only a small amount of insoluble sodium salts of primary phthalates was obtained. This was thoroughly extracted with ether and then

[1] The melting-points recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

saponified in benzene-alcoholic sodium ethoxide. The recovered primary alcohol was crystallised from light petroleum; yield 0·8 g.; M.P. 79°. A collected sample from this and later experiments weighed 1·3 g., and from it the following derivatives were prepared by the methods used by Pollard *et al.* [1931]. Acetate, M.P. 60·7–61°; acid M.P. 83–83·4°; paraffin M.P. 59·7–60° with no definite transition temperatures, showing that it was a mixture of more than 3 paraffins of mean molecular weight corresponding to C_{27} [Piper *et al.* 1931]. The primary alcohol was undoubtedly a mixture of the type which we suggested in an earlier paper [Pollard *et al.* 1931] should be called "ceryl" alcohol.

Ketones. After the removal of the insoluble sodium salts of the primary phthalates the ethereal solution was evaporated to dryness and the residue dissolved in about 200 cc. of boiling alcohol. On cooling 14·5 g. of material, M.P. 70°, crystallised out. This was treated with phthalic anhydride (14 g.) at 120° for 18 hours, and a further small amount of primary phthalates removed as before. On again crystallising the residue from hot alcohol 11·7 g. of material were obtained. The two alcoholic mother-liquors were evaporated to dryness and gave the crude secondary phthalate fractions mentioned below.

The 11·7 g. of crystalline material were stirred at room temperature with 100 cc. of light petroleum (B.P. 40–60°) and the solvent filtered off. This operation was repeated until no more material appeared to go readily into the cold solvent. The residue was dissolved in 100 cc. of warm light petroleum. On cooling 1·98 g. of material crystallised out; M.P. 77·5°. This was recrystallised from alcohol (charcoal), then from light petroleum and finally from carbon disulphide. The yield was 1·1 g.; M.P. 80·6–81°, unchanged in a mixed melt with synthetic 15-nonacosanone (M.P. 80·8°). The oxime was made in the usual way; M.P. 52·5°, unchanged when mixed with synthetic oxime (M.P. 52–53°). (Found: N, 3·3. $C_{29}H_{58}NOH$ requires N, 3·2 %.)

The mother-liquor from the warm light petroleum crystallisation was taken to dryness and the residue repeatedly extracted with cold light petroleum (1 : 10) as before. The final residue was crystallised first from carbon disulphide and then from alcohol (charcoal). A further 0·72 g. of ketone M.P. 80·5° was thus obtained.

The mother-liquors from all the cold extractions with light petroleum were next combined and the solvent removed. The product (M.P. 63–64°) could not be further fractionated by mixed solvents, but as very considerable charring occurred on treatment with sulphuric acid at 120° it was surmised that ketone was still present. It was therefore oximated in the usual way, using 11 g. of hydroxylamine hydrochloride, 11 g. of potassium hydroxide and 200 cc. of alcohol. The mixture was boiled for 12 hours and filtered hot to remove potassium chloride. The filtrate was allowed to cool slowly, when 7·1 g. of practically pure paraffin crystallised out.

The alcoholic mother-liquor was evaporated to 150 cc. and 100 cc. of water were added. The white precipitate was filtered off; weight 1·45 g.; M.P. 50–51°. It was dissolved in a warm mixture of light petroleum (20 cc.) and acetone

(20 cc.) and allowed to cool. 0·1 g. of crude paraffin crystallised out and was filtered off. The mother-liquor was evaporated to dryness and the residue dissolved in warm acetic acid (50 cc.). No precipitate separated on cooling, showing that all the paraffin had been removed. The acetic acid solution was evaporated to 15 cc. and an equal volume of warm acetone added. On cooling 1·1 g. of ketoxime crystallised out; M.P. 51·5°, unchanged in a mixed melt with synthetic 15-nonacosanone oxime (M.P. 52–53°). 1 g. of the ketoxime was saponified for 12 hours in alcohol saturated with hydrogen chloride. 0·75 g. of 15-nonacosanone M.P. 80·6–80·9° was thus obtained. The total yield of ketone was therefore 2·6 g.

Secondary alcohol. The two fractions of the sodium salts of the secondary phthalates were united and saponified in benzene-alcoholic sodium ethoxide, and the secondary alcohol was extracted with hot acetone. The 1·6 g. of white crystalline material which separated on cooling was recrystallised from hot alcohol (charcoal); M.P. 82·3–82·8°. After 3 recrystallisations from pyridine the M.P. was raised to 83·5–83·8°. (Found: C, 81·9; H, 14·3. $C_{29}H_{60}O$ requires C, 82·0; H, 14·2 %.) The presence of *n*-nonacosane and 15-nonacosanone in the wax suggested that this secondary alcohol might be 15-nonacosanol, M.P. 83·6–83·8°, which had not hitherto been found in natural products, but which had been recently synthesised by Piper *et al.* [1931]. This was confirmed by a mixed melt, which showed no depression. The acetate of the natural alcohol was prepared in the usual way, M.P. alone, and mixed with the synthetic acetate, 50·5–51°.

Paraffin fraction. The 7·1 g. of paraffin obtained during the oximation mentioned above melted at 63–64°. On treatment with sulphuric acid at 120–130° very little charring occurred. After washing with water the product was dissolved in hot alcohol and filtered hot to remove traces of carbon. On cooling the paraffin (6·7 g.) crystallised out in small white leaflets. The melting-point 63·7–64° showed that it was a mixture. The material was accordingly fractionated by means of light petroleum [Chibnall *et al.* 1931]. Fractionation was slow, and after some 50 samples had been dealt with we obtained a series of 9 fractions differing slightly in M.P. The melting- and transition-points of the upper, lower and two representative intermediate fractions are given in Table I. Each fraction was melted alongside a sample of synthetic $C_{29}H_{60}$.

Table I. *Fractionation of the paraffin.*

No.	Heating transition-point	M.P.	S.P.	Cooling transition-point
1	57·0–57·3°	63·5–63·7°	63·3°	55·5°
2	56·5–57·0	63·7–63·9	63·5	55·5
3	56·5–57·0	63·9–64·1	63·6	55·4
4	56·3–56·7	64·1–64·3	63·9	55·2
$C_{29}H_{60}$	57·3–57·5	63·4–63·6	63·2	55·8

From the data for mixtures of $C_{29}H_{60} + C_{31}H_{64}$ and $C_{27}H_{56} + C_{28}H_{58}$ given in a previous paper [Piper *et al.* 1931] we conclude that the Brussels sprout paraffin

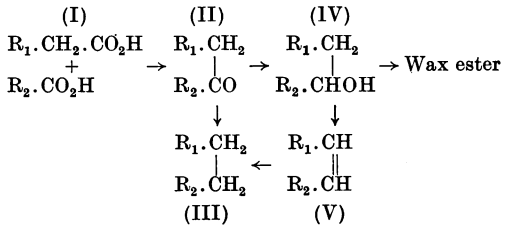
fractions consist of $C_{29}H_{60}$ with 2·5 to 10 % of $C_{31}H_{64}$. If the second paraffin had been $C_{30}H_{62}$ we should not have expected in the case of fraction 4 a fall of nearly 1° in the heating transition-point.

Fatty acids. The small amount of fatty acid recovered from the soaps after saponification was deep green, and required repeated treatment with charcoal in boiling alcohol to obtain a white crystalline product. The M.P. was 79–79·5° showing that the acid was the usual "cerotic" acid of plant waxes.

The constituents of the wax.

It will be seen that the chief constituents of the wax are the three closely allied substances *n*-nonacosane, 15-nonacosanone and 15-nonacosanol. In addition small amounts of mixed higher primary alcohols (ceryl alcohol), mixed higher *n*-fatty acids (cerotic acid) and another paraffin, probably *n*-hentriacontane are present.

In a previous paper [Chibnall *et al.* 1931] it was shown that the chief constituents of apple-peel wax were *n*-nonacosane and *d*-10-nonacosanol. Although the corresponding ketone 10-nonacosanone was not found it was suggested that the original hypothesis of Channon and Chibnall [1929] for the synthesis of higher paraffins in the plant might be extended in the following way:



The presence in the same wax of a paraffin (III) and both its corresponding ketone (II) and secondary alcohol (IV) certainly lends additional support to such an inter-relationship. It was pointed out however that if the ketone (II) arises through the condensation of the molecules of fatty acid (I), then 15-nonacosanone would postulate the presence in the plant at some stage in the synthesis of pentadecoic acid, and, as is well known the presence of this acid in natural products has been repeatedly disproved.

This weighty objection induced us to carry out two further experiments with Brussels sprout leaves.

A. An attempt has been made to demonstrate the presence of the olefin 14-nonacosene, $CH_3.(CH_2)_{12}.CH:CH.(CH_2)_{13}.CH_3$.

B. The composition of the wax has been investigated at three periods in the life history of the leaf. If either the ketone (II) or the secondary alcohol (IV) should be the precursor of the other two products, then it might be expected that the wax of the young leaves would contain relatively more of this substance than the mature or the aged leaves.

Attempted isolation of an olefin.

14-Nonacosene and certain other olefins have been synthesised in this laboratory by Dr Pollard from symmetrical secondary alcohols by the method of Grün *et al.* [1926]. These authors showed that both *cis*- and *trans*-isomerides were produced, one of which had a M.P. 20–30° higher than the other. The higher-melting isomerides crystallise in flakes similar to the corresponding paraffins. They are very soluble in cold light petroleum and are precipitated on the addition of 2 volumes of acetone. The lower-melting isomerides are even more soluble in cold light petroleum, but are not precipitated on the addition of acetone. These solubility relationships suggest that if a higher-melting isomeride were present in the ether extract of Brussels sprout leaves it would be precipitated by acetone with the wax fraction, whereas if the lower-melting—more soluble—isomeride were present it would not be precipitated at this stage, and would pass ultimately into the unsaponifiable fraction of the ether-acetone filtrate.

In the search for the higher-melting isomeride in the wax fraction it was necessary to attempt isolation before treatment with phthalic anhydride, as this reagent might give rise to an olefin by dehydration of the secondary alcohol known to be present. Accordingly the crude unsaponified wax (9·4 g.) was dissolved in 10 volumes of warm light petroleum (B.P. $< 40°$), which was then poured into a dish and left exposed to the air. On evaporation of the solvent the crude wax was left as a fine friable powder. This was then stirred at 15° with 100 cc. of light petroleum for 2 minutes, and the solvent filtered off. The operation was repeated. The combined filtrates were evaporated to dryness, and the residue was taken up in hot alcohol (20 cc.). The material that crystallised out on cooling (0·54 g.) should have contained the major part of the olefin present in the crude wax. The iodine value was 2 and M.P. 63·5–64°. As 14-nonacosene has an iodine value of 62·7 it is clear that the crude wax contained no appreciable amount of olefin.

Dr Pollard is investigating the possible presence of olefins in various leaves, and we have to thank him for permission to publish a brief account of his results showing that the unsaponifiable fraction of the ether-acetone filtrate of Brussels sprout leaves contains no lower-melting isomeride of 14-nonacosene. The unsaponifiable material (15 g.) was treated successively with light petroleum, ethyl alcohol and acetone. 3 g. of crude sterol and 2 g. of residual wax material were separated. The oily residue (10 g.) was dissolved in ethyl acetate (200 cc.) and hydrogenated with hydrogen and palladium in the presence of activated charcoal. The procedure employed readily reduced synthetic olefins to the corresponding paraffins. Although 500 cc. of hydrogen were absorbed the resulting product was still freely soluble in cold acetone, showing that no paraffin had been produced by reduction of an olefin.

The above results show fairly conclusively that no appreciable amount of olefin can have been present in the Brussels sprout leaves.

Changes in the composition of the wax during the life history of the leaf.

Materials used. The seeds which were to provide the plant material for samples 1–3 were sown in open beds on April 17th, 1931. At the same time seeds were sown in boxes which were kept in the open air alongside the beds. Late in May the seedlings growing in these boxes were transplanted into the same open beds, and at a later date provided the material for samples 4–6. The seedlings appeared above the ground about 5–7 days after sowing.

Details of the samples collected are given in Table II. Those of stages 1 and 2 consisted of seedlings which were cut just below the cotyledons, so that

Table II. *Showing the amount of wax in leaves of various ages.*

Stage	Age above ground in days	Condition of plants	Fresh wt. kg.	Dry wt. %	% of fresh wt.			% of dry wt.		
					Total ether extract	Wax	Glyceride fatty acids	Total ether extract	Wax	Glyceride fatty acids
	—	Ungerminated seed	0·1	—	37·8	0·0	33·8	—	—	—
1	10	Seedlings 2 cm. high, consisting of hypocotyl and cotyledon leaves	0·92	12·3	0·64	0·05	0·17	5·2	0·42	1·4
2	22	Seedlings 3–5 cm. high, 1st plumular leaf developing	13·1	11·8	0·83	0·10	0·16	7·1	0·85	1·4
3	43	Young plants 8–10 cm. high with 2–4 plumular leaves	0·92	11·8	0·74	0·13	0·15	6·3	1·1	1·3
4	54	Growing plant, laminae 8–10 cm. long	1·6	11·9	0·75	0·14	0·14	6·3	1·2	1·2
5	126	Mature plants, laminae 15–25 cm. long	22·0	15·7	0·49	0·18	0·09	3·2	1·2	0·5
6	221	Aged plants, laminae beginning to show signs of chlorophyll generation	12·0	Not taken	—	0·21	0·11	—	—	—

the material for analysis included the young plumule, but not the hypocotyl. Those of stages 3–6 consisted of detached laminae without petioles.

Preparation of the wax. To ascertain if the seeds contained any appreciable amount of wax 100 g. were powdered in a coffee mill and then extracted with ether in a Soxhlet apparatus for 24 hours. The ether extract was pale yellow, and on removal of the solvent weighed 37·8 g. 23·5 g. of this material were saponified in the usual way and gave 21 g. of fatty acids of iodine value 112 and 0·63 g. of unsaponifiable material. The latter was dissolved in 25 cc. of acetone and kept at 0° for some days. A small amount of phytosterol, M.P. 134°, crystallised out, but no constituent of the leaf wax.

The fresh leaf material was treated by the method of Chibnall and Sahai [1931] to obtain the ether extract, from which the wax was prepared in the usual way. It will be shown in a later communication that the percentage of

ether-soluble material obtained from leaves previously dried and ground to a powder is sometimes higher than that obtained from fresh material treated by the method quoted above. According to our limited experience this is only the case with leaves having a high surface/weight ratio such as grasses or runner bean, which dry very rapidly (½–1 hour) in an oven at 100°. Fleshy leaves such as the laminae of Brussels sprout, which do not dry rapidly in an oven, and which are fairly completely disintegrated in a meat chopper are best treated by the method quoted above.

It will be seen from Table II that the percentage of wax in the fresh leaf increased steadily with growth, showing that synthesis of wax must have gone on continuously throughout the life history of the leaf. To ascertain if this accumulation of wax had been accompanied by a change in composition, as it might have been, for instance, if paraffins had been formed continuously from ketones or secondary alcohols, the samples of waxes prepared from young (stage 2), mature (stage 5) and old (stage 6) leaves, which weighed 10·9 g., 40 g. and 25·5 g. respectively, were submitted to complete analysis by the method given below.

Analysis of the wax of young, mature and old leaves. In the analysis of the wax described earlier in this paper the ketone was separated from the paraffin partly by fractional crystallisation and partly by oximation. This procedure was laborious, and much material was unavoidably lost. To expedite the procedure the following modification was employed in the present instance. The primary and secondary alcohols were first removed as sodium phthalates in the usual way. The mixture of ketone and paraffin was then reduced with sodium in alcohol [Kipping, 1890], and the resulting secondary alcohol removed as sodium phthalate. The procedure in the case of the wax from the young leaves (stage 2) will illustrate the modified method employed.

The wax (10·9 g.) was saponified in benzene-alcoholic potassium hydroxide for 2 hours, and gave 0·6 g. of fatty acid and 9·0 g. of unsaponifiable material. The latter was treated twice successively with phthalic anhydride in the usual way. 0·74 g. of primary phthalate was obtained, which yielded on saponification 0·22 g. of primary alcohol. The combined secondary phthalates weighed 2·5 g. and gave 1·1 g. of secondary alcohol, M.P. 82·5°. The mixture of ketone and paraffin (5·9 g.) was dissolved in boiling alcohol (250 cc.) and sodium (20 g.) added in small portions at a time during 2 hours. On cooling the reduced ketone and paraffin crystallised out and were removed by filtration. This

Table III. *Analysis of the wax of young, mature and old laminae.*

	Young lamina (Stage 2) %	Mature lamina (Stage 5) %	Old lamina (Stage 6) %
Higher fatty acids	5·4	6·2	7·9
Primary alcohol	2·0	1·3	3·2
15-Nonacosanol	9·5	6·9	11·4
15-Nonacosanone	12·2	11·5	9·9
n-Nonacosane	32·9	26·0	25·1
Total accounted for	62·0	51·9	57·5

material (5·8 g.) was then treated twice successively with phthalic anhydride. 3·57 g. of paraffin and 2·3 g. of secondary phthalate, giving 1·49 g. of secondary alcohol (M.P. 83°), were obtained.

The results of the three analyses are given in Table III. The losses are considerable, due in large part to the nature and number of the operations through which the material is passed. They should, however, be of the same order in each case, so that it seems justifiable to conclude that the composition of the wax has not materially altered throughout the life history of the leaf.

Discussion.

The two well-known leaf-waxes of commerce, candelilla wax from *Euphorbia antisyphilitica* and carnäuba wax from *Copernicia cerifera* occur as epidermal excretions covering all parts of the plant except the roots. By analogy it has generally been assumed that all other leaf-waxes, most of which have hitherto been obtained only in small amounts, are also of epidermal origin, and that their main function in the plant is to reduce transpiration to a minimum.

Chibnall and Channon [1929] have already called attention to the fact that the wax of cabbage leaves, which may amount to 1 % of the dry weight, must be considered an integral part of the cytoplasmic material of the cell, because the leaves were treated in such a way that the wax was obtained from an ether extract of coagulated cell contents which was free from cell-wall material. It can be shown in a similar way that the wax of Brussels sprout leaves is also a constituent of the cell cytoplasm. It follows therefore that the rôle which these two latter waxes play in the physiology of the leaf cannot be directly concerned with transpiration, but must be connected in some way with the normal metabolism of the cells of the mesophyll.

Table IV. *Showing the amount of wax in various parts of seedlings of Brussels sprout.*

		g. per kg. fresh material		
		Dry weight	Ether extract	Wax
Stage 1.	Cotyledon leaves	90	5·7	0·28
	Hypocotyl	90	3·3	0·30
Stage 2.	Leaves	120	8·3	1·0
	Hypocotyl and rootlets	131	4·4	0·4

It is not surprising therefore that we have been unable to demonstrate the presence of wax in the fatty reserve of the seed cotyledons. But at an early stage of germination rapid synthesis of wax must have taken place, for at stage 1 (Table IV), when the young embryos consisted of a short hypocotyl and two rudimentary cotyledon leaves, the amount present in all parts of the embryo was considerable. In subsequent development of the plant the growth of the leaves was accompanied by a slow increase in the amount of wax, as

shown in Table II. If wax synthesis had been confined to new, actively growing, cells the amount present, expressed as a percentage of the fresh weight of the leaf, would have remained constant. Actually there was a definite increase throughout the life history of the leaf, showing that synthesis must have gone on continuously in some, at least, of the fully-grown cells. This slow accumulation suggests that the wax may either play some vital part in the physiological activity of the cell or be an essential metabolic by-product which is innocuous and chemically so inert that it is not further metabolised. The chemical evidence supports this latter suggestion.

Reverting to the suggested inter-relation between the constituents of the wax given on p. 406 it will be seen from the data given in Table III that no *ad hoc.* evidence has been obtained, because the composition of the wax has remained unchanged throughout the life history of the leaf. There is no accumulation of paraffin or secondary alcohol at the expense of the ketone, nor can the total increase in the wax constituents be accounted for by the small fall in glyceride fatty acids (Table II), of which only about 10 % are mixed saturated acids.

If the ketone is indeed the precursor of the paraffin and secondary alcohol, then it is possible that there is an equilibrium between these substances which is not disturbed by changes in the metabolic activity of the cell which accompany growth and senescence. It seems to us, on the contrary, more reasonable to assume that all the wax components, and not only the paraffin, are end-products of metabolism. Such a view does not exclude the possibility that the paraffin, ketone or secondary alcohol may have a common origin at some stage in the plant metabolism. We shall defer further discussion until our search for intermediary metabolites is more complete.

Summary.

The following constituents of Brussels sprout leaf-wax have been definitely identified. *n*-Nonacosane, 15-nonacosanone, 15-nonacosanol, ceryl alcohol, cerotic acid and probably *n*-hentriacontane. Olefins are absent.

As the wax contains a high proportion of paraffin an attempt has been made to throw light on paraffin metabolism by investigating the composition of the wax at various stages throughout the life history of the leaf.

The seeds contained no wax, but synthesis took place rapidly in all parts of the embryo, and went on continuously in the leaf throughout the life history of the plant. The composition of the wax did not alter throughout this period, and no evidence was obtained to support the hypothesis that the paraffin had been formed by reduction of the ketone. It is suggested that all the components of the wax are end-products of metabolism.

We should like to thank Mr Hales, of the Chelsea Physic Garden, for the care bestowed on the plants used.

REFERENCES.

Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Chibnall and Channon (1929). *Biochem. J.* **23**, 176.
—— Piper, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2095.
—— and Sahai (1931). *Ann. Bot.* **45**, 489.
Grün, Ulbrich and Krczil (1926). *Z. angew. Chem.* **39**, 424.
Kipping (1890). *J. Chem. Soc.* **57**, 980.
Piper, Chibnall, Hopkins, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2072
Pollard, Chibnall and Piper (1931). *Biochem. J.* **25**, 2111.