AN X-RAY STUDY OF WAXY COATINGS FROM PLANTS

Comparative Investigations, and the Application of a New Micro-Method for X-Ray Diffraction to Structural Problems with Ancillary Observations under the Polarising and the Electron Microscope

by

D. R. KREGER

(With plates XXIII–XXVIII).

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PART B

INTRODUCTION

In studying the waxy coatings occurring on many plants, the following points may be regarded as most important:

1. the chemical composition of the waxes,
2. the metabolic processes from which the waxes result,
3. the microscopic structure of the wax covers,
4. their submicroscopic structure,
5. the circumstances determining their microscopic and submicroscopic structure,
6. their biological significance and
7. possible relations between properties of the waxes and the natural relationship of the plants producing them.

It goes without saying that knowledge concerning the composition of the waxes (point 1) is necessary before entering into the nature of the metabolism involved in their formation (point 2). Likewise, it is necessary to study the microscopic and submicroscopic structure of the wax covers (points 3 and 4) before anything can be said about the factors governing their structure (point 5). Accordingly, the points 1, 3 and 4 are of fundamental importance in the investigation of this subject.

As a result of the detailed observations recorded by De Bary as early as 1871, a rather extensive knowledge of the diverse microscopic structures of cuticular wax covers (point 3) has been accumulated. The present knowledge concerning points 1 and 4, however, still appears to be very incomplete.

Although more or less extensive data is available concerning the composition (point 1) of a few wax layers, mainly from those of economic importance, nothing is known at all about numerous other ones. This lack of detailed knowledge of the constitution is due to the difficulties connected with the chemical analysis of the mixtures of substances occurring in these waxes, as well as to the variation in composition and to the limited quantities available.

The lack of knowledge concerning point 4, the submicroscopic structure, has been due to the absence of expedients for elucidating those structures satisfactorily. The polarising microscope, generally used in those investigations, does not yield sufficient data for this purpose. It has been found possible in a few cases to indicate the direction of the chain molecules in the wax layer, but no further details as to the crystalline arrangement of the molecules and the shape, dimensions and orientation of the crystallites have been obtained in any case studied. It even remains doubtful whether or not all plant wax covers can be regarded as crystalline structures.

The present state of knowledge of the crystal structures and X-ray diagrams of substances such as are frequently found in natural waxes suggests that X-ray investigation might bring to light new data about both the composition and the submicroscopic structure of the cuticular wax covers. It has been the object of the investigations described here to gain new evidence concerning these two features by means of X-ray diffraction; part A deals with the composition, part B with the submicroscopic structure. In connection with the results obtained, attention is paid in addition to the afore-mentioned points 2 and 7 (in part A, chapter IV and V), and to point 5 (in part B, chapter IV). The biological significance of the waxes will not be discussed.

It may be mentioned at the outset that the importance of X-ray methods in investigation of the composition of natural wax mixtures had already been demonstrated by CHIBNALL, PIPER and co-workers during the years 1930-'37. This research dealt mainly with plant waxes which could be separated from protoplasmic material, although both plant and insect cuticles were included. X-ray methods were used for the identification of components separated from those waxes by means of usual and improved methods of chemical analysis.

In the present work we have not carried out chemical analyses. Our primary aim has been a comparative study, by means of X-ray diagrams exclusively, of a considerable number of different cuticle waxes, taken from the native waxes scraped off the plant epidermises with the intention of ascertaining later to what extent this type of investigation might enable the identification of certain compounds in the waxes. The indications thus obtained might again serve as a starting point of more exact chemical analyses. The advantages of the adopted procedure will be mentioned in part A, I, 1.

Along the above lines, waxes of some 60 plant species were examined, most of which were of unknown composition. The powder diagrams obtained showed a number of similarities and differences in terms of which a classification of the waxes could be given, and it turned out to be possible to identify certain compounds in many waxes with a high degree of probability, without performing chemical analyses. The results also have a considerable bearing on existing theories concerning the metabolism of plant wax formation and give some indication whether the composition of the waxes reflects the natural relationship of the plants producing them.

As regards the investigation into the submicroscopic structure described in part B, it may be mentioned that attempts to elucidate the submicroscopic structure of plant wax covers by means of X-ray diffraction have, hitherto, not been undertaken. We intended to obtain as complete a picture of this structure as possible in the wax layer of a single plant species. For this the wax layer of the sugar cane stem was chosen and in particular the frequently described microscopic rods which occur close beneath the nodes of the stem. In this investigation, a special micro-method for X-ray diffraction has been applied, as will be described below — and polarising and electron-microscopic observations have also been made. This has led to a relatively complete picture of the submicroscopic structure of the rods. In connection with this picture, combined with certain results from part A, the old problem is discussed whether the shape of such microscopic wax elements is determined by organising activities of the living protoplasm or whether it may be explained by mere physico-chemical principles.

For further introductory details we refer to A, I, 1 and 2, and B, I, 1.

During the investigation of the submicroscopic structure of the wax rods, the want of a special micro-method for X-ray diffraction revealed itself. It proved to be impossible to obtain by means of the usual and available X-ray apparatus, diagrams of these tiny specimens showing enough conclusive details. In the literature on X-ray crystallography, no description of such a micro-method was found except a method especially devised for the study of single crystals of microscopic size (KRATKY, 1931). This method is less suitable for studying most other kinds of microscopic objects, however.

It is evident that the study of the submicroscopic structure of biological objects in many cases deals with specimens of microscopic size, and in order to be able to carry out X-ray investigations of such objects, it is desirable to use an appropriate micro-method. Therefore, the problem of adapting X-ray methods to the study of wax rods, in our opinion, was of very general importance, and it seemed justified to undertake an investigation into the possibilities of obtaining X-ray diagrams of single objects of microscopic dimensions. This work, which occupied about two years, has indeed ultimately led to a suitable micro-method for the study of X-ray diffraction of tiny specimens.

This new method has, so far, been successfully applied to the study of the submicroscopic structure of the wax rods of the sugar cane stem. Furthermore, it has proved to be suitable to obtain a fibre diagram from a part of a starch grain. This diagram, essential for elucidating the still unknown crystal structure of the crystallites in starch grains, could not be obtained from native starch before, owing to the small size of the grains.

The investigations bearing on micro-methods for X-ray diffraction will not be recorded here in detail. A short description of the developed apparatus, however, is given in an appendix, while in B, II, 1 and 2 some further details will be found. A preliminary account of the micro-method and its contribution to the elucidation of the crystal structure in starch has already been given (KREGER 1945, 1946, 1947).

Finally it may be mentioned that the design and terminology of this thesis has been adapted to the needs of readers whose acquaintance with X-ray methods is small. Occasional references to the literature on X-ray crystallography cannot, however, always be avoided. As general textbooks we mention: W. H. and W. L. BRAGG (1933), BIJVOET en KOLKMEYER (1938), CLARK (1940) and BUERGER (1942).

The investigations have been carried out in the X-ray department of the Laboratory of Technical Physics, the University College of Technology, Delft, since September 1941.

PART A

A Comparative Study of a Number of Waxy Coatings from Plants on the Basis of their Powder Diagrams

CHAPTER I

METHODS AND APPARATUS

1. *Advantages of applying X-Ray Methods to the Investigation of the Composition of Plant waxes*

The chemical constitution of plant waxes has been studied extensively, as is indicated by the list of publications in this field up to 1929, mentioned by GRÜN and HALDEN (1929). Of the more recent publications which have since appeared, those of CHIBNALL, PIPER and co-workers, in the years 1927-1937, are the most important.

As shown by these investigations, plant waxes generally consist of a mixture of normal aliphatic long-chain compounds, chiefly:

paraffins: $CH_3 . (CH_2)_n . CH_3$,
prim. alcohols: $CH_3 . (CH_2)_n . CH_2OH$,
fatty acids: $CH_3 . (CH_2)_n . COOH$,
ketones: $CH_3 . (CH_2)_n . C = O . (CH_2)_m . CH_3$,
sec. alcohols: $CH_3 . (CH_2)_n . CHOH . (CH_2)_m . CH_3$

and true waxes in the chemical sense, i.e. esters of higher n-prim. alcohols and higher mono-valent n-fatty acids:

$$CH_3 . (CH_2)_n . O . C = O . (CH_2)_m . CH_3.$$

Also glycerides have been found in plant waxes.

Chemical analysis of these mixtures is very laborious and presents many difficulties. These are due, among other things, to the fact that the covers are generally very thin, so that great quantities of plant material must be treated to collect a sufficient amount of wax for chemical analysis. Difficulties also arise in separating some of the constituents and in particular of closely related homologues in the wax. After the separation follows the laborious task of carrying out quantitative analyses and of determining melting points and molecular weights for obtaining information about the number of carbon atoms of the compounds isolated.

The difficulty of the latter separation may be illustrated by the fact that, for quite a long time, certain components which could be regularly isolated from various waxes, were taken for a single substance, whereas afterwards they were found to be a mixture of close homologues. For instance the alcohol "ceryl alcohol", isolated from beeswax, which formerly was considered to be $C_{27}H_{55}OH$, has proved to be a mixture of $C_{26}H_{53}OH$ and $C_{28}H_{57}OH$. The "ceryl alcohol" in the wax from the Brazil wax palm, *Copernicia cerifera*, known as "Carnauba wax", likewise was shown to consist of these two alcohols and to contain, in addition, $C_{30}H_{61}OH$. "Melissyl alcohol" from Carnauba wax, considered before to be $C_{31}H_{63}OH$, turned out to be a mixture of C_{30}, C_{32} and C_{34} primary alcohols [1]. Something like this has appeared with many other components isolated from natural waxes.

The unravelling of a great number of these components which initially were mistaken for pure substances, is due to the work of PIPER, CHIBNALL and co-workers. The importance of this work lies especially in its showing that of certain components in natural waxes, namely the alcohols and fatty acids, only homologues with an even number of carbon atoms occur, whereas with others, namely the paraffins, only those with an odd number of carbon atoms are present. It will be recognised that this knowledge must be of great importance in framing theories about the metabolism of the formation of natural waxes.

These results were obtained by CHIBNALL and PIPER by using X-ray diffraction methods, unlike earlier workers in this field. They were encouraged to apply these methods by the success attained by FRANCIS, PIPER and MALKIN (1930) when using X-ray methods in their researches into the constitution of the long-chain fatty acids in natural products. The latter authors have shown that, for a precise definition of an isolated fraction, it is not sufficient to consider only its melting point and molecular weight. The identity of these fatty acids could only be established with certainty by also taking into consideration the spacing of the crystal lattice-planes (cf. section 2) determined by the ends of the chain molecules, which can be measured by means of X-ray

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[1] From now on, similar abbreviations in which only the number of carbon atoms is given, will be used, instead of complete formulae, to indicate a normal saturated carbon chain, if otherwise there can be no doubt about the formula of the substance in question.

diffraction. In a pure substance both melting point and long crystal spacing agree with their theoretically determined values. If either of the two values deviates, or both, the substance is not pure and, in certain cases, the difference from the theoretical value may lead to conclusions about the composition of the mixture. We shall return to this in chapter III, 4.

The conclusions of PIPER and CHIBNALL were reached as a result of an accurate determination of melting points and long crystal-spacings of synthetised, or highly purified, wax components (1931, 1934) and by using these data as reference standards during their analyses. They have also paid much attention to the improvement of the methods of separation and purification in wax analysis. Accordingly, these investigations owe their value to the combination of improved chemical methods of analysis with X-ray diffraction methods.

In contrast to the above-mentioned investigations we employed X-ray methods exclusively, starting from native waxes scraped off the plant epidermises, as mentioned in the introduction. This investigation initiated from the observation that in diagrams of some native waxes, which had been made to obtain preliminary information about possible similarities and differences between diagrams of waxes from various plant species, distinct long spacing reflections occurred. These reflections made it likely that a study of the diagrams would enable us to draw conclusions about the chain lengths and the nature of certain components in the wax, with the aid of the standard data of PIPER and CHIBNALL, and without carrying out any chemical analyses.

In order to obtain a complete qualitative and quantitative analysis of the waxes the suggested method is, naturally, inferior to accurate chemical analyses combined with X-ray diffraction; its relative simplicity, however, yields certain advantages, which will be summed up below.

It is an advantage that no great quantities of wax need be collected for the investigation. A quantity of about $^1/_{10}$ mm^3, which can easily be obtained by scraping some wax off the plant surface, is enough for making a powder diagram with sufficient detail. Furthermore the required small quantity can also be collected, in a relatively short time, from plants with a thin wax cover for which very few chemical data have been compiled on account of the difficulty of obtaining sufficient amounts of wax for chemical analysis.

Again the scraping of the plant surface ensures that only the waxy coating is investigated and that the samples contain no waxy substances from cutinised membranes or protoplasm. This may be of importance in connection with possible differences between these kinds of waxes. If, for sampling, extraction of great quantities of plant material were required, as in the case of chemical analysis, the above certainty would not exist.

Finally the X-ray diagram reflects the composition and certain structural properties of the wax in a very direct way. This enables a comparison of the wax layers which, in certain respects, will be more valuable than a comparison along the lines of chemical analysis, as, in the latter case, the many treatments might obscure certain differences. Moreover, it would not be easy to achieve a comparison between a great number of waxes along these more laborious lines.

From such a comparison of diagrams it might be possible to deduce general rules, e.g. correlations between the composition of the waxes and the natural relationship of the plants on which they occur, or correlations with environmental circumstances prevailing at the natural habitats of the plants in question. Up to now little attention has been paid to these items. Even if the diagrams do not allow a chemical interpretation, certain differences and similarities between the waxes might be recorded as a contribution to the comparative study of plant cuticle waxes.

2. *The X-Ray Diagram and Crystal Structure of Normal Aliphatic Long-Chain Compounds*

In this section, a short description will be given of the X-ray diagram and crystal structure of the normal aliphatic long-chain compounds occurring in plant waxes, so far as necessary for an understanding of later discussions.

At normal temperatures, these compounds are in a solid crystalline state, so that they yield good X-ray diagrams. The crystals of these compounds are generally of microscopic, or submicroscopic size and it is difficult to obtain larger single crystals. Powder diagrams of these substances, i.e. diagrams made from specimens in which many crystallites lie randomly oriented in the X-ray beam, can easily be obtained by methods briefly described in the next section.

In fig. 1, a sketch is given of a quadrant of a powder diagram of a normal aliphatic long-chain compound, made on a flat film. The diagram shows two sets of reflection circles, namely a set *a* with reflections of normal diameters and a set *b* with reflections of small diameters. In general, the compounds under consideration all give diagrams which show little or no distinction in the reflections belonging to set *a*. Within certain limits, the diameters and intensities of these reflections remain practically unchanged, whatever may be the length of the carbon chain or its characteristic group. On the contrary, the reflections *b* show variations in diameters and intensities, dependent on chain lengths and characteristic groups.

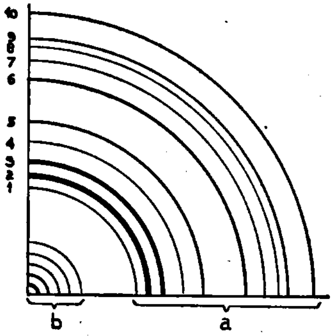

Fig. 1. Diagrammatic representation of a quadrant of the X-ray powder photograph of a normal aliphatic long-chain compound. The intensity of the interference-circles is shown by the width of the lines.

According to Bragg's formula for the relation between the spacings of the crystal lattice-planes in a set of equally spaced, parallel planes and the glancing angle of the radiation reflected from these planes — i.e.

$$n\lambda = 2d \sin \theta,$$

in which λ represents the wave length of the radiation, d the distance or spacing between the planes, θ the glancing angle (i.e. the angle between the incident beam and the planes in the position in which reflection appears, i.e. half the diffraction angle) and n the order of the reflection — the diffraction angle (and accordingly the diameter of the reflection circle) increases with decreasing distance between the lattice-planes.

Hence, the reflections of set *a* belong to relatively short lattice-plane spacings, which are characteristic of the crystals of all compounds under consideration, whereas the reflections of set *b* belong to spacings which vary with the number of carbon atoms and with the characteristic groups of the chain molecules.

In accordance with the results of many investigators, the molecules of the substances under consideration must be visualised as long rods, in which the carbon atoms are linked in zig-zag arrangement. The bonds between the carbon atoms in this zig-zag lie in a flat plane and meet at angles of 109°28′, cf. fig. 3. These rods lie inside the crystal with their longitudinal axes parallel to each other, and, generally, they are packed together in an invariable way with respect to directions perpendicular to their longitudinal axes. In fig. 2 this packing is demonstrated for the paraffin $C_{29}H_{60}$, nonacosane, studied by Müller (1928), by a projection of the molecules on a plane perpendicular to their longitudinal direction. It will be readily understood that, owing to this constant method of packing of the rod-like molecules, the spacings of planes parallel to their longitudinal direction will be the same in different long-chain compounds. This will give rise to a constant set of reflections from these planes. These reflections are those of set *a*. The spacing of the lattice-planes determined by the ends of the chains, however, will increase or decrease as the chains are longer or shorter. Consequently, this spacing is dependent on the number of carbon atoms of the chains; it is represented in the diagram by the reflections of set *b*.

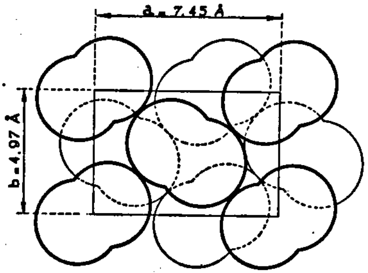

Fig. 2. Diagram showing the crystalline arrangement of the zigzag chains of carbon atoms in a normal long-chain paraffin (Müller, 1928). Projection viewed along the chain direction. Two layers of chains are shown; those of the upper layer are drawn in heavy lines.

We shall distinguish the planes, spacings and reflections belonging to set *a* as side-planes, side-spacings and side-spacing reflections, and those belonging to set *b* as basal planes, long-chain spacings and long-chain reflections.

In contrast to the reflections of set *a*, the reflections of set *b* together represent one spacing, as there is only one set of basal planes. That, nevertheless, we observe more than one reflection is caused by the fact that they are interferences of the first and higher orders from the same set of planes. This also explains why they are almost equally spaced. In mixtures of long-chain compounds, however, each component can give, under certain circumstances, its own set of long-chain reflections, cf. par. 4, chapt. III, and in this case they will represent more than one set of planes. The *a*-reflections generally remain unchanged in mixtures of the substances under consideration.

The chains may be placed normal or inclined to the basal planes when the crystal form is orthorhombic or monoclinic respectively. Generally, one and the same substance may adopt the orthorhombic as well as different monoclinic forms. This polymorphism is of frequent occurrence in n-aliphatic long-chain compounds. In monoclinic forms, the long-chain spacing is shorter than the length of the molecules, as these are placed inclined between the basal planes.

The tilt of the chains, with respect to the basal planes, is confined to definite angles, each angle representing a crystal form. According to SCHOON (1938), this preference for definite angles is a result of the zig-zag structure of the chains, since inclination of the chains involves a mutual shift of the chains in the chain direction. After each mutual shift of adjacent chains over a distance of two carbon atoms, there will occur a certain energetically advantageous and, therefore, preferred interlocking of the zig-zags representing a more or less stable crystal form.

If one of a homologous series of long-chain compounds can assume e.g. three different crystal forms, these forms are also shown by other homologues; though not, generally, by all. Frequently, a particular crystal form occurs either in the higher or the lower homologues of the series exclusively, or either in the even or the odd ones, and certain forms are stable only within a given temperature range. The appearence of a special form is also dependent on the solvent from which the substance has crystallised out and on the purity of the substance.

When plotting graphically the number of carbon atoms against the long-chain spacings in a homologous series, one single straight line is obtained if only the rhombic form is taken into account. In a monoclinic form, even and odd terms give separate lines. This will be recognised, considering that the increase of the long crystal spacing per carbon atom is always the same when the chains are placed normal to the basal planes, whereas in the case of inclined positions alternation of two different increments is found (fig. 3).

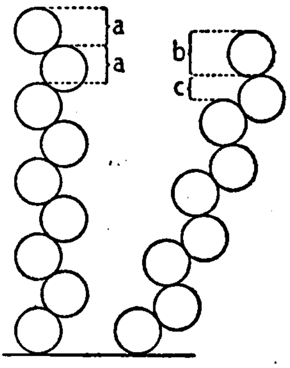

Fig. 3. The increment of the long-chain spacing per carbon atom: a constant amount *a* in chains at right angles to the basal plane, or alternating different amounts *b* and *c* in chains at oblique angles.

The relation between the number of carbon atoms and the long-chain spacing, once established in various polymorphic forms characteristic of a given homologous series, enables the determination of the number of carbon atoms of an unknown homologue from its long-chain spacing measured by means of X-ray diffraction. The results obtained by PIPER, CHIBNALL et al., mentioned before, are based on this principle and so are those of our investigations.

The chain molecules with a chemically active terminal group, such as CH_2OH or COOH deserve special attention. When comparing the diagrams of e.g. long-chain paraffins with those of long-chain fatty acids or primary alcohols of nearly the same chain length as the paraffins, it appears that in the diagrams of the latter substances the long-chain reflections are much nearer the centre than in those of the paraffins. Consequently, the long spacings must be much longer than in paraffins. These spacings, at least if only rhombic forms are taken into account, are nearly twice the spacings in paraffins of the same chain length. The explanation of this phenomenon is given by the fact that in alcohols and acids the chains lie with their particular terminal groups end to end. It is supposed that the opposite electrical charges of these dipole groups are in juxtaposition. Accordingly, the identity periods in the direction of the chains are doubled, which results in smaller diffraction angles of the long-chain reflections. In fig. 4, quadrant I and IV, the two types of arrangement and the corresponding long-chain reflections are shown diagrammatically.

The COOH and CH_2OH double-layers also affect the intensities of the long-chain reflections. Their influence appears to be to diminish the intensities of the even orders of reflection and to enhance the odd orders. This phenomenon can be explained from the particular distribution of scattering matter over the long-chain period, showing an excess at the centre and a deficiency at each end (see e.g. Prins 1926). In fig. 4, IV the weakened even orders are represented by broken lines.

Non terminal characteristic groups in the chain also influence the intensities of the long-chain reflections. Generally, however, they do not influence the spacing. The relation between the position of a side group in the chain and the reflection intensities was originally investigated for a series of ketones by Saville and Shearer (1925). It appears that the excess electron density, localised at the position of the $C = O$ group, has a similar influence on the intensities as the double-layers of CH_2OH and COOH in the primary alcohols and fatty acids if the position of the $C = O$ group is the centre of the chain: The even orders are practically extinguished, whereas the odd orders are clearly visible and show the normal fall of intensity from lower orders to higher, cf. fig. 4, II.

If, however, the $C = O$ group is not localised at the centre of the chain, but at $1/p$ (in which p represents a small integer), the pth order of the long-chain reflections, as well as the orders with numbers which are multiples of p, show the strongest decline in intensity. Accordingly, the position of the $C = O$ group can be determined by studying the long-chain reflections. In fig. 4, III the intensities are shown in the case of $p = 3$. If the $C = O$ group occurs at the penultimate place of the chain, the terminal $C = O.CH_3$ group has a dipole nature and in this case double-layers are formed, as in primary alcohols and fatty acids. In natural waxes, these ketones have not been found.

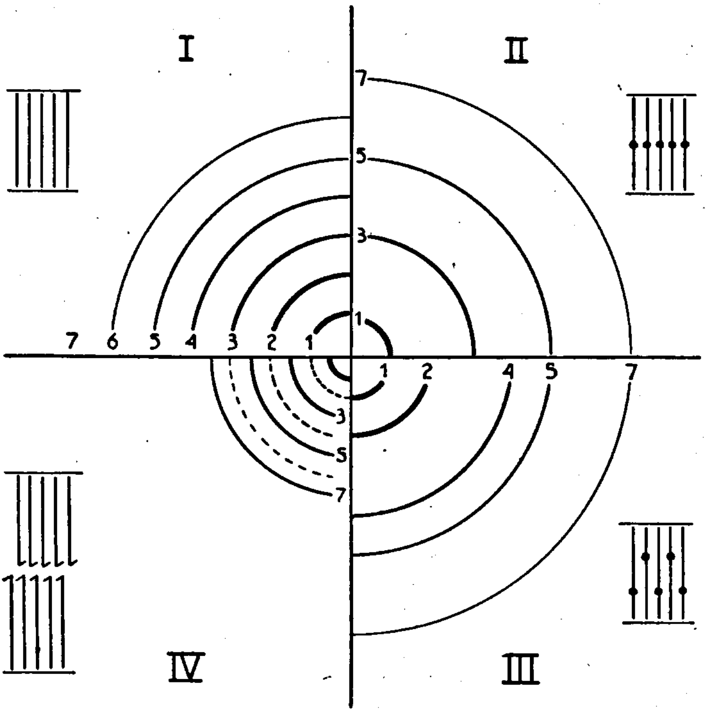

Fig. 4. Diagram showing types of molecules of frequently occurring wax constituents, as placed between the net-planes with long spacing, and their characteristic sets of long-chain reflections. The zig-zag chains are represented by straight lines. The molecules are supposed to possess equal chain lengths and to be crystallised in the rhombic modification.

I. Paraffins. II. Symmetric ketones and sec. alcohols. III. Ketones and sec. alcohols with the side group at 1/3 of the chain. IV. Prim. alcohols and fatty acids.

In view of the conclusions to be drawn later from the diagrams of native cuticle waxes, it is of importance to know what intensity fluctuation appears if the characteristic group is not situated at exactly 1/*p* of the chain length, but at the place of a carbon atom adjacent to the carbon atom at 1/*p*. In other words, to what degree of accuracy will it be possible to determine the position of the side group from regularly distributed extinctions?

As concerns the places $^1/_2$ and $^1/_3$, this has been considered by PIPER, CHIBNALL, et al. (1931) in the case of C_{29}-ketones, with the $C = O$ group at different positions. In fig. 3 of their publication, photographs of diagrams of this series of ketones are shown in which the first 7—10 orders are perceptible. It appears from this figure that, if the keto group does not occupy the centre place in the chain (the place of carbon atom 15), but place 14, the intensity fluctuation is clearly distinguishable from that in the first case: The even orders are extinguished, as in the first case, but also the odd orders are much weakened, and no reflections appear of higher order than the 5th, whereas in the first case the 9th order is still visible. We may conclude that, if the even orders are almost extinguished and strong odd orders are present among which higher orders than the 5th are visible while, in addition, these odd orders show a normal gradual fall in intensity, it can be taken for granted that the keto group occupies the centre place of the chain.

The figure also shows that, if the keto group is found at the place of carbon atom 10, i.e. almost exactly at $^1/_3$ of the chain, or at the place of carbon 9, i.e. less exactly at $^1/_3$, namely at $^1/_{3\cdot3}$, this difference also clearly finds expression in the intensities of the long-chain reflections. In the former instance, the orders 1, 2, 4, 5, 7 and 8 are visible with gradually falling intensities, while 3 and 6 are extinguished, whereas in the latter, only 1, 2 and 5 are clearly visible, while 4 is weak and 3 and 6 are extinguished.

From the foregoing it will be clear that the intensity fluctuation of the long-chain reflections enables the occurrence of a $C = O$ group to be established at exactly $^1/_2$ or at almost exactly $^1/_3$ of the chain length.

Though the figure of PIPER and CHIBNALL discussed above also shows a diagram of a C_{29}-ketone with the keto group at $^1/_{2\cdot5}$, we will leave out of account other positions than $^1/_2$ and $^1/_3$, since our diagrams only give occasion to consider these latter positions.

Secondary alcohols show, according to PIPER and CHIBNALL (1931), the same phenomena as ketones. The molecules show more tendency to incline with respect to the basal planes, however, even though the rhombic form can occur as well.

In **wax esters**, as in paraffins, ketones and sec. alcohols, the long-chain spacing corresponds to the length of one molecule, as has appeared from the investigations of SHEARER (1923), MALKIN (1931) and KOHLHAAS (1938). The question whether in long-chain esters the place of the O—C = O group can also be determined from the intensities, does not appear to be discussed in detail in the literature on the subject.

The esters have been omitted in fig. 4, since sufficient data on these compounds is not available. We suppose, however, that an ester consisting of an acid- and alcohol radical, each of the same length as assumed for the other components represented in fig. 4, would show a set of long-chain reflections, nearly corresponding to those of quadrant IV.

3. *Material and Apparatus*

a. **Choice, sampling and preparation of the waxes.** The choice of plants from which wax was collected, has mainly been determined by the presence of certain wax-bearing plants in the botanical gardens of the Laboratory of Technical Botany, Delft, in the Hortus Botanicus at Leiden and in the vicinity of Delft. In order to be able to ascertain how far similarities and differences between the wax diagrams may reflect natural relationships between plants, wax was collected, if possible, from more than one species out of the families of which wax-bearing plants were at our disposal.

In addition to the native waxes which had been scraped off plant epidermises, certain wax samples have been investigated which were by-products of scent industries and which had been obtained from flowers by extraction with petroleum ether. These waxes were present in the collections of the Laboratory of Technical Botany and the same samples had been investigated by STRAMAN (1926) in that institute.

Furthermore, some of the waxes examined, such as those of red cabbage, apple peel, wheat stems and the flower waxes mentioned above, were particularly investigated because the literature provides data about waxes of these species which might serve to check the reliability of our methods.

Sampling was done by means of glass strips of c. 7.5 mm by 4.5 by 1, which were pushed along stems or leaves by one of the long edges, until a sufficient quantity of wax had collected against the edge. In order to prevent the occurrence of cellulose and other cell wall material in the samples, care was taken that the epidermis was not ruptured during this procedure. The strips were kept in small sample tubes, in which they were fixed between cork and bottom, so that the edge of the strip could not touch the wall of the tube and distribution of the small amount of wax over the tube wall was prevented as far as possible.

For mounting in the X-ray spectrometer, the wax was removed from the glass strip and was placed in the central opening of a small, round, flat, celluloid specimen holder, which was c. 7 mm in diam., 0.24 mm thick and which had a central opening of 0.5 mm diam. For this purpose a mounted needle was used with a flattened point, transformed into a small bent knife. The wax was loosely pressed into the opening with the aid of the outer bent flat of this knifelet. Pressing too tightly may be detrimental to the long-chain reflections, as experience has taught us. In cases where, owing to coarseness or hairs of the plant surface, the glass strips could not be used, plant material was collected and the wax was scratched away out of hollows and between hairs by means of the above-mentioned small knife.

b. The choice of a method of X-ray photography.

Generally, for the examination of long-chain compounds, a very thin layer of the substance is pressed on to a glass plate, or is melted on such a plate, after which it is allowed to solidify. The plate is mounted in an oscillating-type spectrograph perpendicularly to the table. It is oscillated a few degrees about the camera axis during exposure, while the beam strikes the specimen at a small angle. This procedure has certain advantages because the crystallites orientate on the glass plate with the lattice-planes with long spacing parallel to the surface. For further details we refer to MÜLLER (1923) and to the general literature on X-ray crystallography. We did not use this technique because in mixtures such as native waxes, pressure or melting and solidifying may introduce complications and because we intended to observe the side-spacing reflections as well; these may become less clear when this method is followed. For comparing a more extensive number of native waxes which generally are available in only very small quantities, the simpler, common powder method seemed more advantageous.

c. The camera.

We used cameras for flat films such as originally devised by ASTBURY and WOODS (1934), in a somewhat improved design as in use at the Laboratory of Technical Physics, Delft. It is not intended to go into details here, but the following is a brief description of the apparatus. The principle is demonstrated in fig. 5 and a photograph is given in fig. 7; the parts have been correspondingly numbered.

A parallel beam of radiation is obtained by a cylindrical lead-glass capillary (1) with an inner diameter of 0.5 of 0.25 mm, as required, and a length of 4 cm. These pinholes, mounted in a brass cylinder (2), can be slipped into a cylindrical setting (3) of the camera. For powder photographs, the powdered substance is pressed into the central bore (4) of a round glass specimen holder (5*a*) mounted on a ring (6) which latter can be fitted in front of the pinhole.

The film holder (7) can be moved to and fro on a triangular rail (8) in order to adjust the distance specimen-film. The front side of the film

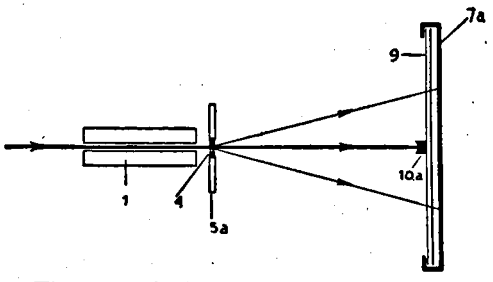

Fig. 5. Principle of X-ray powder photography on flat film.

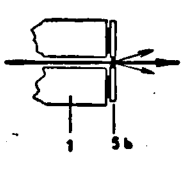

Fig. 6. Method of fitting the celluloid specimen holders.

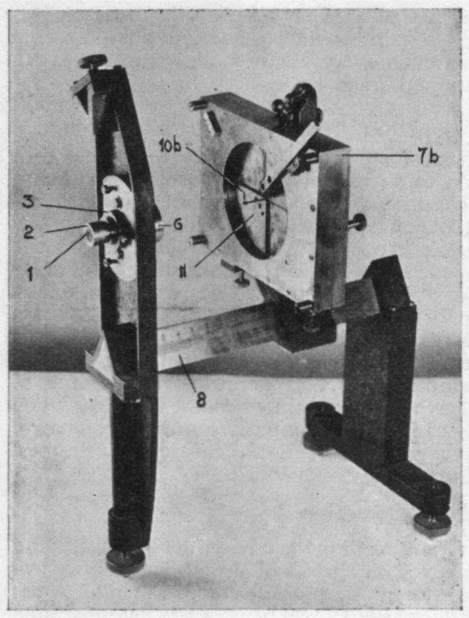

Fig. 7. X-ray camera for flat films.

cassette is closed by aluminum foil 20μ thick (9) and the primary X-ray beam is caught by a lead cup: (10).

Two types of cassette are provided: a normal, so-called open cassette (*7a*) and one (*7b*), generally used in this investigation. The latter is fitted with an arrangement that enables one to make two photographs on one film to facilitate comparison of diagrams. If comparison is not strictly necessary, the latter holder, nevertheless, has the advantage that a film needs to be put in only once for making two photographs. The arrangement consists in a disc (11) in front of the aluminum foil, revolving over 90°. In this disc two opposite quadrants have been cut out, thus making it possible to expose first e.g. the quadrants 1 and 3 of the film and then 2 and 4. In the centre of the disc, the left quadrants are linked through a small disc of about 3 mm diam. which, like the whole revolving disc, is c. 2 mm thick, and serves as primary-beam stop.

For our wax investigations, the standard technique described above was slightly modified as regards two points, namely, the specimen holder and the beam stop.

d. The specimen holder.

The glass specimen holders were replaced by smaller and thinner ones of celluloid, which were punched out of an old unexposed fixed film and have already been described sub *a*. These celluloid holders were used because it is easier to make a small perforation in celluloid than in glass, and small perforations are required because of the limited amounts of wax available. The contents of the perforation in the celluloid holders was c. $^{1}/_{20}$ mm^3, which is c. $^{1}/_{18}$ of the contents of the opening in the usual glass holders and which represents the minimum required volume of wax when pressed together.

Another advantage of the celluloid specimen holders is that they can more easily be made in great quantities than glass ones, so that it is possible to take a new holder for each sample and to preserve the examined specimens in their holders. Moreover, the particulars of the preparation can easily be engraved in the celluloid.

When making a photograph we fixed the celluloid specimen holder with some mounting wax to the terminal plane of the glass capillary, cf. fig. 6. Pinhole and specimen opening must be brought in alignment to prevent the radiation from striking the celluloid. This is in particular necessary when the 0.5 mm capillaries are used, as these have the same diameter as the specimen opening. It is easily achieved when the capillary is set in upright position under a microscope and a beam of light is sent through it.

e. The beam stop and scattered radiation.

In our first photographs, especially when they were made with 0.5 mm pinholes, a central diffuse blackening due to scattered radiation from the air, partly overshadowed the long-chain reflections. It has appeared that this fault could be remedied by placing the primary-beam stop not immediately before the film, but at a distance of c. 1.5 cm in front. For this purpose a lead cup (10b) was mounted on the end of a brass rod of c. 1.5 cm long, which at its other end was screwed into the centre of the revolving disc.

When 0.5 mm pinholes are used, the scattered radiation from the rim of the glass capillary is rather strong, but in powder photographs, if they are made in the way described, the radiation is not very troublesome, as it is partly absorbed in the specimen and in the celluloid of the specimen holder.

f. The distance specimen-film.

The specimen-film distance was usually adjusted at exactly 40 mm. This makes the photographs strictly comparable amongst themselves, and at this distance both the long-chain reflections and the side-spacing reflections are clearly shown in the diagrams. The diameters of the long-chain reflections are indeed on the small side for allowing very accurate measurements of long spacings, and as a rule the reflection of the first order falls away within the area of the central spot. Nevertheless, the presence of various higher orders enables measurements of the long spacings with an accuracy of 0.1—0.4 Å.

Initially, a number of photographs was made at both smaller and greater distances than 40 mm, the latter especially to ascertain the presence of first-order long-chain reflections. In table I the distance is always mentioned when it was not 40 mm.

g. Pinholes.

The photographs were always made first with 0.5 mm pinholes. In most cases it appeared desirable to take a second photograph with a 0.25 mm pinhole, as the long-chain reflections often turn out too broad to allow a sufficiently accurate measurement when a 0.5 mm capillary is used. This, however, requires a nearly 8-fold exposure time. In table I the data which have been obtained from diagrams made with 0.5 mm pinholes only, are marked with an asterisk.

h. The measurement of the diameters of the reflection circles.

The reflection diameters were measured with a ruler subdivided no further than in millimeters. Tenth parts of millimeters were estimated. In practice this estimation appears to be correct to c. 0.1 mm.

Measurement of very weak higher orders of long-chain reflections offers difficulties, as the observation of these faint lines is much hindered by the strong contrasts of brightness between the film and the edge of the ruler. The measurement was carried out as follows: The cellophane envelope, in which the photograph was stored, was pressed tightly to illuminated opal glass, In absence of the ruler, when the weak lines are better visible, two black ink marks were made at opposite points of the reflection circle, as accurately as possible in the middle of the line. The distance between these points on the cellophane is easily measurable.

i. Sources of radiation and exposure times.

The photographs were made with Cu K_α radiation, filtered by a 10μ Ni filter. As a source of radiation the X-ray tube with rotating anticathode of the Laboratory of Technical Physics was generally used. The tube was run at 25 kv and 100 ma from a high tension equipment for rectified alternating current with four rectifiers.

For obtaining sufficiently exposed photographs with a specimen-film distance of 40 mm and a 0.5 mm pinhole, the exposure time amounted to c. one hr. When a 0.25 mm pinhole was used this time became c. 8 hrs.

In some cases Philips Metalix tubes were used, i.e. sealed-off fix-focus tubes, run at 44 kv peak and 25 ma from the same high tension equipment as the former tube. In this case, the exposure times amounted to c. 3 hrs and 30 hrs for the two kinds of pinholes respectively. In contrast to the first tube, these tubes have two windows which can be used simultaneously.

The dimensions of the apparent focus (i.e. the focus seen at an angle of approximately 6° to the surface of the target, which is the direction of the radiation passing through the window of a tube) was c. 0.8 mm by 1 mm in the tube with rotating target and 1.2 by 1.2 mm in the Metalix tube, while the distances from focus to pinhole in both tubes were c. 20 mm and 40 mm respectively.

The total exposure time used for the comparative investigation of waxes, amounted to c. 210 hrs with the rotating-target tube, plus c. 460 hrs (i.e. hours per window) with the Metalix tube.

The total number of photographs made for this investigation was 108.

The film material generally used was Agfa high-contrast X-ray film, coated on both sides.

CHAPTER II

COMPARISON OF THE DIAGRAMS OBTAINED

1. *Classification of the Diagrams*

The differences between the diagrams obtained are considerable both as regards the long-chain reflections and the side-spacing reflections. In certain cases there are no long-chain reflections at all; in others they are present in several higher orders. In some cases their higher orders gradually decrease in intensity, whereas in others the intensities are alternatingly weak and strong or show a less regular fluctuation. The group of side-spacing reflections, though generally corresponding to that characteristic of most n. aliphatic long-chain compounds, in many cases turns out to be different. The attempt will be made to bring some order into the various diagrams obtained from the 60 different waxes.

A superficial glance reveals immediately that the diagrams can be classified into three groups, merely on account of differences in the side-spacing reflections. Furthermore, within each group certain types can be distinguished, in which both the long-chain and side-spacing reflections are used as criteria. As regards one type it appeared desirable to give a division into sub-types.

In order to simplify the discussion which will follow later, we shall provide these groups, types and sub-types with a letter indication. In that discussion (par. 5, chapt. III) we shall enter into the differences between the diagrams in more detail than in the classification given below.

We now pass on to the classification:

The largest group of diagrams shows the general type as demonstrated in fig. 1. The diagrams within this group are, therefore, merely distinguishable on account of their l.c. refl. [1]

This group, on account of its showing the normal set of s.s. refl., is marked "N".

Group N can be subdivided into a number of types, which include:

1. a type showing l.c. refl. which correspond to spacings of 35—45 Å. This type, marked "Ns", can be divided into the subtypes:

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[1] From now on the terms long-chain reflections and side-spacing reflections will be abbreviated: "l.c. refl." and "s.s. refl."

PLATE XXIII.

Fig. 8. Wax diagrams of:		Fig. 9. Wax diagrams of:	
I. *Cotyledon orbiculata,*	type Nsa	I. *Secale cereale,*	type DG
II. *Xanthosoma violaceum,*	„ Nsb	II. *Eucalyptus globulus,*	„ DG
III. *Allium porrum,*	„ Nsc	III. *Papaver somniferum,*	„ DP
IV. *Saccharum officinarum,*	„ Nl	IV. *Picea pungens,*	„ DP

The quadrants are slightly enlarged from the original photographs made at 40 mm distance from the specimens. The original of fig. 8, IV was made at c. 35 mm and is enlarged to the same scale as the others. The quadrants separated by a narrow white line occur on the same film.

*Photographs obtained by using 0.5 mm-pinholes, (see chapt. I, 3).

Nsa, in which all orders of the l.c. refl. generally shown in the diagrams, i.e. 4 to 7 orders, are present in gradually falling intensities,

Nsb, showing weak and strong l.c. refl. in regular alternation and gradually falling intensities, in which the weak series is, occasionally, practically extinguished,

Nsc, showing weak and strong l.c. reflections with a less regular intensity fluctuation.

2. a type in which the l.c. refl. correspond to long spacings of 70—80 Å, marked "Nl".
3. a type in which no l.c. refl. can be observed, marked "No".

Examples of these types are shown in the quadrants of fig. 8, except of type No.

A second group does not show the normal diagram of fig. 1, as the s.s. refl. show deviations from this type.

The group is indicated by "D".

Within this group two types are distinguished;

1. a type in which the two strongest s.s. refl. are slightly closer together than in group N, while the other ones show deviating intensities. The l.c. refl. are present in alternating weak and strong intensities.
   This type, found in cereals and in *Eucalyptus,* is indicated by "DG".
2. a type showing 3 strong s.s. refl. at about the same place as the two strongest in group N and showing only 2 additional s.s. refl., which are weak and not conterminous with any of the weaker s.s. refl. in the diagrams of group N.
   The l.c. refl. are clear, but the orders 3 and 6 are missing.
   The type is found in certain *Papaveraceae* and in *Picea pungens* var. *glauca,* and has been indicated by "DP".

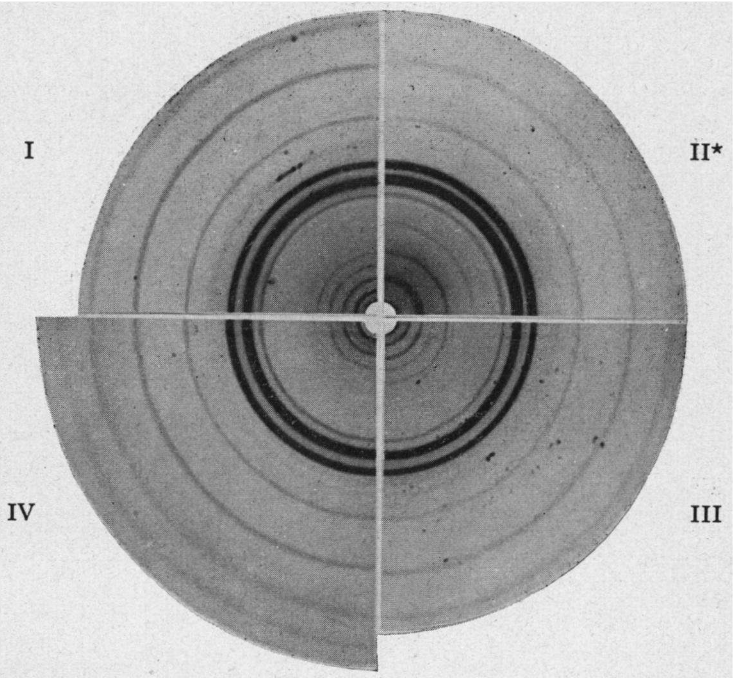

Fig. 8

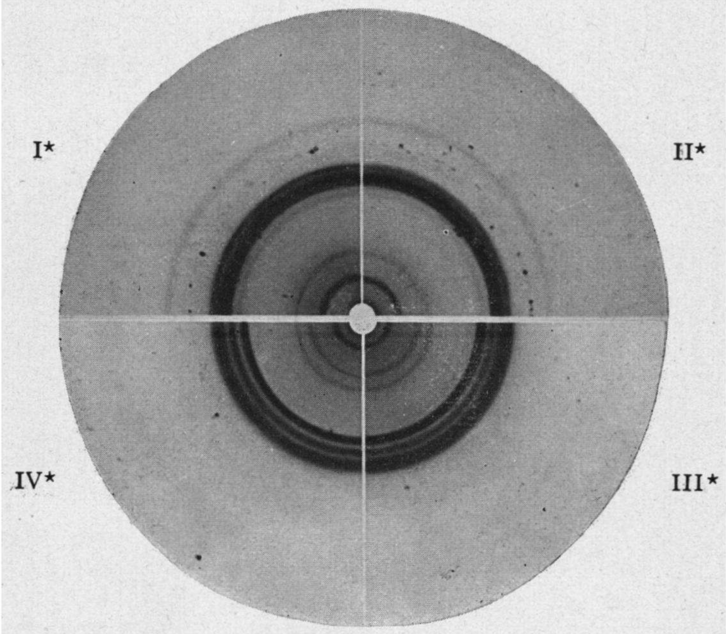

Fig. 9

PLATE XXIV

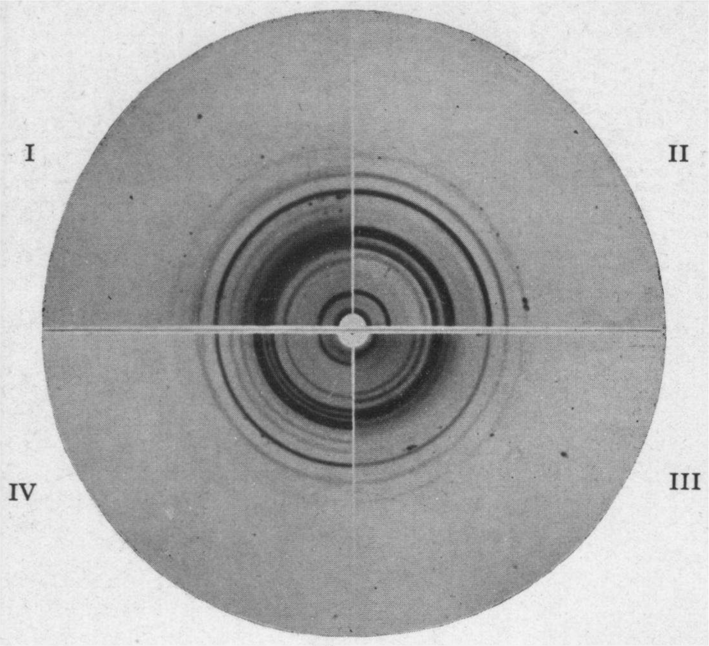

Fig. 10

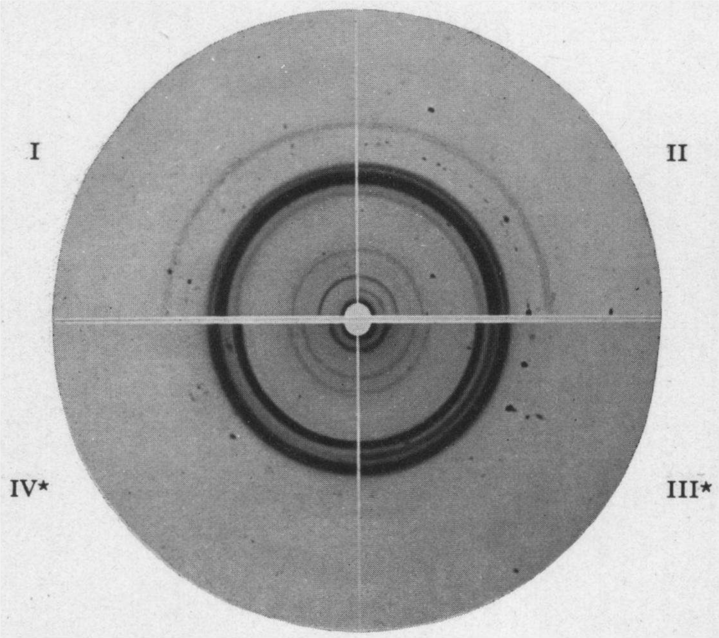

Fig. 11

PLATE XXIV.

Fig. 10. Wax diagrams of:

I.	*Echeveria globosa*,	type MC
II.	*Kalanchoë marmorata*,	„ MS
III.	*Ricinus communis*,	group M
IV.	*Benincasa cerifera*,	„ M

Fig. 11. Wax diagrams of:

I.	*Hordeum vulgare*,	type DG
II.	*Triticum vulgare*,	„ DG
III.	*Glaucium flavum*,	„ DP
IV.	*Argemone mexicana*,	„ DP

For further details see letter-press to Plate XXIII.

Examples of these types are shown in the quadrants of fig. 9.

In addition, two separate instances of diagrams, which will be dealt with later, have also to be included in this group. They show some resemblance to DP, but have been marked "D" only in table I.

A third group shows the characteristic paraffin diagram, mixed with a diagram without any resemblance to the former.

This group is marked "M".

The group includes two types:

1. a type observed in most *Crassulaceae* and marked "MC",
2. a type found in succulent *Compositae* and in one *Crassulacea*, marked "MS".

These types often show l.c. refl. indicating much shorter spacings than these reflections usually do. They probably originate from a non-waxy component in the wax.

Among this group the diagrams of *Ricinus*- and *Benincasa*-wax are to be classed, the former showing most resemblance to MC and the latter to MS, though they differ too much from these types to be included in either of them. They are marked "M" only in table I.

The diagrams of the third group are shown in fig. 10.

Finally, in fig. 11 some diagrams are shown, which may serve to demonstrate differences which may occur between diagrams of the same type originating from allied species: I and II are different in that the first l.c. refl. in II is clearly stronger than in I; III and IV are different in that the second s.s. refl. in III is stronger than in IV.

TABLE I.

I. Survey of data on all wax diagrams obtained. Explanation is given in the next section.

Family	Species	$2f_{40}$ (in mm)	d	Intensity	n	l (in Å)	l (average)	Type of diagram	Origin of the wax
Palmae	*Copernicia cerifera*	2.8	43.6	s	1	43.6	**44.0**	Nsa	commercial
	(70)	5.5	22.3	f	2	44.6	±0.4		wax
		8.5	14.6	ff	3	43.8			
Crassulaceae	*Cotyledon*	5.7^5	21.37	s	2	42.7		id.	leaf
	orbiculata	8.6	14.36	f	3	43.1	**42.9**		
		11.6	10.69	ff	4	42.8	±0.2		
		14.5	8.59	fff	5	42.9			
Rosaceae	*Rosa centifolia*	3.3	37.6	s	1	37.6		id.	petals
	(70)	6.4^5	19.1	f	2	38.2	**37.9^5**		(scent industries)
		9.7^5	12.6^8	ff	3	38.0	$\pm 0.2^5$		
Violaceae	*Viola odorata*	3.2	38.5	s	1	38.6		id.	petals
	(65)	6.4	19.3	f	2	38.6	**38.6**		(scent industries)
		9.7	12.8	ff	3	38.4	±0.1		
		12.8	9.7	fff	4	38.8			
Oleaceae	*Jasminum*	5.9	20.9	s	2	41.8	**41.5**	id.	petals
	grandiflorum	9.0	13.7	f	3	41.2	±0.4		(scent industries)
Araceae	*Xanthosoma*							Nsb	leaf
	violaceum	2.9^5	41.7	s	1	41.7			
	(75)	8.9^5	13.8	f	3	41.4	**41.4***		
	Colocasia	15.0^5	8.2^7	ff	5	41.3^5	±0.2		
	antiquorum	21.5	5.8^7	fff	7	41.1			
	(75)								
Cruciferae	*Brassica oleracea*	6.3	19.5^7	f	2	39.1^4		id.	leaf
	var. *capitata*	9.5	13.0^1	s	3	39.0^5	**39.0**		
	Crambe maritima	12.7^5	9.7^3	ff	4	38.9	±0.1		
		16.0	7.8^0	sf	5	39.0			

Mimosaceae	*Acacia dealbata* (100)	2.8	43.6	s	1	43.6	43.6 ±0.1	id.	flowers (scent industries)
		8.4^5	14.6	f	3	43.8			
		14.3	8.7	ff	5	43.5			
Liliaceae	*Allium porrum*	5.9	20.89	s	2	41.8	41.6 ±0.2	Nsc	leaf
		8.9	13.88	s	3	41.6^5			
		12.0	10.34	f	4	41.3^5			
		15.0	8.32	f	5	41.5			
	Allium fistulosum	8.9	13.88	s	3	41.6^5	41.6* ±0.2	id.	leaf
		12.0	10.34	f	4	41.3^5			
		15.0	8.32	f	5	41.5			
	Aloë vera	4.7^5	26.2	f	3	78.6	41.7 ±0.1	id.	leaf
		5.9	20.9	s	2	41.8			
		7.5	16.5	ff	5	82.4			
		8.9	13.9	sf	3	41.7			
		11.9	10.4	sff	4	41.6			
Umbelliferae	*Foeniculum vulgare*	6.3	19.5^2	s	2	39.1	39.1* ±0.1	id.	stem
		?		ff	3				
		12.7^5	9.7^3	f	4	39.0			
		16.0	7.8	ff	5	39.0			
		22.5	5.6	fff	7	39.2			
Rosaceae	*Pyrus malus*	6.4	19.2	s	2	38.5	38.7^5 ±0.2	id.	apple peel
		9.6	12.7	ff	3	38.7			
		12.8	9.7	f	4	38.8			
		16.0	7.8	ff	5	39.0			
Gramineae	*Saccharum officinarum* (70) *Coix lacrima* *Andropogon sorghum* *Eulalia japonica*	4.9^5	24.9	s	3	74.7	75.2 ±0.4	Nl	stem
		6.5	18.8^3	f	4	75.7			
		8.2	15.0^6	sf	5	75.3			
		9.9^5	12.4^3	ff	6	74.6			
		11.4^5	10.8^2	sff	7	75.7			

TABLE I (continued)

Family	Species	$2f_{40}$ (in mm)	d	Intensity	n	ι (in Å)	l (average)	Type of diagram	Origin of the wax
	Saccharum officinarum	3.1	39.7	s	1	39.7	40.0 ±0.2	id.	wax from aged press cake (from cane-sugar manufacturing process)
		4.5	26.9	f	3	80.6			
		6.15	20.0	sf	2	40.3			
		7.8	15.8	ff	5	79.0	79.6 ±0.8		
		9.2	13.4	sff	3	40.2			
	Saccharum spontaneum (65) *Phragmites communis*	5.3^5	23.1	s	3	69.4	70.1 ±0.4	id.	stem
		7.1	17.4	f	4	69.7			
		8.8	14.1^5	sf	5	70.7			
		10.5	11.7^5	ff	6	70.6			
		12.4	10.0	sff	7	70.0			
	Arundo donax	4.7^5	25.9	s	3	77.7	78.2 ±0.4	id.	stem
		?		f	4				
		7.8^5	15.7	sf	5	78.5			
		?		ff	6				
		11.0	11.2	sff	7	78.4			
Liliaceae	*Yucca filamentosa*	4.9^5	24.9	s	3	74.7	74.6 ±0.2	id.	leaf
		8.2^5	14.9	sf	5	74.5			
Iridaceae	*Iris florentina* *I. germanica*	4.8	25.6	s	3	77.0	77.0 ±0.1	id.	leaf
		8.0	15.4	sf	5	77.0			
Musaceae	*Musa paradisiaca* (65)	4.1	30.3	s	2	60.5	60.5 ±0.2	id.	young petiole
		6.1	20.2	s	3	60.4			
		8.1^5	15.1^5	ff	4	60.6			
	Strelitzia reginae (50)	3.7	33.4	s	2	66.9	65.2 ±1.2	id.	flower-stalk and bracts
		5.7	21.6	s	3	64.8			
		7.7^5	15.9	ff	4	63.6			

Liliaceae	*Hyacinthus* sp.	no long spacings						No	petals (scent ind.)
Cannaceae	*Canna flaccida*								leaf and flower-stalk
Bromeliaceae	*Aechmea miniata*								leaf
Cactaceae	*Cereus peruvianus*								phylloclade
	C. macrogonatus								”
	Opuntia robusta								”
	O. crassa								”
Gramineae	*Triticum vulgare*	6.0	20.5	f	2	41.0	40.7 ±0.3	DG	stem and leaf-sheath
	Avena sativa	9.1	13.6	s	3	40.8			
	Secale cereale	12.3	10.1	ff	4	40.4			”
		15.4	8.1	sf	5	40.5			
		21.6	5.84	sff	7	40.9			
	Hordeum vulgare	6.0		f	2		id.	id.	”
	Elymus arenarius	9.1		s	3				leaf
	E. sybiricus	15.4		sf	5				stem
Myrtaceae	*Eucalyptus globulus*	8.5	14.53	s	3	43.6	43.5^{5} ±0.3	id.	leaf
		14.3	8.70	sf	5	43.5			
Papaveraceae	*Papaver somniferum*	6.4	19.2^{5}	s	2	38.5	38.6* ±0.2	DP	fruit
	Glaucium flavum	12.8	9.7	f	4	38.8			”
	Argemone mexicanum	16.2	7.7	ff	5	38.5			leaf
Abietaceae	*Picea pungens* var. *glauca*	23.0	5.5	fff	7	38.5			needles
Rosaceae	*Rubus biflorus*	7.0	17.6	s	2	35.2	35.5 ±0.2	D	woody stems
		14.1	8.9	f	4	35.6			
		17.5	7.1^{5}	ff	5	35.5			

TABLE I (continued)

Family	Species	$2f_{40}$ (in mm)	d	Intensity	n	l (in Å)	l (average)	Type of diagram	Origin of the wax
Cruciferae	*Lepidium latifolium*	7.0 14.2 17.7	17.9 8.7^5 7.1	s f ff	2 4 5	35.8 35.0 35.5	**35.6*** ±0.3	id.	stem
Myricaceae	*Myrica cerifera*	5.3 8.3	23.2^5 14.9	s s	2 3	46.5 44.7	**45.6** ±1.0	id.	fruit
Crassulaceae	*Echeveria globosa* *Pachyphytum oviferum* *Cotyledon Desmetiana* *C. glauca* *C. gibbiflora* *Graptopetalum amethystinum*	7.6 15.4	16.2^5 8.1	s f	1 2	16.2 16.2	**16.2** ±0.1	**MC**	leaf and stem » » » » »
Compositae Crassulaceae	*Senecio Galpinii* *Kleinia repens* *Kalanchoë marmorata*	3.9 8.1 12.3 16.5	31.5 15.2 10.1 7.6	f s ff sf	1 2 3 4	31.5 30.4 30.3 30.4	**30.4** ±0.3	**MS**	leaf and stem » »
Cucurbitaceae	*Benincasa cerifera*	7.6 15.4	16.2^5 8.1	s f	1 2	16.2^5 16.2	**16.2** ±0.1	**M**	fruit
Euphorbiaceae	*Ricinus communis*	5.4 9.2	22.8 13.4	s f	3 5	68.4 67.0	**67.7** ±1.0	id.	stem

2. *Tabular Survey of the Diagrams.*

A survey of all wax diagrams made, is given in the preceding table I. This table comprises a number of important data on the diagrams, with particular reference to the l.c. refl.

The s.s. refl. are not mentioned in this table. The types of the diagrams, as indicated in the foregoing section, are, however, included, and these infer certain data about the s.s. refl. More details of those reflections will be summed up in the discussion of the individual diagrams in sect. 5 of chapt. III; cf. table IV*a*, *b*, *c*, etc.

In the successive columns of table I are mentioned:

1. the family of the plant from which the wax has been collected.
2. the plant species.
3. the diameters of the l.c. refl. circles at a distance of 40 mm from the specimen, indicated by $2f_{40}$. If those diameters have been converted from diagrams made at other distances than 40 mm, the actual distance of photographing is given in brackets behind the species name in column 2.
4. the spacing *d*, calculated from the diameters mentioned in the foregoing column according to the BRAGG law given in Ch. I, 2. In this calculation, the order of the reflections, *n*, has been put equal to 1, because it is not evident without further comment that a set of l.c. reflections consists of higher orders belonging to one spacing. The reflections might belong to different periods, as may happen in mixtures. See also the explanations to columns 6 and 7.
5. the reflection intensities. These have been indicated as follows: s = the strongest reflection, f = the second strongest reflection, ff = the third strongest refl. etc. If strong and weak reflections alternate, the strong series is marked by s in all orders and, accordingly, is indicated by s, sf, sff, etc., while the weak series is indicated by f, ff, fff, etc.
6. the supposed order numbers *n*, presuming that all l.c. refl. in a diagram belong to the same spacing.
7. the long-chain spacing *l*, calculated for each l.c. reflection from $l = nd$.

   It appears that, if the reflections are assigned the order numbers mentioned in the foregoing column, generally, all l.c. refl. in a diagram point to practically equal spacings. Hence it must be concluded that the supposed order numbers are to be regarded as correct and that, generally, all l.c. reflections in a diagram do in fact belong to one spacing. For, if this were not the case it would be impossible te calculate equal values of *l* from the reflections by assigning them small whole order numbers, except under very special circumstances. In two cases, however, (wax of *Aloë* and sugar cane wax from press cake) it was impossible to consider all l.c. refl. as belonging to one series of reflections of higher order, cf. chapt. III, 5.

8. mean value of *l*, computed from the *l*-values of the individual l.c. reflections. The long spacings in this column marked with an asterisk have been obtained from photographs made with 0.5 mm-pinholes.
9. type of diagram. The sequence in which the types of diagrams are summed up in the table is the same as in the foregoing section. Generally, the sequence of plants within a type or subtype is the taxonomical one after v. WETTSTEIN.
10. data on the origin of the waxes.

CHAPTER III

INTERPRETATION OF THE DIAGRAMS

1. *The Possibility of a Chemical Interpretation of the Reflections belonging to the Long Crystal-Spacings*

It has been shown in the preceding section that the l.c. refl. exhibited by many of the diagrams generally consist of interferences which, within each set, all arise from one long-chain spacing. From this fact by itself, the conclusion can be drawn that a certain chain length predominates in the wax. For if the waxes contained very divergent chain lengths, present in nearly equal percentages, no l.c. refl. would be expected. This may be understood by considering the possibilities of constitution of the crystallites in the wax:

In the first place it could be imagined that in the wax molecules of divergent lengths are united to crystallites in such a way that each crystallite consists of molecules of equal length. Whether these molecules of equal length are also of the same nature or, in other words, whether e.g. sec. alcohol and paraffin molecules of the same length are mixed in one crystallite, is of no account. In this case groups of crystallites could be distinguished, each group possessing its own characteristic chain length. Each of these crystallite groups will give its own series of l.c. refl., though of low intensity, since its corresponding crystallite group represents only a small proportion of the wax. Furthermore, the reflections from the different groups of crystallites will lie closely together as they all have comparatively small diffraction angles. Owing to these two circumstances, the reflections will not be resolved unless very narrow pinholes and long exposure times are used.

In the second place we may imagine that the molecules of divergent lengths do not occur in separate crystallites, but that they are found mixed in each crystallite. In this case a regular packing can be spoken of only with respect to directions at right angles to the chain direction. Owing to the difference in chain length, the ends of the molecules cannot lie in equally spaced planes and, on account of this lack of periodicity in the chain direction, no long chain reflections will appear.

If, however, molecules of a certain length occur in greater quantity than those of other lengths, we may expect l.c. refl. for both possibilities discussed above for the constitution of the crystallites. With the possibility discussed first, the crystallite group consisting of the molecules of the predominating length will reflect more strongly than the other groups. If this group represents a sufficiently large proportion of the wax, its reflections will become perceptible. In the second case discussed, the molecules of the predominating length might give rise to a certain periodicity in the chain direction of the crystallites. Hence in this case l.c. refl. may be expected too.

In anticipation of section 4, we mention here that if 2 or 3 components, differing only slightly in chain length, make up the bulk of the wax, long-chain reflections appear which belong to one spacing.

In the following discussion we shall enter into the question whether it is possible to draw conclusions concerning the abovementioned predominating chains in the waxes in virtue of the l.c. refl. ascribed to them.

From the values of the long-chain spacings as given in table I, it may be seen that two groups of long-chain spacings can be distinguished; a small group of spacings lies within a range of c. 70—80 Å, whereas most others have values of c. 35—45 Å. It is a plausible inference that the predominating components of the waxes show little variation in chain length and that, in the cases of the longer spacings, the molecules are placed, with a terminal characteristic group end to end, so that the period of identity in the chain direction embraces two molecules. In the waxes with shorter spacings the period might correspond to the length of one molecule (see chapt. I, 2). In the former instances the reflections could be ascribed to prim. alcohols, fatty acids or wax esters, whereas in the latter cases the presence of paraffins, ketones or sec. alcohols is to be suspected. Further data concerning the nature of the commight be derived from the intensities of the l.c. refl. on account of the details concerning the intensities mentioned in chapt. I, 2 (cf. fig. 4). However, it may be possible to draw more profound conclusions, in which it will automatically appear wether or no the above suppositions are correct.

As mentioned in chapt. I, 2, there is a linear relationship between the number of carbon atoms and the long-chain spacing in all the above-mentioned substances. This relationship, once established, allows the determination of the number of carbon atoms in a compound of unknown chain length from its long-chain spacing. For this purpose, however, it is necessary to know the crystal modification in which the compound is present and it should not be mixed with neighbouring homologues, since these affect the long spacing. As to the natural waxes, we know nothing about the crystal form in which the components are present and the waxes are mixtures. Both these circumstances seemingly present insuperable difficulties in drawing further conclusions from the l.c. spacings measured.

The literature on long-chain compounds, however, yields certain data concerning the crystal forms appearing in wax components under certain circumstances. These data enable us to make an assumption about the crystal form appearing in the waxy mixtures studied. It will be shown in the next paragraph that, in all probability, this crystal form is always the rhombic one, which obviously will much enhance the possibility of drawing further conclusions from the long spacings observed. Additional support to this supposition will be given in par. 3 by the fact that the chain lengths found, as well as the distribution of these chain lengths, turn out to be in good agreement with certain known phenomena if the rhombic form, or at least a rectangular position of the chains with respect to the basal planes, is taken as a starting point.

The literature referred to, also yields data concerning the l.c. spacings in certain mixtures of l.c. compounds in the rhombic state. These data will be discussed in par. 4.

It will appear that the above-mentioned circumstances enable us to draw conclusions about the chain lengths of the predominating components in many of the waxes with a comparatively high degree of certainty.

2. *The Crystal Forms of the Components in the Native Wax*

In the present section, we shall endeavour to ascertain which crystal forms may be assumed to exist in native waxes. For this purpose, the work of PIPER, CHIBNALL et al. (1931, 1934) was found to be a valuable source of reference.

As far as the paraffins are concerned, the odd-number paraffins with 11 and more C atoms generally show, according to PIPER et al. (1931), the so-called A form, which is rhombic. In the even-number paraffins the state of affairs is more complicated, but these paraffins have not been found in natural waxes. All long-chain products from these waxes formerly taken for even paraffins, have proved to be odd paraffins, cf. CHIBNALL, PIPER et al. (1934*a*). Therefore, the even-number paraffins will be left out of account in this discussion, and we may assume that paraffin chains in plant wax mixtures are placed at right angles to the lattice-planes with long spacing.

As to the ketones, it appears from a table given by PIPER et al. (1931) that C_{29} ketones with the keto group at different places in the chain, show the same l.c. spacing as C_{29} paraffins. Thus the chains of ketones may also be expected to lie perpendicular to the planes with long spacing. The same applies to a number of sec. alcohols mentioned in this table: The symmetrical C_{23}, C_{27} and C_{31} sec. alcohols show the same long spacings as the corresponding paraffins. The non-symmetrical C_{29} sec. alcohol d-n-nonacosanol-10 also shows the same long spacing as C_{29} paraffin. Some other sec. alcohols mentioned in this table show slightly shorter spacings than the corresponding paraffins, indicating an oblique position of the chains. There are reasons, however, to suppose that, in a less pure state, their position generally tends to perpendicularity with respect to the planes with lang spacing, as this has also been observed in components to be mentioned later.

It is not clear in the literature whether even-number ketones and sec. alcohols are absent in natural waxes. The way, however, in which CHIBNALL and PIPER (1934*b*) have placed these compounds in their scheme of the metabolism of the formation of plant- and insect waxes, justifies the surmise that they assume these compounds to occur as odd-number terms only, just as with paraffins. The conclusions to be drawn later from our photographs are in harmony with this view.

With the prim. alcohols and fatty acids it is assumed, in accordance with the results of CHIBNALL, PIPER et al. (1934*a*), that odd-number long-chain homologues of these series do not occur in natural products. Consequently, the crystal forms of these need not be discussed here.

A description of the crystal modifications of long-chain n-prim. alcohols and of certain long-chain esters is given by Malkin (1930, 1931) and of long-chain n-fatty acids by Francis et al. (1930). The investigations of these authors have shown that even-number prim. alcohols and all long-chain fatty acids and esters crystallise from most organic solvents in the monoclinic B-form, at least at normal temperatures. Impurities, however, frequently cause a substance which would normally crystallise as a B-modification to adopt the (rhombic) A-form, or another monoclinic form, the C-form.

As far as the alcohols are concerned, it has appeared from our investigations into the submicroscopic structure of the wax rods of the sugar cane stem (which, on account of chemical analyses and in correspondence with our X-ray diagrams, are to be considered as consisting mainly of prim. alcohols, cf. par. 5) that here the alcohol is present in the rhombic form. We assume, therefore, that the same will hold for other cases in which alcohols constitute a considerable proportion of the wax.

Free fatty acids are found in epidermal waxes in comparatively small quantities only. It is therefore not to be anticipated that their presence will much affect the l.c. refl. It has already been mentioned above that, when impure, they often adopt the rhombic form; so they may be present in the wax in this form.

Finally, as regards the esters, there is no evidence available about the crystal forms of the esters characteristic of natural waxes; i.e. esters of monovalent long-chain primary alcohols and corresponding fatty acids both with chain lengths of 20—35 carbon atoms. The ethyl esters, however, having 19, 20, 21, 22 and 23 C atoms recrystallise, after having been melted, with the chains perpendicular to the planes with long spacing. This may be deduced from the long spacings of these compounds mentioned by Malkin (1931), when comparing these spacings with those of rhombic paraffins with the same number of C atoms. This comparison is given below:

number of C atoms:	19	20	21	22	23
spacings of esters in Å:	27.1	28.6	29.8	31.3	32.4
„ „ paraffins: in Å:	26.2	27.5	28.6	30.3	31.2
differences:.	0.9	1.1	1.2	1.3	1.2

The ester spacings are found to be c. 1.1 Å longer than those of the paraffins. This can only be explained if the ester chains are perpendicular to the basal planes, as in the paraffins and if, in addition, they contain the oxygen atom which shows no double bond built in like a carbon atom. This oxygen atom will cause the greater length of the ester chains. The paraffin spacings mentioned above have been obtained by extrapolation from fig. 12, which will be discussed in the next section.

From the data mentioned in this paragraph, the conclusion may be drawn that, in all probability, paraffins, ketones, sec. alcohols, prim. alcohols and fatty acids occur in native waxes in the rhombic form or, at least, that their chains are placed at right angles to the lattice-planes with long spacing. Even in esters this crystal form cannot be precluded.

3. *The Relation between the Long Crystal-Spacings deduced from the Diagrams and the Chain Lengths of the Wax Components*

If the conclusions drawn at the end of the preceding section, is in accordance with the facts, it will be possible to determine the number of carbon atoms of the main wax components from the relation between the number of carbon atoms and the long spacing as empirically established for carbon chains in rhombic packing. It goes without saying that this linear relationship is different when the long spacing embraces twice the chain length from that obtained if only the single chain length is involved; in the former case, the increment of the spacing per carbon atom in the molecule is twice as great as in the latter. Furthermore, it is not to be expected that in the former case the spacing will be exactly twice as long as in the latter when chains of equal length are compared. The distance between the opposite chain ends with dipole groups, measured in the chain direction, will not be the same as that between opposite CH_3 groups. The latter occur at both ends in "single" chains and at one end in "double" chains. For these reasons, it is necessary to draw a separate graph for each of these possibilities when it is intended to show the relation graphically. This has been done in fig. 12 and fig. 13, the numbers of carbon atoms being plotted horizontally, the long spacings vertically. The empirical points through which the lines have been drawn and the points of the line corresponding to the long spacings measured in the waxes and mentioned in table I, are marked as given in the legend of fig. 12. In order to prevent superposition of the dots representing the latter points these have been put above or beneath the line. Their actual situation is their vertical projection on the line. The short thick line through each dot indicates the limit of accuracy of its position. In certain cases, one dot represents the waxes from a group of plants which all show the same spacing. For each dot, the origin of the

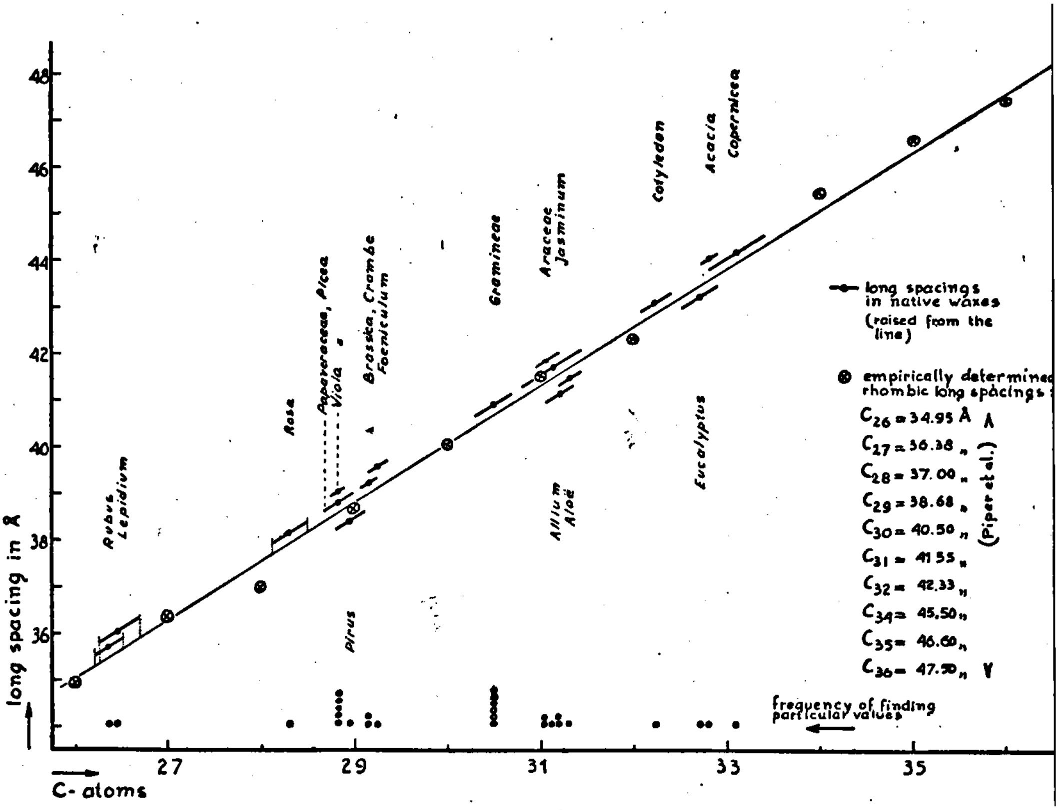

Fig. 12. The long spacings of 30—45 Å from Table I, plotted on a curve representing the relation between the long spacing and the number of carbon atoms for normal carbon chains in rhombic packing in which the long spacing embraces the single chain length. Further explanation is given in the text.

wax(es) in question is mentioned in the figures. The dots at the bottom of the figure, beneath each dot on the line, show, by their number, for each spacing found the number of waxes possessing the spacing in question. At the same time their distribution along the abscis gives a clear picture of the distribution of all chain lenghts found.

The data used for plotting the empirical points in fig. 12, have been taken from PIPER, CHIBNALL et al. (1931), table I, in which the long spacings of a series of highly purified or synthetised long-chain paraffins in the rhombic form are mentioned. These paraffins and their long spacings are tabulated on the right side in our figure 12.

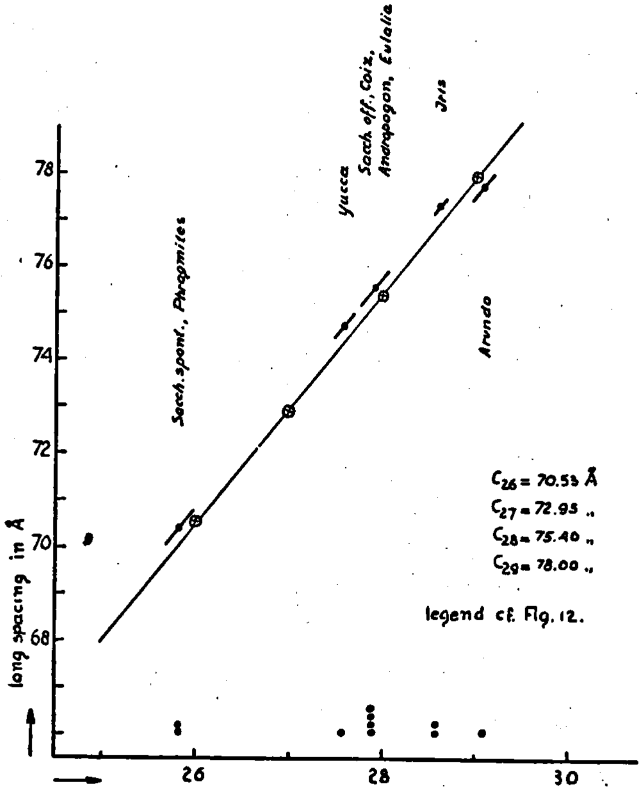

Fig. 13. The long spacings of 70—80 Å mentioned in Table I, plotted on a curve representing the relation between the long crystal-spacing and the number of carbon atoms for normal carbon chains in rhombic packing in which the long spacing embraces twice the chain length.

The data used for plotting the empirical points in fig. 13, have been taken from PIPER, CHIBNALL et al. (1934). The spacings of the odd-number terms are those of C_{27} and C_{29} prim. alcohols as mentioned in table I of these authors. The even-number prim. alcohols do not crystallise in the rhombic form when in a pure state. Hence their rhombic long spacing cannot be measured on pure substances. A slight impurity, however, forces them to crystallise in the rhombic modification. Consequently, for drawing the graph, we used long-chain spacings of even-number terms which have been measured on preparations of even-number alcohols containing a slight amount of a neighbouring homologue. These values, viz. those for C_{26} and C_{28} prim. alcohol, have been taken from table III of the above-mentioned publication, in wich the long spacings are given for a series of mixtures of C_{26} and C_{28} prim. alcohols of different composition. Of these, the spacings for the mixtures of 95% C_{26} + 5% C_{28} and 5% C_{26} + 95% C_{28} have been used. The values are found to lie on the same line as those of the odd-number alcohols, which in a pure state do show the rhombic form. Accordingly, the admixture does not essentially affect this spacing. The spacings are listed on the right side in the figure.

If, now, attention is paid to the spacings measured in our wax diagrams, it is conspicuous, especially in the graph of the single chains, that, almost without exception, the values found lie in the close neighbourhood of the empirical spacings of one of the chains with 29, 31 or 33 C atoms. In a few cases only, the spacing found differs more than 0.3 Å from one of these spacings. As regards the double chains, the above phenomenon appears as well, though less conspicuously. This may be due to the circumstance that the number of waxes with doubled periods is rather small. In any case a clear culmination of dots is present near C_{28}.

It is a plausible inference that in waxes where the long spacing lies so closely to the spacing of a pure rhombic wax component, this component, or at least a component of the same chain length, occurs in predominating amount. This is the more probable as, in this case, the distribution of the dots in the diagram is in good agreement with the established exclusive occurrence in natural waxes of the odd-number homologues of those compounds which crystallise as single-chain layers, and of the even-number homologues of those which crystallise as double-chain layers.

Furthermore, this coincidence of accumulations of dots with points which represent the spacings of rhombic pure wax constituents is a new argument that, indeed, the chains in the waxes are placed vertically to the net-planes with long spacing. For, if the chains in the waxes were inclined to these planes, a graph of this kind would not show, even if it revealed any accumulations of dots at all, a distribution of these accumulations at intervals corresponding to chain-length increments of 2 carbon atoms; it would show smaller intervals and the accumulations would not coincide with the empirical points.

When, again, packings of oblique chains and vertical chains are compared, both with the same long spacing, the oblique chains are longer than the vertical ones. The chain lengths we find in the waxes if the vertical position is postulated, are in good agreement with the chain lengths which are known to occur most frequently in natural waxes. In this connection it may be mentioned that in a survey of constituents of 18 plant and insect waxes, examined by CHIBNALL, PIPER et al., the chain lengths C_{29} and C_{31} predominate as far as the paraffins are concerned, whereas in prim. alcohols and fatty acids C_{28} and C_{30} prevail (see CHIBNALL and PIPER 1934*b*). The same feature is demonstrated in our graphs. If oblique positions of the chains were present in our waxes, the long spacings observed would indicate chain lengths in these waxes which would, naturally, be longer than those actually established and the conformity with the data reported above would not appear. This implies a further argument in favour of perpendicularity of the chains to the basal planes.

To sum up, in this section it is recorded that if the rhombic relation between long spacing and chain length is used for the determination of the chain lengths in the waxes we examined, the results are in good agreement with certain known data. If, on the other hand, oblique positions of the chains with respect to the basal planes are postulated no such agreement can be achieved. Thus further arguments are obtained in support of the assumption made at the end of the preceding section, namely, that the chain molecules in the waxes are generally placed vertically to the basal planes.

4. *The Long Crystal-Spacings in Synthetic Mixtures of Wax Constituents*

Before, however, drawing conclusions from the long spacings measured individually, it is desirable to know how these spacings and the corresponding reflections behave in mixtures of wax constituents. Also about this point PIPER, CHIBNALL et al. (1931, 1934) provide a number of important data. They determined the long spacings of synthetic mixtures of 2, 3 or 4 paraffins, prim. alcohols or fatty acids having chain lengths such as occur in natural waxes, mixed in different proportions. Also other characteristics of these mixtures, such as melting points and temperatures of transition from one modification into another, have been determined. These latter data, though valuable in wax analyses, are of less importance for our investigation, and only the data which are useful for our purposes will be summarised below. Attention will be confined to data which refer to rhombic spacings.

As far as the paraffins are concerned, the following conclusions can be drawn:

1. Only one long spacing appears in binary mixtures, unless the chain lengths differ by 4 or more carbon atoms.
2. In binary mixtures consisting of substances with chain lengths differing by 1 or 2 carbon atoms, at a proportion of about 99 : 1, the spacing is always a little longer than that of the main component in a pure state, no matter whether the contaminating chain is the longer or the shorter one.
3. At a proportion of 10 to 20% of a contaminating shorter chain, the long spacing approximates that of the pure longer chain. With a longer contaminating chain, the spacing increases steadily up to 80% impurity.

TABLE II.

Long crystal-spacings for binary mixtures of odd-number normal paraffins and even-number normal prim. alcohols in the rhombic form, after PIPER, CHIBNALL et al. (1931, 1934).

a			*b*		
Paraffins			Primary alcohols		
% C_{29}	% C_{31}	long sp. in Å	% C_{26}	% C_{28}	long sp. in Å
100	0	38.68	100	0	— B-form only
99	1	39.12	95	5	70.53 ↑
97.5	2.5	39.25	90	10	71.1
95	5	39.15	75	25	72.7
90	10	39.45	60	40	73.1
85	15	39.7	50	50	73.5
80	20	39.75	40	60	74.6 A-form only
70	30	40.55	30	70	75.4
60	40	40.9	25	75	75.2
50	50	41.2	20	80	75.75 ↓
40	60	41.2	10	90	75.4
30	70	41.45	5	95	75.4
20	80	41.5	0	100	— B-form only
10	90	41.7			
5	95	41.85			
2.5	97.5	41.8			
1	99	42.0			
0	100	41.5			

In table II*a*, the variation of the long spacing in mixtures of C_{29} and C_{31} paraffins is shown, as given by PIPER, CHIBNALL et al. (1931). When drawing conclusions from our diagrams it has been presumed that the same changes in the spacings take place in binary mixtures of other adjacent odd-number homologues of which the long spacing embraces one molecule, a generalisation also accepted by PIPER and CHIBNALL.

4. The number of orders in the set of l.c. refl. decreases to about 4 as the proportions approximate equimolarity. The intensity of the first of the four remaining orders, however, increases and, consequently, the gradual fall of intensity is steeper. It should be noted that these details have been observed in photographs obtained from preparations on a glass plate, in which the lattice-planes with long distance are parallel to the glass surface, cf. chapt. I, 3. These photographs always show more higher orders than do our powder photographs. In certain cases, however, the phenomenon is also observed in our photographs.
5. Examination of a few complicated mixtures has revealed no data which have led to generalisations.

Points 2 and 3 mentioned above apply to odd-number paraffins. Mixtures of two even-number paraffins or of an odd- and even-number paraffin show a somewhat different curve. The even-number chains, however, will not be considered, as there is no evidence as yet that they occur in natural waxes. For the same reason the odd-number chains will be left out of account in the next discussion of prim. alcohol mixtures.

In primary alcohols the following points are of importance:

1. Presumably the same holds true as mentioned sub 1 in paraffins, though **Piper** et al. only mention the fact in connection with paraffins.
2. Binary mixtures of adjacent even-number prim. alcohols show a tendency to crystallise in the (rhombic) A-form. When pure, these alcohols crystallise in the B-modification. When 5% of the longer chain has been added to the shorter one the B-form disappears but, at the other end of the series of mixtures, B- and A-forms appear together at 5 and 10% contamination of the longer chain by the shorter one. The A-form alone appears when 20% of the shorter chain is present.
3. Between the extreme compositions of the mixture in which the A-form exists, there is a fairly uniform rise in spacing from the low end to about equimolar proportions. Above equimolar concentrations the spacing approximates that of the longer component.

In table II*b* the long spacings of mixtures of C_{26} and C_{28} prim. alcohol are given, as taken from **Piper**, **Chibnall** at al. (1934). When drawing conclusions from our diagrams we presume that similar shifts of the long spacing occur in other binary mixtures of adjacent even-number rhombic prim. alcohol homologues.

4. The diagrams of complicated mixtures of prim. alcohols often show many sharp l.c. refl. of higher order. Hence this phenomenon can never serve as an indication of purity in alcohols. Acid- and ester mixtures, on the contrary, are easily distinguished from the pure constituents by their showing few and vague higher orders.
5. In equimolar ternary mixtures of successive even-number prim. alcohols, the long spacing is c. 1.6 Å longer than that of the middle term. At a proportion 40 : 40 : 20 the spacing nearly equals that of the middle term and at a proportion 20 : 40 : 40 it is about 1.5 Å shorter than that of the longest. (The first number mentioned in both ratios refers to the shortest chain.)

Considering the above-mentioned data, it should be born in mind that, when drawing conclusions from the long spacings measured in our diagrams, coincidence of a measured long spacing with a spacing of a pure substance is by no means an indication that this substance, or a substance of the same chain length, is present in the wax in a pure state. The adjacent shorter homologue may be present up to 30% in the mixture, and even up to 60%, at least in paraffins, if the measured spacing is only 0.6 Å shorter than that of the pure substance. In the case of coincidence, or of differences up to c. 0.3 Å, it can be taken for granted, however, that the chain length in question is present in the wax in a predominating amount in binary mixtures, and in a considerable quantity in more complicated mixtures.

As to these complicated mixtures, we assume that amongst those of our waxes showing distinct l.c. refl., no, or very few waxes occur in which the bulk of the wax is made up of more than two different chain lengths. Otherwise, the general agreement of measured spacings with those of pure substances would, presumably, on account of point 5 under alcohols, be less conspicuous than it actually is.

Furthermore it is presumed that, in general, vague l.c. reflections with few higher orders are characteristic of rather complicated mixtures, while the appearance of a relatively great number of sharp higher orders has been considered to be a feature indicating that one or two chain lengths predominate. Though it is emphasised by Piper et al., as mentioned sub point 4 above, that in prim. alcohols also complicated mixtures often reveal many sharp higher orders, we suppose this will be less the case in powder specimens like ours than in oriented specimens on a glass mount like those of Piper et al.

In connection with point 1 (under paraffins) it is worth while mentioning that our diagrams, with the exception of two, show no double reflection series. We have, therefore, to assume that in those waxes which show clear l.c. refl. the bulk of the components do not differ by more than 3 C atoms. This implies that, when exclusively odd-number or exclusively even-number chains occur in a wax, the bulk of the chains are adjacent odd- or adjacent even-number homologues.

Certain data which would be of importance for our investigation are lacking. There is, for instance, little information about l.c. refl. in natural wax esters and nothing is known about the character of these reflections in mixtures of such esters. Also data about mixtures of compounds belonging to different homologous series are lacking. As CHIBNALL et al. always first saponified their preparations and separated the compounds in question, they saw no point in studying such mixtures. Mixtures of ketones and of sec. alcohols have also not been investigated.

Consequently, when ascribing certain phenomena in the l.c. refl. of a number of our diagrams to mixtures such as those mentioned above, this will be done under assumptions which, though plausible in our opinion, have not been verified empirically. In a few cases, however, there are available in the literature data about the chemical composition of the waxes in question, which prove to be affirmative as regards those assumptions. (See under *Brassica* and apple peel wax in the next section.) The lack of evidence is not, in fact, serious, though it involves an element of uncertainty.

5. *Discussion of the Individual Diagrams*

In the present section, each diagram will be dealt with separately to ascertain what conclusions it allows regarding the main components of the wax in question. The following general reminders may precede the discussions:

If data about the composition of certain waxes are available in the literature, these data will only be cited as far as is necessary for comparison with the conclusions drawn from our diagrams. This implies that, as a rule, only details of the predominating components of a wax will be cited. These results of other investigators are only considered to be useful as a check to our interpretations, at least as far as the chainlengths are concerned, if they have been obtained by chemical analyses amplified by X-ray measurements.

For the numerical values of the long spacings we refer to table I, and for their relation to the chain length to fig. 12 or fig. 13. For convenience they will be repeated in the next discussions.

Conclusions concerning the proportions of chains of different lengths are always based on table II. The same variation of spacings as that shown in this table is supposed to occur in similar mixtures of longer or shorter odd or even-number homologues.

The intensity fluctuation of the long-chain reflections is given in table I and can be observed for certain diagrams in fig. 8, 9, 10 and 11, while in chapt. II, 1, it has been used as a criterion for the classification of the diagrams. In the next discussions, additional details about the intensities will be mentioned and for the sake of convenience the general particulars on which the classification is based will be repeated. For the interpretation of the intensities the details mentioned in chapt. I, 2, will serve as a starting point, in which attention may be especially called to fig. 4. In those cases in which the intensity fluctuation does not correspond to one of the examples given in fig. 4, the interpretation will be discussed more thoroughly.

Various types of sets of side spacing reflections may be observed in fig. 8, 9, 10 and 11. In the following discussions, the diameters and intensities of these reflections will be recorded in table IV*a*, *b*, *c* etc. for all types and particular cases.

The sequence in which the waxes will be discussed is the same as that of table I.

Finally, before starting the discussions, the nomenclature of certain long-chain compounds which will be mentioned frequently, is recalled in table III.

TABLE III.

Nomenclature of n-long-chain compounds frequently mentioned.

paraffins		prim. alcohols	
general formula: C_nH_{2n+2}		general form. $C_nH_{2n+1}OH$	
n-tricosane	n = 23	n-tetracosanol	n = 24
n-pentacosane	25	n-hexacosanol	26
n-heptacosane	27	n-octacosanol	28
n-nonacosane	29	n-triacontanol	30
n-hentriacontane	31	n-dotriacontanol	32
n-tritriacontane	33	n-tetratriacontanol	34
n-pentatriacontane	35		
For the names and formulae of ketones and sec. alcohols frequently found in nature, cf. table VI, p. 73. The general formulae are mentioned in Chapt. I, 1.			

We now pass on to the discussion of the diagrams.

Group D.

All diagrams belonging to this group show the series of s.s. refl. characteristic of n-long-chain paraffins and allied substances, cf. figs. 1 and 8. Their diameters and relative intensities as measured on the diagram of *Saccharum spontaneum* wax. are given in table IV*a*. In other diagrams of this group they may show slight deviations, both in intensities and diameters.

TABLE IV*a*.

Side-spacing reflections in group N.

number:	1	2	3	4	5	6	7	8	9	10
$2f_{40}$:	28.0	31.3	35.5	41.0	46.1	57.6	62.1	66.1	68.5	75.6
intensity:	f	vs	s	vf	f	m	vf	vf	f	f

vf = very faint, f = faint, m = mean, s = strong, vs = very strong.

Type Nsa, cf. fig. 8, I and fig. 4, I.
The higher orders of l.c. refl. show no extinctions. There is a gradual intensity fall. This is considered indicative of paraffins.

Wax of *Copernicia cerifera*, "Carnauba wax", a commercial preparation from the collection of the Laboratory of Technical Botany, Delft.
The l.c. refl. are vague. The long spacing (44.0 Å) corresponds to that of fairly pure C_{33} paraffin. This coincidence, however, may be accidental; the vagueness of the reflections and the small number of higher orders indicating fairly divergent chain lengths, make the diagram inconclusive.
The data mentioned by GRÜN and HALDEN (1929) and CHIBNALL et al. (1934*a*) also point to chains of various lengths. According to the latter authors C_{26} to C_{34} prim. alcohols are present of which C_{30} to C_{34} predominate.

Wax of *Cotyledon orbiculata*, from the leaves, cf. fig. 8, I.
The photograph shows 4 sharp l.c. refl., indicating a long spacing (42.9 Å) corresponding to that of a paraffin mixture of c. 70% C_{31} and 30% C_{33}. The sharpness of the reflections shows that there is little variation in chain length and that, therefore, these components are to be considered to predominate in the wax.

Flower wax of *Rosa centifolia*, by-product of scent industries, present in the collection of the Laboratory of Technical Botany, Delft and originating from the firm of Roure-Bertrand Fils, Grasse who obtained it from the flowers by extraction with petroleum ether. The same preparation has been investigated by STRAMAN (1926) along the lines of chemical analysis in the above-mentioned laboratory.

There are 3 sharp l.c. refl. showing a sharp decrease in intensity, The long spacing (37.9 Å) corresponds to 65% C_{27} + 35% C_{29} paraffin.

For this wax, STRAMAN (1926) mentions c. 75% paraffin, without indicating the chain length. PROPHÈTE (1926) found a considerable quantity, c. 50%, paraffin in wax of rose petals, the paraffin having chain lengths from C_{16} to C_{30}, of which C_{27} in the greatest proportion, namely 15%. Naturally, in interpreting PROPHÈTES work, the even number paraffins are to be left out of consideration.

Flower wax of *Viola odorata.* Same origin as wax of *Rosa.*

There are 4 sharp l.c. refl., the spacing (38.6 Å) of which indicates fairly pure C_{29} paraffin in which, however, c. 30% C_{27} may occur as a maximum. In addition, there is a faint reflection with a diameter of $2f_{40} = 31.5$ mm, the meaning of which could not be ascertained.

According to STRAMAN this wax contains 47% paraffin, 10% higher alcohol, 35% saturated and 6% unsaturated fatty acid, of which the paraffin has been identified as hentriacontane. This is in contradiction with our diagram which clearly indicates n-nonacosane.

Flower wax of *Jasminum grandifolium,* of the same origin as the wax of *Rosa.*

There are sharp 2nd and 3rd higher order l.c. refl. The long spacing (41.5 Å) points to almost pure n-hentriacontane. However, n-nonacosane may be present for c. 20%. That no more orders are visible is due to too short an exposure of this diagram.

STRAMAN reports for this wax 50% paraffin, identified as hentriacontane.

Type Nsb, cf. fig. 8, II and fig. 4, II.

Either the even orders are extinguished, or odd and even orders show strong and weak intensities respectively, while the spacing indicates "single" chains.

In symmetric ketones and symmetric sec. alcohols the even orders are almost extinguished in the lower orders. No other wax components with single chains which might cause this phenomenon being known, we must conclude that these compounds make up the bulk of the wax if the even orders are extinguished. Though esters of fatty acids and prim. alcohols both having chain lengths like that of frequent occurring natural glyceride fatty acids, i.e. C_{16} and C_{18}, may also show a similar long spacing and intensity fluctuation, the occurrence in the wax of these compounds can be precluded, as they have not been found in plant waxes in considerable amount, cf. CHIBNALL and PIPER (1934*b*), table I.

If the even orders are clearly visible, though weaker than the odd orders, we suppose that the ketones and sec. alcohols are mixed with paraffins, for the latter do show even orders. We were able to check this view by comparing the known data about the composition of *Brassica* wax, see below. The less the even orders are weakened the more paraffin we suppose to be present.

Waxes of *Xanthosoma violaceum* and *Colocasia antiquorum*, originating from the leaves and petioles, cf. fig. 8, II.

The orders 1, 3, 5 and 7 are clearly visible, whereas the even orders are extinguished. The long spacing (41.4 Å) is in agreement with that of practically pure n-hentriacontane. Both waxes give identical diagrams. On account of the extinction of even orders, the occurrence of n-hentriacontane-16-one or n-hentriacontane-16-lo is to be concluded. Presence of analogous symmetrical products with C_{29} chains up to c. 20% cannot be excluded.

Waxes of *Brassica oleracea* var. *capitata* f. *rubra* (red cabbage) and *Crambe maritima*, collected from the leaves.

The diagrams are alike. They show strong 3rd and 5th order l.c. refl. and a trace of the 7th order, whereas the orders 2 and 4 are present with diminished intensity. The long spacing (39.0 Å) is in agreement with that of nonacosane, mixed with a very slight amount of hentriacontane or heptacosane. The weakened even orders point to a mixture of symmetric ketones and(or) symmetric sec. alcohols with paraffins. Therefore, the main components of the waxes are, most probably, n-nonacosane and n-nonacosane-15-one or-ol, while a small amount of C_{31} or C_{27} chains may be present.

CHIBNALL et al. have examined the waxes of various *Brassica* species. These waxes, however, were obtained by extraction and according to the investigators originated from cytoplasmic material.

In the leaves of *Brassica oleracea* var. *capitata*, CHANNON and CHIBNALL (1929) have found n-nonacosane-15-one and a paraffin, which later (CHIBNALL et al. 1934*a*), was found to contain 95% n-nonacosane and 5% n-hentriacontane. The correspondence with our results is triking.

We note further that they found 220 gm. ether extract of cytoplasmic material, from 220 kg cabbage leaves, to contain 12.3% of unsaponifiable matter consisting mainly of the paraffin and ketones mentioned above. On account of the strong l.c. refl. in our diagram of the cuticle wax, this wax apparently contains a much higher amount of the compounds in question than did the ether extract. SAHAI and CHIBNALL (1932) isolated from leaves of *Brassica oleracea* var. *gemmifera* n-nonacosane, n-hentriacontane, n-nonacosane-15-ol, primary alcohol and fatty acid.

For *Brassica campestris*, CHIBNALL et al. (1934*a*) mention 95% C_{29} + 5% C_{31} paraffin, n-nonacosane-15-ol and no ketone.

All these data are in agreement with our results, and are a valuable support to our methods of interpreting the l.c. refl.

Flower wax of *Acacia dealbata.* For the origin of the wax cf. the wax of *Rosa.*

The orders 1, 3 and 5 of the l.c. refl. are sharp and clearly visible, while the even orders are missing.

The long spacing (43.6 Å) points to a chain length of C_{33}, possibly mixed with c. 25% C_{31} chains. The absence of even orders indicates a symmetrical ketone or sec. alcohol which can only be n-tritriacontane-17-one or -ol, though the occurrence of this compound in natural waxes has not yet been established. The C_{31} chains, if present, must belong to similar products, as no even orders are visible.

STRAMAN (1926), by chemical analysis of this preparation, found 52% hentriacontane, c. 24% higher alcohol, c. 14% saturated and 8% unsaturated fatty acid. We are of the opinion that this high percentage of paraffin is very unlikely, as it would reveal itself in the X-ray diagram. STRAMAN makes no mention of ketones or sec. alcohols.

Type Nsc, cf. fig. 8 III and fig. 4, III.

The intensity fluctuation is less regular than in the preceding types.

Waxes of *Allium porrum* and *Allium fistulosum*, collected from the leaves, cf. fig. 8, III.

There are a 2nd, a 3rd, a 4th and a 5th order l.c. refl. The long spacing (41.6 Å) is in agreement with a chain length of C_{31} mixed with a small amount of C_{29} or C_{33}.

In *A. porrum* wax, the first two reflections are fairly and almost equally strong, while the next two are weaker. This implies that the 2nd and 4th order are weaker than in the case of a normal intensity fall.

In *A. fistulosum* the 2nd order is absent and the 4th order is very weak.

Hence, in both waxes the even orders are weakened as compared with a normal fall in intensity. Therefore we have to assume a mixture of n-hentriacontane-16-ol or -one and n-hentriacontane, while in *A. fistulosum* wax the amount of paraffin must be considerably less than in *A. porrum* wax.

It is not evident why in *A. fistulosum* wax the 4th order is faintly visible, whereas the second order is absent. The reverse would be normal.

It may be noticed that there is only a difference of degree between the *Allium* waxes and those of the type Nkb in that in the former, especially in *A. porrum*, the weakening of the even orders is less conspicuous than in the waxes of the latter type.

The *Allium* wax diagrams show very faint reflections which indicate a small amount of gypsum. These would not have been recognised as such if this phenomenon had not been much more conspicuous in *Aloë* wax.

Wax of *Aloë vera*, originating from the leaves.

The orders 2, 3 and 4 are present. They are sharp and show a gradual fall in intensity, while the spacing (41.7 Å) indicates hentriacontane with a small amount of C_{29} or C_{33} paraffin.

Furthermore, there are 2 weak l.c. refl., placed in brackets in table I, which cannot be included in the same series as the others. If, namely, we suppose that double chains are present, so that the order numbers mentioned above are to be multiplied by 2 and the faint reflections are to be considered as 3rd and 5th order, there would be weak odd orders and strong even orders. In the case of "double" chains exactly the reverse should occur, cf. fig. 4, IV. It is likely, however, that these faint reflections are to be taken for the strong 3rd and 5th order reflections of C_{30} prim. alcohol (which would be in agreement with the long spacing they indicate), which alcohol might be present as admixture in the paraffin mentioned above. The amount of alcohol is probably rather small as its strong orders are much weaker than the paraffin reflections. Its weak orders will coincide with the paraffin reflections. Additional evidence can only be obtained from experiments with synthetical mixtures and from chemical analysis of the wax.

Apart from the wax reflections, the diagram shows reflection circles which indicate the occurrence in the wax of a fairly coarse-grained crystalline material. The d-values of these reflections in Å: 7.75, 4.3, 3.08, 2.88, 2.68, are characteristic of gypsum in the modification $CaSO_4 . 2H_2O$. Wax samples from both the upper and the lower side of the leaf, samples collected at intervals of several months and samples collected from different plants invariably produced these reflections, whereas wax samples obtained from other succulent plants in the same greenhouse, growing in the neighbourhood of the *Aloë* specimens in question, did not. The substance, therefore, may be regarded as an integral constituent of the *Aloë* wax layer.

Wax of *Foeniculum vulgare*, collected from the stems.

The orders 2, 4 and 5 are clear and sharp, while the 7th order is faintly visible. They show a gradual intensity fall. The 3rd order is weakened and the 6th is absent. The long spacing (39.1 Å) is in agreement with that of C_{29} paraffin with a small amount of C_{27} or C_{31} chains.

The weakening of the third order and the absence of the sixth indicate the presence of a ketone or sec. alcohol with the side group at $^1/_3$ of the l.c. period, cf. fig. 4, III. If, however, this latter component were present exclusively, the third order would be extinguished. We presume the weak third order to be an indication that the ketone or sec. alcohol is mixed with paraffin of the same chain length. Hence, and on account of the long spacing, we conclude to the presence of n-nonacosane.

The ketone or sec. alcohol, in all probability, will be n-nonacosane-10-ol in its optically active d-form. For this substance has been demonstrated to be present in other plant waxes: It has been found in apple peel wax, cf. CHIBNALL et al. (1931) and in the fruit of *Ginkgo biloba* from which KAWAMURA and FURUKAWA isolated an alcohol "ginnol", which has been identified by CHIBNALL et al. as the above-mentioned substance, cf. CHIBNALL and PIPER (1934*a*).

Wax of *Pyrus malus*, scraped from the surface of an apple.

The orders 2, 4 and 5 are visible and show a normal intensity fall, whereas the 3rd order is weakened. The long spacing (38.7 Å) is in agreement with that of C_{29} paraffin, possibly mixed with 10 to 20% C_{27}. Consequently, there is analogy with the preceding wax. Here, as in the preceding case, we presume the weak 3rd order to be indicative of the presence of n-nonacosane-10-ol accompanied by n-nonacosane, the paraffin preventing the 3rd order from being completely extinguished.

CHIBNALL, PIPER et al. (1931) investigated apple wax obtained by extraction of great quantities of apple peel. They have examined this wax very carefully as it could be obtained in fairly large quantities and could be used for developing improved methods of separating paraffins, prim. alcohols, ketones and sec. alcohols, all of which had already been demonstrated in this wax by previous workers. They have isolated: the paraffins C_{29} and C_{27}, d-n-nonacosane-10-ol and the primary alcohols C_{26}, C_{28} and C_{30}, whereas ketones were lacking. In order to give some idea of the proportions of these constituents we mention that they found 57 gm. wax to contain c. 50 gm. unsaponifiable matter, which again consisted of c. 23 gm. paraffin, c. 8 gm. prim. alcohol and 8 gm. sec. alcohol (13 gm. phtalate of each). The long spacing of the paraffin fraction was 38.7 Å, which practically equals the spacing of the native wax we examined. Hence the prim. alcohols and the ester do not seem to affect this spacing. It may be, however, that these substances are absent in the scraped off true cuticle wax and are present only in the extraction product, though we consider this to be unlikely.

As in the case of *Brassica* and *Crambe*, the above-mentioned comparison of results demonstrates a comparatively great reliability of the native wax diagram as a source of information about the main wax components.

Type Nl, cf. fig. 8, IV and fig. 4, IV.

The diagram shows l.c. refl. in which odd and even orders are alternatively strong and weak and which indicate a long spacing which corresponds to twice the length of a molecule.

Wax of *Saccharum officinarum*, scraped from the stems.

The orders 3 up to and including 7 are distinct, of which the even orders show diminished intensities. The long spacing (75.2 Å) differs only c. 0.2 Å from that of C_{28} prim. alcohol.

In case of a binary mixture, this indicates a mixture of 75% C_{28} + 25% C_{26}. However, the amount of C_{26} may also be much smaller. In case of a ternary mixture, it points to a proportion $C_{26} : C_{28} : C_{30} = 40 : 40 : 20$, cf sect. 4, point 5 under alcohols. In the latter case, however, one cannot expect such sharp higher orders, at least not in powder preparations, cf. sect. 4. Hence we prefer the first possibility.

A detailed examination of the wax of *Saccharum officinarum* was performed by WIJNBERG (1909). Both wax scraped from the stems and waxy products from the press cake of the cane-sugar industry were investigated. As regards the scraped wax, the results were compared with those of earlier investigators. Of these we only mention AVEQUIN (1840) and LEWY (1845). AVEQUIN has given a most detailed and accurate description of the wax. For the melting point after repeated extraction of the wax with cold ethyl alcohol both WIJNBERG and AVEQUIN arrived at 81.5—82°. This is the melting point of a mixture of C_{28} prim. alcohol with 30—20% C_{26} alcohol, cf. PIPER et al. (1934), and which is in close agreement wih the conclusions from our diagram.

The elementary analysis carried out by LEWY, which after WIJNBERG is the most reliable of various earlier analyses, yielded a proportion of C, H and O close to that of $C_{26}H_{54}O$. WIJNBERG, however, does not consider it very likely that indeed the wax is made up of this alcohol. He presumes that it is a mixture containing C, H and O in the same proportion as the above alcohol, and he demonstrates by a method in which the scraped wax is treated with soda lime (the amount of hydrogen liberated is a measure of the quantity of prim. alcohol) that it consists of prim. alcohol only up to c. 45%. By combustion analysis, the acid obtained from the sodium soap formed during the process was identified as $C_{30}H_{62}O_2$. On account of both this result and the melting point of the acetate of the alcohol from wax from press cake (see the next wax), WIJNBERG definitely concludes that the alcohol is C_{30} prim. alcohol. Furthermore, 35% of the wax was reported to consist of a body not affected by soda lime which, on account of combustion analysis, proved to be $C_{33}H_{68}O$ and which was not identified further, but could not be a prim. alcohol, ester, fatty acid, or paraffin.

We are inclined, at least for the scraped wax, to question the correctness of the chain lengths WIJNBERG found: in the first place, because they do not tally with the long spacing we have measured (The possibility that the chains are not placed perpendicularly to the basal planes is to be precluded on account of the discussion in sect. 2 of this chapter and the investigation described in part B), in the second place because the melting point of the wax given by WIJNBERG and AVEQUIN agrees so well with the X-ray information, and in the third place because also the melting point given by WIJNBERG for the acid obtained from the sodium soap mentioned above (85—87°), is in agreement with the m.p. of a mixture of C_{28}

with 20—40% C_{28} fatty acid (PIPER et al. 1934), whereas C_{30} acid has a m.p. of 93.6—94° (PIPER *ib.*). We refer also to what is mentioned about this question under *Saccharum spontaneum.*

Wax of *Saccharum officinarum*, extraction product from aged press cake from manufacturing process at Java, refined with chlorine and present in the collection of the Laboratory of Technical Botany, Delft.

The orders 2, 3, 4, 5 and 6 are clearly visible with normal sharpness. Curiously, here the even orders are strong and the odd orders show diminished intensities, while in all other instances of alternating strong and weak l.c. refl. the reverse is the case. In *Aloë*, however, we have met with a similar aberrant situation.

The spacing (79.6 Å) is in agreement with that of a mixture of prim. alcohols $C_{30} + C_{28}$ at a proportion 65 : 35. In connection with the inverse intensities, however, it is doubtful whether the wax actually has this composition.

The preparation we used had been made by WIJNBERG from a shipment of aged press cake and had also been examined by him. WIJNBERG, on the analogy of his conclusion about the scraped-off sugar cane wax, concluded that a high proportion of n-triacontanol is present, which conclusion is, unlike his conclusion for the scraped-off wax, in reasonable agreement with the spacing found.

It is striking that this wax from press cake shows a long spacing which is fairly different from that of the scraped-off wax. Leaving out of account the inversion of the intensities, there are two possible explanations of this difference: In the first place, the cuticle wax constituents of the sugar cane variety grown in Java may have chain lengths which vary from those of the variety growing in the greenhouses at Delft. In the second place, the constituents of the cuticle wax of sugar cane might be essentially different from those occurring in the cell walls and the protoplasm. These latter constituents may be expected to be present in the wax from press cake (which cake contains the precipitated components of the press juice), whereas, naturally, these constituents will be absent in the scraped-off wax.

On account of the appearence of a difference in chain length between the scraped-off wax of *Saccharum officinarum* and that of the wild sugar cane species *S. spontaneum*, a species which will be treated next, a similar difference might occur between varieties of the cultivated sugar cane. This suggestion supports the first of the above possibilities.

Finally, the problem of the remarkable inversion of reflection intensities remains. As an explanation of this phenomenon it may be supposed that the long spacing found must be halved and that the observed strong reflections are interferences of the first, second and third order of a long spacing of 40 Å, in correspondence with a paraffin mixture $C_{29} + C_{31}$ in the proportion 75 : 25, while the weak reflections in between are the strong 3rd and 5th order interferences of a mixture of primary alcohols such as mentioned already.

If this supposition were true, the paraffins would, on account of their stronger reflections, presumably constitute the main proportion of the wax; and then the question of the origin of the paraffins arises. As to this question, in our opinion there are two possibilities: Firstly, the paraffins may be of protoplasmic origin or, secondly, they may have been formed as a result of bacterial activity in the press cake, which was stored in the open air in a tropical climate during several months.

As long as no data are available about the l.c. refl. in artificial mixtures of long-chain paraffins and prim. alcohols and without further chemical investigations, the conclusions about the composition of this wax cannot be accepted without reserve.

Waxes of *Coix lacrima*, *Andropogon sorghum* and *Eulalia japonica*, collected from the stems.

The diagrams are identical to those of the cuticle wax of *Saccharum officinarum*. Hence the conclusions about the composition of the waxes are the same as for that wax.

Waxes of *Saccharum spontaneum*, collected from leaves and stems, and *Phragmites communis*, collected from the stems.

The orders 3 up to and including 7 of the l.c. refl. are clearly visible. The even orders show diminished intensities. The long spacing (70.1 Å) indicates a mixture of prim. alcohols $C_{26} + C_{24}$ in the approximate proportion 65 : 35.

The two diagrams are almost identical with that of the cuticle wax of *Saccharum officinarum*, the only difference being that the long spacings correspond closely to a chain length of two C atoms shorter.

It would be worth while determining the melting point of these waxes after purification through repeated extraction with cold alcohol, as has been done by WIJNBERG and AVEQUIN for the wax of *S. officinarum*. If the melting point turned out to be in good agreement with our X-ray evidence about the composition of the wax, which easily can be ascertained through the melting point curves of long-chain alcohol mixtures given by PIPER, et al. (1934), this would support out conclusions about the constitution of *S. officinarum* wax and the waxes under discussion.

Wax of *Arundo donax*, collected from the stem.

The diagram is similar to that of the preceding species. The long spacing (78.2 Å) is different, however, and indicates a mixture of prim. alcohols $C_{30} + C_{28}$ in the approximate proportion 45 : 55.

Wax of *Yucca filamentosa*, collected from the leaves.

The orders 3, 4 and 5 are perceptible, of which 4 is weakened, while 5 is somewhat broadened as compared with the diagrams of the preceding gramineous waxes. The long spacing (74.6 Å) indicates a mixture of C_{28} and C_{26} prim. alcohols in the proportion 60 : 40. The lack of sharpness of the 5th order and the absence of the 7th may be indicative of a ternary mixture, which cannot be forecast any further, however.

Waxes of *Iris florentina* and *I. germanica*, collected from the leaves.

The orders 3, 4 and 5 are present of which 4 is weak, while 5 is slightly broadened, as in the preceding case. The long spacing (77.0 Å) is in agreement with that of a mixture of the prim. alcohols C_{30} and C_{28} in the proportion 40 : 60. As in the preceding case, the possibility of a ternary mixture is to be suggested on account of the vagueness of the 5th order and the absence of the 7th. In this case, we may think of a mixture of $C_{26} + C_{28} + C_{30}$ prim. alcohols at equimolar proportions, which according to PIPER, c.s. (1934) has a long spacing of 77.05 Å.

Waxes of *Musa paradisiaca*, collected from the young leaf stalk, and *Strelitzia reginae*, collected from pedicels and bracts.

Both diagrams show 3 vague l.c. reflections, of which the first two are almost equally strong, while the third is very weak. The long spacing of *Musa* wax is 60.5 Å and that of *Strelitzia* wax is 65.2 Å.

If this wax is composed of "double" chains, the spacings correspond to C_{22} and C_{24}; in the case of "single" chains they correspond to C_{46} and C_{50}, as extrapolation in fig. 12 and 13 shows. It is not obvious how these diagrams should be interpreted.

CHIBNALL and PIPER, (1934*a*) mention that wax of *Musa sapientum* on saponification yielded a large amount of prim. alcohol and little acid. The alcohol was found to be a mixture of $C_{28} + C_{30} + C_{32}$ in the proportion 2 : 4 : 4. The acid has not been identified. The wax yielded no ketone, sec. alcohol or paraffin.

GRESHOFF and SACK (1901) found in that wax an ester consisting of an alcohol melting at 78° and an acid melting at 71°. The melting point of C_{18} n-fatty acid, i.e. stearic acid, is 69° while the melting points of C_{26} and C_{28} prim. alcohol are 79.5° and 83.2°. We therefore think of the possibility that the character of the l.c. refl. in this diagram must be due to esters of alcohols of a length normally occurring in natural waxes and of fatty acids like those occurring in natural glycerides, i.e. of the lengths C_{16} and C_{18}, which esters would have a length nearly corresponding to that of the spacings measured.

An additional reason for which we are inclined to suggest esters as main components of these waxes is that we know no other typical wax constituents which allow an explanation of the observed l.c. refl.

Type No.

The diagrams of this type show no l.c. refl. This phenomenon is regarded as indicative of a fairly complicated composition of the wax. About two of the waxes belonging to this type, the literature provides certain data:

Flower wax of *Hyacinthus* sp., originating from the factory of "Orange" at Amsterdam. For further data see *Rosa.*

In this wax STRAMAN (1926) found almost equal proportions of paraffin, alcohol, saturated- and unsaturated acid. It is in accordance with the absence of long-chain reflections that no component predominates.

Wax of *Opuntia* sp. was examined by CHIBNALL, c.s. (1934*a*). It was found to contain prim. alcohols (mainly C_{28} and C_{30}), paraffins (mainly C_{33} and C_{35}) and fatty acids of c. C_{22}. The diversity of the chain lengths may account for the absence of l.c. refl.

Group D.

The diagrams of this group show abnormal sets. of s.s. refl.

Type DG, cf. fig. 9, I and II, and fig. 11, I and II.

The set of side-spacing reflections is distinct from that in the normal wax diagrams in that the reflections 2 and 3 (cf. table IV*a*) lie closer to each other and are less sharp. The diameters ($2f_{40}$) of these reflections are 31.7 and 35.0 mm. Refl. 5 is notably broadened, while 6 and 8 are very faint. The other reflections are absent. Hence, the packing of the chains is somewhat different from the normal packing. As the reflections seem generally to be a little broadened, the crystallite dimensions in directions perpendicular to the chains may be somewhat smaller than in normal waxes.

The l.c. refl. are distinct and show strong odd- and weak even orders of which the latter in some cases are almost extinguished.

The long spacing (40.7 Å) of the waxes of cereals belonging to this group always corresponds to that of a mixture of C_{29} and C_{31} chains at a proportion 65 : 35. The intensities, as in the type Nsb, indicate the presence of symmetrical sec. alcohols or (and) ketones of the above-given lengths, these substances presumably being mixed with paraffins of the same length, since the even orders are fairly well visible, at least in certain cases.

The waxes of *Triticum* (fig. 11, II), *Avena* and *Secale* (fig. 9, I) show clear even orders, though they are decreased in intensity as compared with the odd orders. Hence, in these cases, presumably, ketones and (or) sec. alcohols are mixed with paraffin.

The waxes of *Hordeum* (fig. 11, I) and the two *Elymus* species show outstandingly weak even orders. In these cases, therefore, presumably, little or no paraffin in present.

POLLARD et al., (1933), from 14 kg dried leaves of young *Triticum* plants in the stage just before the appearance of the ears, isolated 62 g of wax, which they reported to be cytoplasm wax. From this they separated 40 g of unsaponifiable matter which mainly consisted of n-octacosanol and which was found to contain little paraffin and no ketone or sec. alcohol. Not even a trace of the latter substances could be detected.

It should be mentioned here that their results do not agree with ours at all. We have no reason for questioning the results of POLLARD et al. as they were deduced from X-ray measurements carried out in the investigations of CHIBNALL, PIPER et al. On the other hand, we do not see any plausible possibility of interpreting our diagrams other than the interpretation we have given above. Hence, for the presence, we have to assume a striking difference between the cuticle wax of the full-grown stems and the cytoplasm wax of the young leaves of wheat plants. It goes without saying that further evidence concerning this question is highly desirable.

Wax of *Eucalyptus globulus*, collected from the leaves, cf. fig. 9, II.

This diagram shows close correspondence to that of the cereals discussed above. The s.s. reflections except 5 are absent, however. The long spacing (43.5 Å) indicates a chain length C_{33}. The even orders are absent. Therefore, we conclude to the presence of n-triacontane-17-one or -ol, compounds which have not been detected in natural waxes before.

Type DP, cf. fig. 9, III and IV and fig. 4, III.

The side spacing reflections are different from those of the normal wax diagrams. Their diameters and intensities are given in table IV*b*.

TABLE IV*b*.
Side-spacing reflections in type DP.

number:	1	2	3	4	5
$2f_{40}$:	28.0	31.2	34.0	57.0	60.5
intensity:	vs	m-f	s	f	f

Refl. 3 is considerably broadened. In the diagram of *Glaucium* refl. 2 is faint, whereas in the other diagrams it has an average intensity. In *Papaver* refl. 4 and 5 are united into one broad line.

The l.c. refl. show the orders 2, 4, 5 and 7 in decreasing intensities. The orders 3 and 6 are absent. This applies to all diagrams of type DP.

In all cases the long spacing (38.6 Å) is in agreement with a chain length C_{29}. The possibility of presence of C_{27} chains up to c. 25% may, however, not be excluded. The absence of the orders 3 and 6 indicates a side group at $^1/_3$ of the chain. We conclude therefore that the waxes of the *Papaveraceae* studied and of *Picea pungens* mainly consist of d-n-nonacosane-10-ol or -one, the first substance having been found in other waxes before, though mixed with paraffins (cf. *Pyrus* and *Foeniculum*). It should be remembered, however, that in the latter the side spacings are normal.

There are two possible causes for this difference: In the first place, the paraffins present in the latter waxes might force them to crystallise in the normal packing, whereas the sec. alcohol when in a pure state does not crystallise in the normal packing, which might be due to its asymmetric C atom and the exclusive occurrence of the optically active d-form in the natural wax.

In the second place, quite a different substance might be present. The first possibility we consider the most probable, however, as it is in good agreement with some well-establishhed phenomena.

The other diagrams of group D.

In group D, three diagrams cannot be included in one of the types DG or DP, though they each have certain characteristics in common with one of these types:

Wax of *Rubus biflorus* var. *quinqueflorus*, collected from the woody stems.

As to the s.s. refl. the diagram shows a certain likeness to the normal wax diagram. Reflections 2, 3 and 6 of the normal wax diagram are present whereas the others are missing. In addition; there are faint reflections $2f_{40} = 28.0$, 29.5 and 33.6 mm, which only become visible when 0.25 mm pinholes are used.

As to the l.c. refl. there is correspondence with DP; they show a clear 2nd order and rather weak orders 4 and 5, whereas 3 is missing. The long spacing (35.5 Å) corresponds to that of a mixture of $C_{25} + C_{27}$ chains in the proportion 65 : 35.

On account of the absence of the third order, we presumably have to do with a mixture of ketones or sec. alcohols with the side group at c. $^1/_3$ of the chain length, viz. n-pentacosane-8 -ol or -one and n-heptacosane-9-ol or -one.

Since in natural products compounds which possess an asymmetric C atom generally occur in the optically active d-form and as, according to CHIBNALL et al., the alcohol in apple peel wax and *Ginkgo* wax is actually present in the d-form, it is very likely that the wax of *Rubus* also contains the optically active compounds in the d-form exclusively. That the packing is different from that in type DP might be due to the presence of two compounds and to substances which do not reveal themselves in the X-ray diagram.

Wax of *Lepidium latifolium*, collected from the stems.

The s.s. refl. show some correspondence with those of the type DP. They are tabulated in table IV*c*.

TABLE IV*c*.
Side-spacing reflections of *Lepidium* wax.

number:	1	2	3	4	5
$2f_{40}$:	29.9–	33.4–	36.5–	55.5–	63.0
intensity:	vs	s	s	f	f

The l.c. refl. are similar to those of *Rubus*. Their intensities, however, as compared with the s.s. refl., are stronger.

We must infer the presence of the same components as in *Rubus* wax. However, it is remarkable that, though the long spacing and the long-chain intensity fluctuation are the same, the s.s. refl. in the two diagrams are so different. This suggests that other components also play an important rôle, and these we suppose to be present especially in *Rubus* wax, its l.c. refl. being weaker than those of *Lepidium* wax, where they might cause *Rubus* wax to cristallise in a packing more nearly normal.

Wax of *Myrica cerifera* fruits.

The s.s. refl. are tabulated in table IV*d*.

TABLE IV*d*.
Side-spacing reflections of *Myrica* wax.

number:	1	2	3	4	5	6	7	8
$2f_{40}$:	27.9–	30.0–	32.0–	34.6–	45.5–	57.5–	61.5–	68.0
intensity:	s	m	s	s	f	f	f	f

The diameters of refl. 1, 4, 6 and 7 correspond, to a certain extent, to those of 1, 3, 4 and 5 of type DP.

The l.c. refl. allow no conclusions to be drawn.

Group M.

In addition to the normal set of paraffin reflections the diagrams of this group show another set of reflections which is quite different from the former. This second set of reflections is not the same in all diagrams belonging to group M; within the types of this group it is the same. These reflections indicate the presence of wax components which, presumably, are no normal aliphatic long-chain compounds. As to the nature of these substances we suggest cyclic paraffins or resins. We suppose that in each diagram the deviating set of reflections belongs to one admixed substance, though to support this, chemical investigations are required. Their reflections being stronger than the normal wax reflections, these admixtures most probably occur in the waxes in predominating quantities.

The diagrams allow no conclusions to be drawn about the chain lengths or nature of the components of the long-chain fraction in the wax.

Type MC, cf. fig. 10, I.

This type has been found in certain *Crassulaceae*, which are mentioned in table I.

The diameters and intensities of the reflections belonging to the additional component are given in table IV*e*.

TABLE IV*e*.
Reflections from the deviating component in type MC.

number:	1	2	3	4	5	6	7
$2f_{40}$:	7.6–	13.1–	15.4–	18.2–	21.0–	25.1–	28.2
Intensity:	s	f	m	f	vs	f	f

Reflection 1 and 3 may be regarded as the first and second order belonging to a spacing of 16.2 Å.

Type MS, cf. fig. 10, II.
This type has been found in two succulent Compositae and in a Crassulacae, which are mentioned in table I.
The diameters and intensities of the reflections from the additional component are given in table IV*f*.

TABLE IV*f*.

Reflections from the deviating component in type MS.

number:	1	2	3	4	5	6	7	8	9
$2f_{40}$:	3.8	5.9	8.1	12.2	16.5	18.6	22.4	25.0	28.2
intensity:	m	vf	s	f	m	s	vs	vf	vf

Reflection 1, 3, 4 and 5 may be taken for the orders 1—4 belonging to a long spacing of 30.4 Å.

The other diagrams of group M.
The next diagrams both show certain features in common with the types described above, but they cannot be included in these types.

Diagram of *Benincasa cerifera* wax, collected from the fruit cf. fig. 10, IV.
For the reflections from the additional component see table IV *g*.

TABLE IV*g*.

Reflections from the deviating component in *Benincasa* wax.

number:	1	2	3	4	5	6	7	8
$2f_{40}$:	5.4	7.6	15.4	18.5	20.0	21.9	25.8	28.3
intensity:	vf	s	m	s	s	vs	f	f

Refl. 2 and 3 may be regarded as the first and second order reflections belonging to a long spacing of 16.2 Å, which spacing is also shown in type MC. Refl. 4 and 6 are similar to 6 and 7 of type MS respectively.
Furthermore, the three sets of reflections listed in table IV*e*, *f* and *g* above, show correspondence as to the last two weak reflections of each set.

Diagram of *Ricinus communis* wax, collected from the the stems, cf. fig. 10, III.

The reflections belonging to the additional component are given in table IV*h*.

TABEL IV*h*

Reflections from the additional component in *Ricinus* wax.

number:	1	2	3	4	5	6	7	8
$2f_{40}$:	5.4	9.2	14.3	16.5	18.5	19.5	22.0	24.9
intensity:	s	f	m	m	m	s	vf	vf

Reflection 1 and 2 may be regarded as the orders 3 and 5 belonging to a long spacing of 66.7 Å. The intensity of these reflections suggest that they indeed belong to the unknown component and not to the normal long-chain fraction of the wax. On account of the low intensity of the s.s. refl. of the normal long-chain fraction in the wax, only its strongest reflections being visible, it cannot be expected that l.c. refl. of this fraction appear, as the intensity of the latter reflections is always much less than the intensity of the strongest s.s. refl.

Ricinus wax was optically examined by WEBER (1942). Both the birefringence of flow and the birefringence in a preparation smoothed out on glass, proved to be different from that in the other waxes WEBER examined. This different character and the good solubility in ether, as well as the high melting point, led WEBER to the supposition that, apart from the typical wax components, it also may contain cyclic compounds and branched chains. We suppose that all waxes of the M group show similar deviations when examined either optically or as regards their solubility and melting points, because of the presence of non-waxy components.

6. *Survey of the Chemical Results mentioned in Section 5.*

The conclusions drawn about the composition of the waxes in the preceding section, will be briefly summarised and discussed from certain general points of view.

In table V, those kinds of waxes are listed whose diagrams enable an estimation of the predominating components to be made. In this table, these components are mentioned after every plant name. The ratios after certain compounds indicate the estimated proportions of these compounds in the wax in question, as determined by using the data from table II in sect. 4.

The proportion of compounds given in brackets is uncertain, but it is supposed to be less than 30 per cent. of the total main components in the case of prim. alcohols and less than 50 per cent.

in the case of paraffins, including the possibility of total absence. This will be elucidated in what follows:

It will be clear from table II that in binary mixtures of adjacent homologues the long spacing is little sensitive to amounts of the shorter chain up to c. 30 per cent. of the mixture. Therefore, conclusions about the amount of the shorter chain based upon a small deviation of a measured spacing from the theoretical spacing of a pure longer chain are, in our opinion, to be regarded as rather uncertain. Further uncertainy is caused by the fact that the spacings of pure substances as given by PIPER and CHIBNALL, exhibit slight deviations from the values they should possess theoretically: they deviate from the curve, cf. fig. 12. Hence, if only a small difference is observed between a spacing measured in a wax and a spacing which theoretically belongs to a pure substance, we are able to conclude only that this substance is present in the wax in a high proportion, but the slight deviation does not enable an estimation to be made of the amount which is present of a contaminating shorter chain. For, as shown by the empirical points, if the wax consisted of the pure longer chain exclusively, a slight deviation from the theoretical spacing might equally occur.

If the deviation meant above is greater than c. 0.6 Å the ratio of the quantities of the longer and of the shorter chain can be determined with more certainty, and if the deviation becomes more than c. 1 Å, the long spacing becomes very sensitive to slight differences in the proportion. In this case, we always have to deal with mixtures in which the amount of the shorter chain is greater than c. 70 per cent. in paraffins and c. 45 per cent. in prim. alcohols.

In the third column of table V, after those waxes about which data are provided in the literature, these data are quoted so far as they refer to the bulk of the wax. An asterisk after the name of the authors indicates that the data are based upon X-ray measurements as an amplification of chemical analysis. It is only these data which we regard as a check of our results, since chain length determinations without X-ray measurements have proved to be less reliable, cf. sect. 1, chapt. I.

The X-ray method does not allow for the distinction between ketones and sec. alcohols. For this reason, in the names indicating these substances, both the endings "ol" and "one" have been used, followed by a question mark, except in cases where exact data from the literature are available.

In certain cases, there may be some doubt about the position of a CO or CHOH group in a chain. When this is the case, the number indicating the position is followed by a question mark.

TABLE V.

Survey of those waxes of which the diagrams give indication of certain compounds as their main constituents. Further explanation is given in the text.

Wax from:	Main constituents	Results of former investigators
Cotyledon orbiculata	n-hentriacontane, n-tritriacontane, 7:3	
Rosa centifolia (flower wax)	n-heptacosane, n-nonacosane, 65:35	PROPHÈTE (1926): paraffins C_{16}-C_{30}
Viola odorata (flower wax)	n-nonacosane, (n-heptacosane)	STRAMAN (1926): n-hentriacontane
Jasminum grandiflorum (flower wax)	n-hentriacontane, (n-nonacosane)	STRAMAN (1926): n-hentriacontane
Xanthosoma violaceum	*n-hentriacontane-16-one?,-ol?	
Colocasia antiquorum	id.	
Brassica oleracea var. *capitata*	n-nonacosane n-nonacosane-15-one?,-ol?	CHANNON and CHIBNALL* (1929): n-nonacosane 95% n-hentriacontane 5 % n-nonacosane-15-one (in wax of the protoplasm)
Crambe maritima	id.	
Acacia dealbata (flower wax)	*n-tritriacontane-17-one?,-ol? (*n-hentriacontane-16-one?,-ol?)	STRAMAN (1926): n-hentriacontane
Allium porrum	n-hentriacontane *n-hentriacontane-16-ol?,-one?	
Allium fistulosum	*n-hentriacontane-16-ol?,-one?	
Aloë vera	n-hentriacontane (n-triacontanol) *$CaSO_4 . 2H_2O$	
Foeniculum vulgare	n-nonacosane, (n-heptacosane) d-n-nonacosane-10-ol?,-one?	
Pyrus malus	id.	CHIBNALL* et al. (1931): n-nonacosane n-heptacosane d-n-nonacosane-10-ol

Saccharum officinarum	n-octacosanol, (n-hexacosanol)	WIJNBERG (1909): n-triacontanol
Coix lacrima *Andropogon sorghum* *Eulalia japonica*	id.	
Saccharum spontaneum	n-hexacosanol, n-tetracosanol, 65:35	
Phragmites communis	id.	
Arundo donax	n-triacontanol, n-octacosanol, 45:55	
Yucca filamentosa	n-octacosanol, n-hexacosanol, 6:4	
Iris florentina	n-hexacosanol, n-octacosanol, n-triacontanol, 1:1:1	
Iris germanica	id.	
Triticum vulgare	n-nonacosane, n-hentriacontane n-nonacosane-15-ol?,-one? *n-hentriacontane-16-ol?,-one?	POLLARD* (1933): n-octacosanol (in wax of the protoplasm)
Avena sativa *Secale cereale*	id.	
Hordeum vulgare	n-nonacosane-15-ol?,-one? *n-hentriacontane-16-ol?,-one?	
Elymus arenarius *Elymus sybiricus*	id.	
Eucalyptus globulus	*n-tritriacontane-17-ol?,-one?	
Papaver somniferum	d-n-nonacosane-10-ol?,-one?	
Glaucium flavum *Argemone mexicana* *Picea pungens glauca*	id.	
Lepidium latifolium	*d-n-heptacosane-9?-ol?,-one? *d-n-pentacosane-8?-ol?,-one?	
Rubus biflorus	id.	
Musa paradisiaca *Strelitzia reginae*	esters of C_{28}-C_{30} prim. alc. and C_{16}-C_{18} fatty acids?	

Compounds which, so far, had not been found in natural waxes are marked with an asterisk.

A number of waxes, viz. those of group M in table I, contain, apart from typical wax components, a great quantity of a substance which presumably does not belong to the normal long-chain constituents. Consequently, the typical wax reflections are weak and the diagrams exhibit no long-chain reflections. For that reason no suggestions about the long-chain compounds in those waxes can be given. In the various waxes belonging to this group, the non-waxy component is not always the same. These components may be cyclic paraffins or resins. There is no instance in which they have been identified, however.

In a number of waxes made up of typical long-chain compounds, these compounds could not be identified any further owing to lack of distinctness of the long-chain reflections, or on account of their total absence. These latter features have been supposed to be indicative of a fairly complicated composition of these waxes.

With a view to further investigations, attention may be called to the following problems arising from the preceding section:

1. whether in the waxes of *Papaveraceae* and of *Picea* the appearance of an abnormal set of side-spacing reflections is associated with the exclusive occurrence of an optically active d-modification of the main constituent.
2. whether in wheat a fundamental difference does exist between the protoplasm wax of the young leaves and the cuticle wax of the stems of full-grown plants.
3. whether the inversion of the long-chain intensity fluctuation in the diagram of sugar cane wax from aged press cake, as compared with the diagram of scraped-off sugar cane wax, may indeed be explained by the presence of a mixture of paraffin and prim. alcohol in the former, and whether a similar explanation applies to the fluctuation in the case of *Aloë* wax.
4. what compounds in the waxes showing M diagrams give rise to the abnormal set of reflections.
5. how typical wax esters reveal themselves as long-chain reflections, and how long-chain reflections behave in mixtures of compounds belonging to different homologous series.

CHAPTER IV.

THE SCHEME OF CHIBNALL AND PIPER FOR THE METABOLISM INVOLVED IN THE FORMATION OF NATURAL WAXES, DISCUSSED IN CONNECTION WITH THE CONCLUSIONS DRAWN FROM THE DIAGRAMS

CHIBNALL and PIPER (1934*b*) have made certain hypotheses about what metabolic processes may lead to the formation of the various compounds they have been able to identify in natural waxes. They have embodied their postulates in a scheme that will be discussed briefly below, after which we shall ascertain whether our results, as summarised in the preceding section, are consistent with this scheme. For this purpose we have to pay, of course, special attention to the compounds which had hitherto not been found in natural waxes and which are marked with an asterisk in table V. As to the considerations that have led CHIBNALL and PIPER when framing their scheme, we must refer to their publication.

The scheme can be reproduced best in two parts, the first of which is represented as follows:

$$\begin{tikzcd}
& \mathrm{R_1\,.\,CH = CH\,.\,R_2\,.\,COOH} \arrow[dl, "\mathrm{I}"'] \arrow[dr, "\mathrm{II}"] & \\
\mathrm{R_1\,.\,CH_2\,.\,CH_2\,.\,R_2\,.\,COOH} & & \mathrm{R_1\,.\,CHOH\,.\,CH_2\,.\,R_2\,.\,COOH} \arrow[d, "\mathrm{III}"] \\
& & \mathrm{R_1\,.\,CO\,.\,CH_2\,.\,R_2\,.\,COOH}
\end{tikzcd}$$

It refers to the hypotheses according to which hypothetical unsaturated n-fatty acids (hypothetical with respect to their length, as they are only known in nature from the series of glyceride fatty acids, which are much shorter than wax fatty acids) undergo reduction to give the saturated wax fatty acids (I); and according to which from the same fatty acids hydroxy-acids and ketonic acids are formed by hydration (II) and subsequent oxidation (III)

The second part of the scheme may be represented as follows:

$$\begin{tikzcd}
\mathrm{R\,.\,CH_2\,.\,CH_2\,.\,CH_2OH} & \mathrm{R\,.\,CH_2\,.\,CH_2\,.\,COOH} \arrow[l, "\mathrm{IV}"'] \arrow[d, "\mathrm{V}"] & \\
& \mathrm{R\,.\,CO\,.\,CH_2\,.\,COOH} \arrow[r, "\mathrm{VII}"] \arrow[d, "\mathrm{VI}"] & \mathrm{R\,.\,CO\,.\,CH_3} \arrow[d, "\mathrm{VIII}"] \\
\mathrm{R\,.\,CH_2OH} & \mathrm{R\,.\,COOH} \arrow[l, "\mathrm{IV}"'] & \mathrm{R\,.\,CH_2\,.\,CH_3}
\end{tikzcd}$$

It is, amongst other things, based upon the hypothesis that prim. wax alcohols are formed from the corresponding acids by a process of reduction (IV).

A fatty acid can undergo oxidation to give the β-keto-acid (V), from which by hydrolysis, under separation of acetic acid, a fatty acid is formed with two carbon atoms less (VI) than the initial fatty acid. This process, known as β-oxidation, and first established by KNOOP (1904) in feeding experiments with animals, is a generally accepted principle to explain the formation in the organism of a series of shorter fatty acids from a longer one.

The β-keto-acid presumably may also be decarboxylated to give a methyl ketone with one carbon atom less than the original fatty acid (VII), and by reduction of the ketone (VIII) a paraffin might be formed with one carbon atom less than the initial fatty acid.

From the hydroxy-acids and ketonic acids mentioned in the first part of the scheme, by way of the processes V, VII and VIII, secondary alcohols and ketones like those occurring in waxes might be formed.

When starting from even number unsaturated acids, the scheme accounts for the presence of a series of even-number fatty acids and prim. alcohols as well as odd-number paraffins, sec. alcohols and ketones in natural waxes.

CHIBNALL and PIPER refrain from making any suggestions about the mechanism of the biological synthesis of unsaturated fatty acids. The original suggestion of Emil FISCHER, viz. that fatty acids arise from the condensation of sugars, they suppose to be questionable, however. Their investigations have not shown fatty acids and alcohols with numbers of carbon atoms being multiples of six to occur more frequently in waxes than those with other numbers, as it might be expected if a formation from sugars took place. Our results agree in showing that multiples of six are not of more frequent occurrence.

In addition, it should be mentioned that certain intermediate compounds playing a part in the scheme, and having chain lengths such as characteristic of waxes, have not been found in plant or animal products; e.g. the unsaturated acids and hydroxy-acids in the first part of the scheme and the methyl ketones in the second part. In the shorter series of fatty acids from natural glycerides, however, unsaturated acids are of frequent occurrence. In this series also hydroxy-acids have been found, while methyl ketones with chain lengths from C_7 up to and including C_{13} have been found in essential oils. A ketonic acid such as appears in the first part of the scheme, has been found by CHIBNALL and PIPER et al. (1934*a*) in the insect wax of *Coccus cacti*, but no further incidence of such acids with chain lengths longer than those of the natural glyceride fatty acids, is known.

If, now, we consider the compounds in table V which were hitherto unknown in natural waxes, it will appear that the formation of these compounds can be accounted for by the scheme without giving rise to special difficulties. There are certain points which deserve attention, however:

In the first place, the considerations of CHIBNALL and PIPER concerning the positions of the double bonds in the hypothetical unsaturated fatty acid chains, as related by way of the scheme to the positions of CO or CHOH groups in the ketones and sec. alcohols found, deserve attention.

In the second place, we note the frequency of the occurrence of ketones and/or sec. alcohols appearing in our results, especially of the symmetrical ketones and sec. alcohols; whereas CHIBNALL and PIPER have found these symmetrical substances and in general all these "secondary products" to be of "an extremely limited distribution in the plant kingdom", (somewhat to their surprise).

In connection with the discussion of these points, in table VI are summed up all products with CO or CHOH in the chain definitely identified in natural waxes by CHIBNALL, PIPER et al., and those the occurrence of which is very likely on account of our diagrams.

As for the first point, it will be clear that the first two compounds mentioned in the first column of table VI, according to the abovementioned schemes, can be derived from the series of unsaturated acids:

$$CH_3 . (CH_2)_{13} . \overset{15}{CH} = \overset{16}{CH} . R_1 . COOH,$$

in which it is supposed that the hydroxyl group is attached to C atom 15.

The three next compounds can be derived from acids of the unsaturated series:

$$CH_3 . (CH_2)_{17} . \overset{19}{CH} = \overset{20}{CH} . R_2 . COOH,$$

in which it is supposed that the hydroxyl group is attached to C atom 20.

TABLE VI.

The n-long-chain compounds possessing a CO or CHOH group which, according to the present evidence, can occur in natural waxes.

Upper part of the table: Components definitely identified in waxes by Chibnall, Piper et al.

*Plant species in the wax of which the substance in question is indicated by our diagrams.

Lower part: New components indicated by our diagrams.

A question mark expresses uncertainty whether either CO or CHOH is present, or, when placed after a figure, whether the position denoted by this figure, is quite correct.

Formula	Name	In wax from:
$CH_3 . (CH_2)_{13} . \overset{15}{CO} . (CH_2)_{13} . CH_3$	n-nonacosane-15-one	*Brassica* species
$CH_3 . (CH_2)_{13} . \overset{15}{CHOH} . (CH_2)_{13} . CH_3$	n-noncosane-15-ol	id.
	id. -ol?,-one?	**Crambe*, *cereals
$CH_3 . (CH_2)_{18} . \overset{20}{CHOH} . (CH_2)_8 . CH_3$	d-n-nonacosane-10-ol id. -ol?,-one?	apple cuticle, *Ginkgo* **Foeniculum*, **Papaveraceae*, **Picea*
$CH_3 . (CH_2)_{18} . \overset{20}{CO} . (CH_2)_{11} . COOH$	13-keto-n-dotriacontanoic acid	insect wax of *Coccus cacti*
$CH_3 . (CH_2)_{18} . \overset{20}{CO} . (CH_2)_{13} . CHOH$	15-keto-n-tetratriacontanol	id.
$CH_3 . (CH_2)_{14} . \overset{16}{C?} . (CH_2)_{14} . CH_3$	n-hentriacontane-16-ol?,-one?	*Araceae*, *Allium*, *Elymus*, cereals
$CH_3 . (CH_2)_{15} . \overset{17}{C?} . (CH_2)_{15} . CH_3$	n-tritriacontane-17-ol?,-one?	*Acacia*, *Eucalyptus*
$CH_3 . (CH_2)_{16} . \overset{18?}{C?} . (CH_2)_6 . CH_3$	d-n-pentacosane-8?-ol?,-one?	*Rubus*, *Lepidium*
$CH_3 . (CH_2)_{17} . \overset{19?}{C?} . (CH_2)_7 . CH_3$	d-n-heptacosane-9?-ol?,-one?	id.

The numbers above the formulae denote the position of the side group reckoning from the end of the chain at which no β-oxidation can take place.

Accordingly, Chibnall and Piper conclude that all long-chain wax products known to them, can be derived from one homologous series:

$$CH_3 . (CH_2)_{13} . \overset{15}{CH} = \overset{16}{CH} . (CH_2)_2 . \overset{19}{CH} = \overset{20}{CH} . R_3 . COOH$$

with the double bonds between the carbon atoms 15 and 16, and 19 and 20 respectively, reckoning from the end of the chain at wich no x-oxidation can take place.

The process implies that at one of the double bonds hydration and at the other one hydrogen uptake takes place. Otherwise it would lead to chains possessing two sidegroups.

The authors lay no stress on the exact position of the double bonds on account of the uncertainty regarding the point of attachment of the hydroxyl group on hydroxylation.

If, now, we endeavour to derive from the same unsaturated series the newly found wax components mentioned in the lower part of table VI, this appears to be possible for the first and the last only: The first we can arrive at by supposing the OH group to become attached to carbon atom 16, the last by supposing this group to become attached to carbon atom 19.

If, however, the oxygen is found at the places 17 or 18, as in the second and the third of the compounds mentioned in the lower part of the table, we have to suppose other positions of the double bonds: The occurrence of the oxygen at place 17 necessitates a double bond between C atoms 16 and 17, or 17 and 18; the occurrence of the oxygen at place 18 demands a double bond between C atoms 17 and 18 or 18 and 19.

By assuming a third double bond in the hypothetical initial homologous series between the C atoms 17 and 18, the two substances discussed last can be derived from the series as well. Hence, if it is desirable to consider all wax components as metabolic products formed from one hypothetical series of unsaturated acids according to the scheme of Chibnall and Piper, we would replace the double unsaturated series they have suggested by the triple unsaturated series:

$$CH_3 . (CH_2)_{13} . CH = CH . CH = CH . CH = CH . R . COOH,$$

with double bonds between the C atoms 15 and 16, 17 and 18, and 19 and 20 respectively, reckoning from the end of the chain at which no β-oxidation can take place. This allows for the derivation of both the new components indicated by our diagrams and those already known, from the same series.

As to the second point to be discussed, and briefly defined above already, the following can be said:

It is striking that our diagrams suggest ketones and sec. alcohols to be of a much more widespread occurrence in plant waxes than CHIBNALL and PIPER have been able to establish; they found symmetrical products in *Brassica* species only and d-n-nonacosane-10-ol in wax of the apple cuticle and of *Ginkgo*, cf. table VI. It is remarkable that it is the positions $^1/_2$ and $^1/_3$ which seem to be preferred above others. At present we do not see a plausible explanation of the preference for the position $^1/_3$. The comparatively frequent cases, however, in which we have been forced to assume a symmetrical product with a CO or CHOH group as a main wax component — often mixed with a paraffin of the same length — raise the question whether formation of these components by condensation of fatty acids of half the length of the compounds in question, in accordance with the principle:

$$2\,R.COOH \rightarrow R.CO.R + H_2O + CO_2,$$

is likely. After reduction, the ketones thus formed might yield sec. alcohols and paraffins.

This possibility, already suggested by CHANNON and CHIBNALL (1929) in connection with their finding n-nonacosane-15-one in cabbage leaves, and by CLENSHAW and SMEDLEY-MACLEAN (1929) on account of their isolation of hentriacontane from spinach leaves, was later rejected by CHIBNALL and PIPER (1934*b*) in favour of the new scheme. Important arguments against that suggestion have, among other things, been firstly the circumstance that n-nonacosane-15-one requires the odd-number C_{15} acid n-pentadecanoic acid as its precursor, whereas there is no evidence as yet that odd-number n-fatty acids of that length occur in natural products and, secondly, that symmetrical ketones and sec. alcohols in the waxes investigated by CHIBNALL et al., proved to be confined to the only instance of n-nonacosane-15-one and -ol in *Brassica* species.

On account of the many instances in which our diagrams are found to be indicative of the presence of the above-mentioned products, including also symmetrical C_{31} and C_{33} chains, the natural glyceride fatty acids, in our opinion, deserve new attention as possible precursors of wax constituents. As arguments in favour of this possibility we mention the symmetry of the compounds in question and the possibility of deriving hentriacontane-16-one, for the occurrence of which we found many indications, from the widespread C_{16} acid, palmitic acid. On the other hand, objections arise owing to the impossibility of deriving in the same way the C_{29} and C_{33} symmetrical ketones and sec. alcohols from such acids (as they require odd number acids) and the lack of indications of the presence of the condensation product of stearic (C_{18}) acid, the C_{35} ketone.

A possibility of removing these objections might be found in the so-called ω-oxidation, i.e. the oxidation of a terminal methyl group of a n-fatty acid. This type of oxidation was observed by VERKADE and V. D. LEE (1934) in feeding experiments with animals. On administration of e.g. capric acid (C_{10}) in the form of tricaprin the excretion of the dicarboxylic acid sebacic acid, possessing the same number of carbon atoms as capric acid, has been demonstrated.

It has been shown by FLASCHENTRÄGER et al. (1934) that ω-oxidation may be followed by a breakdown of the chain at the end at which this oxidation has taken place, in a similar way as described above for β-oxidation.

Though ω-oxidation has been observed neither in longer chains nor in plants, we might still imagine C_{29} and C_{33} ketones to arise from C_{31} and C_{35} ketones (the latter two formed from palmitic and stearic acid) by oxidation of both terminal methyl groups of these latter ketones to carboxyl groups (ω-oxidation) and subsequent β-oxidation, decarboxylation and reduction, according to V, VII and VIII in the scheme of CHIBNALL and PIPER. This might be represented as follows:

$$\begin{array}{cl} CH_3 . (CH_2)_2 . R . CO . R . (CH_2)_2 . CH_3 & \\ \downarrow & \\ COOH . (CH_2)_2 . R . CO . R . (CH_2)_2 . COOH & \\ \downarrow & \mathrm{V} \\ COOH . CH_2 . CO . R . CO . R . CO . CH_2 . COOH & \\ \downarrow & \mathrm{VII} \\ CH_3 . CO . R . CO . R . CO . CH_3 & \\ \downarrow & \mathrm{VIII} \\ CH_3 . CH_2 . R . CO . R . CH_2 . CH_3 & \end{array}$$

It would be premature, however, to lay much stress upon the given account for the symmetry and frequency of the compounds in question as long as no further evidence is available about biochemical processes in living plants involving chains possessing lengths characteristic of plant wax constituents.

We hope to have demonstrated in the preceding discussion how the X-ray diagrams of native waxes may give support to certain theories about metabolic processes in their formation.

CHAPTER V.

THE WAX DIAGRAMS CONSIDERED IN CONNECTION WITH THE NATURAL RELATIONSHIP OF PLANTS

From the tabular survey in chapt. II, 2 and the results compiled in chapt. III, 6, certain conclusions may be drawn as to how far the composition of the cuticle wax of plants is correlative with their natural relationships. The number of waxes investigated being rather small, these conclusions can only be of a preliminary character, however.

In many cases, correspondence exists between the wax diagrams of species belonging to a same plant family, e.g. among the two groups belonging to the *Gramineae*, among the *Araceae*, *Iridaceae*, *Musaceae*, *Papaveraceae*, *Cruciferae* and *Crassulaceae*. In some cases the diagrams are quite identical.

On the other hand, exceptions from the characteristic type of diagram occur in some families: *Lepidium* wax is different from that of the two other *Cruciferae* investigated. *Cotyledon* wax is different from that of the other *Crassulaceae*, and so is *Kalanchoë* wax; the diagrams of both waxes are the same as those of the *Compositae Senecio* and *Kleinia*. *Yucca* wax, in contrast to the other waxes of *Liliaceae*, belongs to the waxes with "double" chains and shows correspondence to those of the *Iridaceae*.

In the two latter cases the similarity between the composition of the waxes of plants of different families is accompanied by a certain correspondence in morphological characters of the plants under consideration. It is a matter for further investigation, however, whether it is a general phenomenon that morphological similarities crossing the natural relationships coincide with correspondence in the composition of the waxes. Below, we shall return to this question when discussing the wax of succulents.

A great similarity can also exist between wax diagrams of plants belonging to widely divergent families. This is, for instance, the case with the almost identical diagrams of the waxes of *Picea* and *Papaver*, and with the diagrams of *Eucalyptus* and the cereals, cf. fig. 9. In the latter instance, there is a chain length difference of two carbon atoms, however. The fact that, occasionally, correspondence is found between waxes of plants from widely divergent families, may be associated with a relatively limited variability of the wax constitution as compared with the immense variability of plant forms.

Besides the correspondence in the waxes of allied species belonging to the same family, a more general correlation may be observed. Thus it appears that the diagrams of the type Nl, which are characteristic of prim. alcohols, are found in Monocotyledons exclusively, viz. in *Gramineae*, *Liliaceae* and *Iridaceae*.

Finally, it is striking that waxes with M-diagrams are found in succulent forms predominantly, especially in those succulent forms which belong to the *Crassulaceae* and *Compositae*. The wax of certain *Cactaceae*, however, does not show this type of diagram, nor do the waxes of *Cotyledon orbiculata* and *Aloë speciosa*. M-diagrams are also found in non-succulent forms such as *Cucurbita* and *Ricinus*, though these are M-diagrams other than those meant above. In the family of the latter (*Euphorbiaceae*) many succulent forms are found, however. Hence, it should appear from further investigations how far indeed M-diagrams are correlated with succulence.

We may conclude that:

1. The composition of the cuticle wax cannot be regarded as characteristic of the plant species.
2. Closely allied species generally produce waxes of corresponding, or identical composition, slight differences being mainly confined to the proportions in which the constituents are mixed.
3. There are plants of widely divergent families which nevertheless have cuticle waxes of almost the same composition.
4. Cuticle waxes which predominantly consist of prim. alcohols seem to be confined to Monocotyledons.
5. There are certain indications that morphologic features common to plants of different families, are often accompanied with correspondence in the constitution of the cuticle wax.

Summary of part A.

The present knowledge concerning the constitution of plant cuticle waxes was based mainly on investigations of those waxes which occur in thick layers and are of economic importance. On account of the slight thickness of most plant wax layers and the difficulties involved in chemical wax analysis, only a few of the thinner layers had been examined.

In earlier work on natural waxes (CHIBNALL, PIPER et al.), X-ray methods have proved to be an invaluable aid in identifying the components isolated by chemical analysis of such products.

We observed that X-ray diagrams of native plant cuticle waxes scraped off the plant surfaces, often show marked differences and distinct so-called long-chain reflections. Therefore, it seemed worth while to undertake an investigation of those waxes without applying any chemical treatments, by merely studying their X-ray diagrams.

The initial aim of the investigation has been to compare the X-ray diagrams of a number of cuticle waxes, with the intention to establish whether certain similarities and differences between the waxes are correlated with the natural relationship of the plants producing them.

The second purpose was to ascertain whether this comparatively simple method of investigation might allow the identification of any constituents of the waxes and, if so, what new data it would be able to provide about the constitution of the waxes examined.

In chapter I, the advantages and drawbacks inherent in the relative simplicity of the method have been pointed out in comparison with the usual methods of wax investigation. This is followed by a description of features of the X-ray diagram as connected with the crystal structure of those n-aliphatic long-chain compounds which, according to earlier investigations, are characteristic of plant waxes. These features will, naturally, be of much importance for the identification of certain compounds in the waxes. Finally, a description is given of the methods of collecting the waxes and of the apparatus used for obtaining the diagrams.

In chapter II a classification is given of the diagrams obtained; they have been divided into three main groups subdivided into a number of types, according to certain criteria. In addition, a tabular survey is given in which important details of all diagrams are listed. These details especially concern the so-called long-chain reflections exhibited by most diagrams, which might enable certain compounds to be identified in the mixtures.

Subsequently, in the first four sections of chapter III, the point is discussed whether the diagrams do allow such a qualitative chemical interpretation. It has turned out that if distinct long-chain reflections appear, they generally give indication of the predominating constituents in the wax with a comparatively high degree of certainty. This favourable outcome is due to:

1. the evidence gained that, as a general rule, the constituents in the wax can be assumed to be crystallised in the rhombic modification,
2. the availability of certain data concerning the long crystal-spacing in synthetic mixtures of rhombic wax constituents, as collected by CHIBNALL, PIPER et al.,
3. the knowledge of the intensity fluctuation in the long-chain reflections of various n-alphatic long-chain compounds as dealt with in chapter I, 2.

In chapter III, 5, along the lines indicated in the preceding sections, it has been ascertained for each individual diagram what conclusions are justified concerning the constitution of the wax under discussion.

It has turned out that in 37 of the 60 kinds of waxes examined, certain compounds, which presumably constitute the bulk of the wax, could be identified with a, more or less, high degree of certainty. In 28 of these instances, data about the composition of the wax were entirely lacking.

In general, the diagrams proved to be indicative of n-long-chain compounds belonging to either of the series of paraffins, prim. alcohols, ketones or sec. alcohols as main constituents of the wax, whereas only two diagrams gave reason to assume the presence of esters as main constituents. In 13 waxes it was concluded substances were present which, although belonging to one of the abovementioned series, had hitherto not been found in natural waxes. This concerns 4 different compounds, cf. table V.

In some waxes, the diagram turned out to be indicative of the presence of a high percentage of products which presumably cannot be referred to the n-aliphatic long-chain compounds, but which could not be identified any further. The diagram of *Aloë* wax pointed to the presence of a fair amount of gypsum ($CaSO_4 . 2H_2O$) in this wax, a fact hitherto not recorded.

Finally, in section 6 of chapter III, a survey is given of all chemical data obtained in the preceding section. In this survey, in many cases evidence is recorded of the occurrence of symmetric ketones or sec. alcohols, whereas CHIBNALL and PIPER found these products to be of an extremely limited occurrence in the waxes they examined.

Similar conclusions hold true for products with a CO or CHOH group at c. $^1/_3$ of the chain length.

In chapter IV, the results are discussed in connection with the theories concerning the metabolic processes active in natural wax formation as postulated by CHIBNALL and PIPER. In general these theories are consistent with the formation of all new components indicated by our diagrams. It was clear, however, that the hypothetical series of initial double unsaturated n-fatty acids from which, according to these theories, all long-chain products identified in waxes might originate, must be replaced by a triple unsaturated series to allow for the derivation of some of the new components indicated by our diagrams.

Furthermore, the frequent occurrence of symmetric ketones and sec. alcohols gives no support to the formation of these substances according to the scheme of CHIBNALL and PIPER, but directs attention to the possibility of a formation from natural glyceride fatty acids of half the length of the compounds in question, as previously suggested by CHANNON and CHIBNALL, and CLENSHAW and SMEDLEY-MACLEAN.

The last chapter inquires into the question how far the composition of the waxes reflects the natural relationships of the plants producing them. It was found that closely allied species generally produce waxes of similar or identical composition, but there are also instances of close agreement in this respect between species belonging to widely divergent plant groups.

PART B.

Investigation into the Submicroscopic Structure of the Wax Rods of Sugar Cane Stems

CHAPTER I

SOME INTRODUCTORY ASPECTS OF THE MORPHOLOGY AND SUBMICROSCOPIC STRUCTURE OF WAXY COATINGS OF PLANTS

The waxy coatings on plant cuticles show various structures when examined microscopically. These structures were studied by DE BARY on a wide variety of plant species as early as 1871. This work may still be referred to as the most extensive and detailed on this subject. DE BARY has distinguished four principal microscopic structures of wax coatings, which, on account of the occurrence of intermediate forms, cannot always be strictly differentiated, however. These forms are:

1. the heaped wax layers, consisting of a compact layer of very fine needles or grains superimposed at random,
2. the singular granulated layers, consisting of a single layer of wax grains, which may be separated by comparatively wide interspaces, but may also be joined to form a thin continuous brittle layer,
3. the rodlet layers, consisting of a single layer of wax rods which are c. 1—4μ thick, and are placed nearly perpendicularly to the epidermis, either closely together or in separate groups and, if so, sometimes on definite epidermal cells exclusively,
4. the crusty wax layers, mostly thin, brittle, sometimes cracked, homogeneous, glazy coatings, but sometimes also thicker or even very thick, layered covers.

Out of the many examples of each form mentioned and described by DE BARY, those wax layers of which we have studied the X-ray powder diagrams in part A, will be mentioned below:

According to DE BARY, the wax layers of *Elymus arenarius*, *Secale cereale* and of various species of *Eucalyptus*, consisting of fine needles, and those of *Kleinia* and *Ricinus*, consisting of grains, belong to the first group mentioned above.

The wax layers of *Iridaceae*, *Liliaceae* and of *Brassica* belong to the second group. This is the form which is found most frequently.

The wax layers of *Musa*, *Canna*, *Strelitzia*, *Saccharum*, *Coix* and *Eulalia* belong to the third group, while also those of *Benincasa cerifera* and of *Cotyledon orbiculata* may be included here.

The layers of *Opuntia*, *Cereus* and of the *Myrica* fruit belong to the fourth group. Also the well-known thick wax layers of the wax yielding palms *Copernicia cerifera*, *Klopstockia cerifera* and *Ceroxylon andicola* are classed in this group.

In particular some of the wax layers of groups 3 and 4 show remarkable structural peculiarities when examined microscopically; for these details we refer to DE BARY and to the examples to be discussed in Chapter IV, 4.

Wax deposits are not found on the cuticle only, but they are often present in the outer walls of the epidermal cells as well, especially in the cutinised cell-wall layers. This wax cannot be observed microscopically without taking certain measures; e.g. on heating the wax appears as droplets on the planes of intersection of the cell-walls. The presence of wax in the cutinised layers is not always associated with the presence of wax on the cuticle surface.

DE BARY has also given his opinion about the submicroscopic structure of the wax covers. We quote:

„Für die gehäuften Ueberzüge, zumal die feinen Nädelchen von *Secale* u.a., ist es dem Augenschein nach wahrscheinlich, dass ihre einzelnen Formelemente krystallinischer Natur sind. Die grösseren Stäbchen und vor allem die Schichtenüberzüge zeigen vielfach einen Bau, welcher an den von geschichteten und gestreiften Zellmembranen lebhaft erinnert. Es ist daher wenigstens die Vermutung zulässig, dass sie mit diesen den organisierten Formelemente zugehören. Eingehendere Untersuchungen über ihre Molecularstructur werden hierüber noch zu entscheiden haben".

The possibility that rodlet layers and crusty wax covers owe their morphological characteristics to organisation by the living matter was rejected by WIESNER (1876). After some hesitation, cf. WIESNER (1871), he expressed as his definite opinion that the wax covers are crystallised excretions and that it is their crystalline nature which accounts for their structure. This opinion was based on the fact that the waxes are birefringent and that they separate in crystals from their solutions. According to WIESNER the birefringence can only be attributed to their crystalline nature, the plasticity of the substance preventing internal strains.

In part, the controversy finding expression in the above-mentioned opinions is unreal, as we know at present that the so-called organised matter is to be regarded as crystalline as well, at least to a certain extent. This crystallinity had already been postulated by NÄGELI's theory of crystalline micelles (1858) and has been corroborated by means of X-ray diffraction after about half a century. Inasmuch, however, as the concept of "organised" implies a direct influence of the living matter on the growth of the rodlets and of certain crusty wax layers, the controversy between the ideas of the above-mentioned authors remains: A true growth was considered likely by DE BARY, whereas WIESNER advocated excretion and crystallisation on the cuticle surface exclusive of any direct protoplasmatic activity in the formation of the various structures. Only unequal distribution of the wax on the plant surface would, according to WIESNER, be a matter of organisation.

At the present time, still little clarity exists as to the problems of the excretion of the wax and the formation of the various structures. In chapter IV we shall return to these points.

As to the crystallinity of the waxes, there is no reason to doubt at present that in the native state they are to be regarded as crystalline; for as far as investigated, they render clear X-ray diffraction spectra without exception, cf. part A of our investigations. Irrespective of this knowledge there remain, of course, many problems concerning their submicroscopic structure.

Among the heaped wax covers, undoubtedly the small, randomly heaped needles, which form these covers, are to be regarded as fine crystals, as DE BARY suggested. The isotropic grains of the granular wax layers might be built up like sphaerocrystals, as WIESNER (1876) supposed.

More difficulties arise in forming a picture of the submicroscopic structure of the microscopic units of bigger size, such as the parallel rods of the rodlet layers or e.g. the prisms described by DE BARY for the crusty wax layer of *Klopstockia cerifera*. These bodies give rise to the problem whether they are to be regarded as individual crystals or as aggregates of submicroscopic crystallites. If they are individual crystals it would be of interest to know the position of the crystal axes with respect to the cell-wall and if they are aggregates of submicroscopic crystallites the questions of the form and orientation of these crystallites arise. Evidence concerning these details would be the first requirement for obtaining insight into the formation of these wax layers. In addition, of course, a precise knowledge concerning the structure of the underlying cell-wall layers is desirable. Furthermore, it is a question how the various wax constituents are distributed in the wax layer: whether every single crystallite consists of one single substance or if chain molecules of different lengths associate to form impure crystallites (cf. part A, III, 1).

Research with regard to the above-mentioned details in plant wax covers is only at its beginning. This is due to the fact that the study of birefringence does not yield conclusive evidence as to these questions. Nevertheless it has enabled certain conclusions to be drawn.

As a comparatively recent study of birefringence of plant waxes, in particular of wax in cutinised membranes, we mention the work of MADELEINE MEYER (1938). This investigation, involving the cutinised cell-wall layers of *Clivia*, *Gasteria*, *Yucca* and *Dasilyrion*, has led to the conclusion that in these layers the wax is present in tangentially orientated platelets, enclosed in a network of cutin and cellulose chains. The position of the molecules in the platelets is with their longitudinal direction at right angles to the cell-wall. The carbon chains in the plate-shaped wax excretions on the leaves of *Crassula argentea* have, according to this author, the same position.

When our investigation of the structure of the wax rods of sugar cane had already begun, the thesis by WEBER (1942) on the optics and structure of plant waxes appeared. It deals principally with the birefringence of a number of different waxes in different solvents. Also, as a result of examination under the polarising microscope, WEBER gives a schematic representation of the structure of the wax rods of the stem of *Phragmites communis*. These rods show much correspondence to those of the sugar cane stem as regards both their habit and constitution (see A, III, 5). According to WEBER, the molecules in the wax rods are placed with their longitudinal axis perpendicular to the longitudinal direction of the rods, and are orientated radially; so they are parallel to the epidermis. In our opinion the radial direction of the molecules is insufficiently demonstrated by WEBER, to which point we shall return in chapter III, 1. A different picture is given by WEBER for the chips of the wax cuticles of *Crassula argentea*, *Copernicea cerifera*, *Opuntia Ficus-indica* and the wax scales of the abdomen of bees. Here the position of the molecules is perpendicular to the epidermis, in accordance with the result of MADELEINE MEYER.

X-ray investigations of the submicroscopic structure of wax coatings have, so far, not been undertaken, presumably because of the drawback of the smallness of these objects in this type of investigation. Since, generally, the study of X-ray diffraction allows conclusions more far-reaching about the submicroscopic structure than does the study of birefringence, we thought it desirable to make an attempt in this direction.

CHAPTER II

X-RAY INVESTIGATION OF THE WAX RODS

A. INVESTIGATIONS BY MEANS OF STANDARD APPARATUS

1. *Material and Methods*

As has already been mentioned, it is a disadvantage in the investigation to be undertaken that X-ray diffraction methods in general, and in particular the standard equipment at our disposal as described in part A, I, 3, do not lend themselves very well to the study of small objects. It was, accordingly, desirable to select as thick a wax layer as possible as an object for study. For this reason the rodlet layer of *Saccharum officinarum* was chosen, which is in places as thick as c. 0.1 mm, the species being, moreover, under cultivation in one of the hothouses of the botanical garden of the Laboratory of Technical Botany. There are thicker wax layers, such as e.g. those of *Klopstockia cerifera*, of which DE BARY reports a maximum thickness of 5 mm, but these were not available. Furthermore, a rodlet layer seemed particularly suitable as it was not expected to have a very complex submicroscopic structure.

The wax layer covers the sugar cane stem as a thin continuous layer which was classed by DE BARY in the singular granulated layers. At the upper end of every internode this layer increases in thickness and gradually alters into a rodlet layer to form a white ring beneath the nodes. Here the wax layer consists of rodlets, c. 1—4μ thick and 50 to often more than 100μ long, which grow perpendicularly to the epidermis and mostly have a bent tip. Within the white ring, two parts of the layer may be distinguished: a lower layer next to the epidermis, which is about 50μ thick and consists of closely adjoining straight rodlets, the majority of which do not protrude above this layer, and an upper part formed by rodlets protruding from the lower layer. In the upper part the rodlets are twisted and bent (see fig. 17), thus imparting to the layer a white and downy appearance. The longer, twisted rodlets are c. 3—4μ thick. When lifted and seen from below, the layer facing the cuticle shows the surface of the cuticle in relief and is shiny. One gets the impression that the rodlets are fused at the base to form an extremely thin continuous layer. According to DE BARY, groups of rodlets which are much thicker than the rodlets described above, occur on top of the so-called short cells in the epidermis of the outer side of the leaf sheaths.

The epidermis was not investigated in detail. For a description we refer to De Bary and to Wijnberg.

The thickest wax rings, as a rule, are found on the basal stem internodes. Chips were cut off these wax rings by means of a razor, both with and without a part of the epidermis and underlying cell layers. In the latter case the wax separates without difficulty from the cuticle.

We intended to make X-ray diagrams of these chips by irradiating in both the parallel and a perpendicular direction with respect to the longitudinal direction of the rodlets, expecting that the diagram in one or either case would show orientation in the specimen. The method of taking X-ray diagrams described in A, I, 3, was employed in which the common film holders were used, cf. fig. 5.

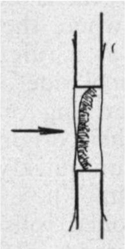

Fig. 14.
Mounting of the specimen when irradiated parallel to the longitudinal direction of the rods.

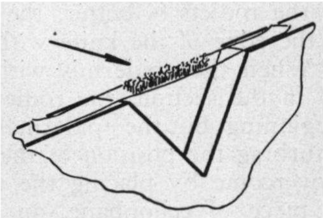

Fig. 15.
Mounting of the specimen when irradiated perpendicularly to the longitudinal direction of the rods.

For the diagrams to be made with the beam parallel to the longitudinal direction of the rods, chips, either with or without epidermis, were put in the central opening of a round glass specimen holder, usually with the epidermis side facing the pinhole. The opening was closed on both sides with thin cellophane films (fig. 14). For the diagrams to be made with the beam perpendicular to the longitudinal direction of the rodlets, a strip, c. 5 mm long and 1 mm broad, was cut out of a chip of wax mounted on cellophane. As a specimen holder a lead plate, 1 mm thick with a V shaped incision, was used. The strip of cellophane with the wax was placed on the edge of the lead plate over the dentition and attached to the lead at both ends with glue (fig. 15). The specimens were fixed in front of the pinhole opening in such a way that the X-ray beam struck a part of the wax layer where the parallel arrangement of the rodlets was the least disturbed.

It is evident that it is of the utmost importance that the rodlets struck by the beam are as parallel to each other as possible, the aim of our investigation being to ascertain the orientation of the crystals in one rodlet. The orientation effect in the diffraction from each rodlet will only express itself in the diffraction of all rodlets together, if these effects of the individual rodlets coincide by their parallel position. The wax layer is photographed as a whole only because the examination of a single rodlet would involve difficulties which have not yet been overcome.

It is rather difficult to avoid disturbances in the parallel position of the rodlets when preparing the specimens. The coherence of the rodlets in the wax layer is relatively small and when one tries to cut a piece of it from the epidermis with a razor, the layer often falls apart into small packets of rodlets. In places where the coherence between the rodlets is better, the layer curls as a rule and forms rolls on the edge of the knife with the epidermal side of the layer outside. When the epidermis with some underlying cell layers is included in the section, the rodlets stay in their original position in the beginning, but the epidermal layer dries up quickly and curls, thus disturbing the position of the rodlets again. These difficulties can be overcome by placing the above-mentioned rolls on a well damped piece of cellophane, on which they tend to flatten out. When this flattening out is carefully enhanced by means of a needle and the water is subsequently allowed to evaporate, the rolls stretch and at the same time adhere to the substratum, which can be previously smeared with a small drop of MAYER's albumine fixative for better adherence. Out of the cellophane with a chip of wax on top of it, a small strip can be cut without disturbing the wax layer too much. This strip is mounted as described above.

In connection with the choice of pinhole diameters the following points have to be considered: For organic specimens such as waxes, the exposure required for obtaining a diagram with sufficient details is rather long and becomes longer as the specimen is smaller. The blackening caused by the scattered radiation from the air thus becomes stronger than in diagrams which require only short exposure times. Moreover, a small object such as the wax layer, which cannot cover the pinhole completely, will not screen the radiation from the glass, as is the case in the powder diagrams mentioned in part A. This scattering from the glass edge will also cause additional homogeneous blackening. The narrower the pinhole is, the better the specimen is able to catch the whole primary beam, and the more favourable is the ratio of the intensities of the radiation diffracted by the object and the scattered radiation from the air. The intensity of the scattered radiation from the glass edge also decreases with respect to that of the primary beam when a narrow capillary is chosen, provided its length remains the same. An additional advantage of a narrow pinhole is that a smaller part of the wax layer is irradiated than when a wider capillary is used. This advantage is evident because a small part of the wax layer in which the rodlets are well parallel can be found more easily than a larger part. For these reasons it was desirable to use the narrowest of the available capillaries, i.e. those of 0.25 mm. As the required exposure is very long, however, the 0.5 mm-capillaries were used for provisional photographs. In this case, however, the long-chain reflections vanish completely in the homogeneous central blackening.

We made 17 diagrams of 4 different specimens with the incident beam perpendicular to the longitudinal axis of the rodlets, 18 diagrams of 12 different specimens in which the beam was parallel to this axis and two from 2 different specimens in which the irradiation was at 45° to the axis.

The distance from specimen to film varied between 20 and 40 mm. The exposure times when using the Philips Metalix tube at 25 kv and 40 ma with CuK_α radiation and 10μ Ni filter, were 15—35 hrs when 0.25 mm-capillaries, and 3—5 hrs when 0.5 mm-capillaries were used. The film was Agfa high contrast X-ray film, coated on both sides. The aggregate hours of exposure during this investigation amounted to c. 370 hrs (per window) on the Philips Metalix tube.

2. *Discussion of the Diagrams Obtained*

All diagrams taken with the incident beam parallel to the longiudinal axes of the rodlets exhibit hmogeneously blackened rings ike the normal powder diagram of the wax. This proves that the crystallites in the wax layer as a whole, do not show any preferential orientation when viewed from the above-mentioned direction of the X-ray beam. It does not exclude a particular arrangement of the crystallites in the individual rodlets when viewed in this direction, however. The latter point will be considered more closely in chapter III.

The diagrams taken with the incident radiation perpendicular to the long axes of the rodlets, on the contrary, show rings with regions of enhanced intensity, which are symmetrically located with respect to the meridian and the equator of the diagram. The diagram exhibiting these maxima most clearly is reproduced in fig. 16. Fig. 17 shows the pinhole and specimen to scale in their mutual position.

The maxima in the photograph shown (see also the diagrammatical representation in fig. 18 pl. XXV) indicate that, when viewed in the direction of the incident beam, the wax crystallites are not arranged at random, but that they are orientated with one of their axes in a constant direction. From the symmetry of the diagram it must be concluded that this direction of orientation is perpendicular to the direction of the incident radiation. In addition, the fact that the first mentioned diagrams do not show such maxima at all, proves that this direction of orientation is parallel to the direction of the incident beam in those diagrams, i.e. parallel to the longitudinal axis of the rodlets.

The diagrams obtained by irradiation at 45° angles to the longitudinal axes of the rodlets, also show similar maxima; they are, however, not symmetrical with respect to the equator. This disappearance of the symmetry with respect to the equator on oblique irradiation also points to a direction of orientation which is parallel to the longitudinal axes of the rodlets.

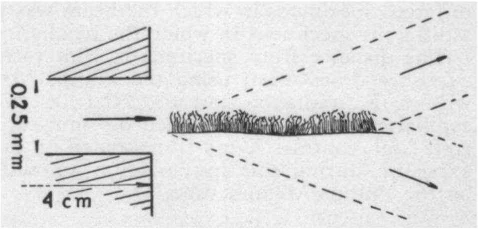

Fig. 17.
Pinhole aperture and specimen used to obtain the photograph shown in fig. 16, presented to scale.

The described arrangement of the crystallites, i.e. orientation with one of their axes in a constant direction, but otherwise arranged at random, is characteristic of a so-called fibre structure. The diagrams obtained with the beam perpendicular to the longitudinal axes of the rodlets may be regarded as so-called fibre diagrams. The direction to which one of the axes of every crystallite is more or less parallel, i.e. the longitudinal axis of the rodlets, is called the fibre direction. For an explanation of the characteristics and the interpretation of fibre diagrams we must refer to the textbooks on X-ray crystallography (see the introduction).

The length of the arcs of enhanced intensity in the rings depends on the degree of departure from perfect orientation of the crystallites. The greater the angular dispersion in the crystallite positions with respect to the fibre direction the longer the arcs become and the more the diagram resembles a powder diagram. The more perfect the orientation the shorter are the arcs to which the maxima contract. For a more detailed definition of this relation we refer to KRATKY (1933). The fact that in the diagram presented in fig. 16 the arcs are very elongated, indicates that in the wax layer there exists a considerable dispersion in the positions of the crystallites with respect to the fibre direction. This does not imply, however, that also in the individual rodlets such a great

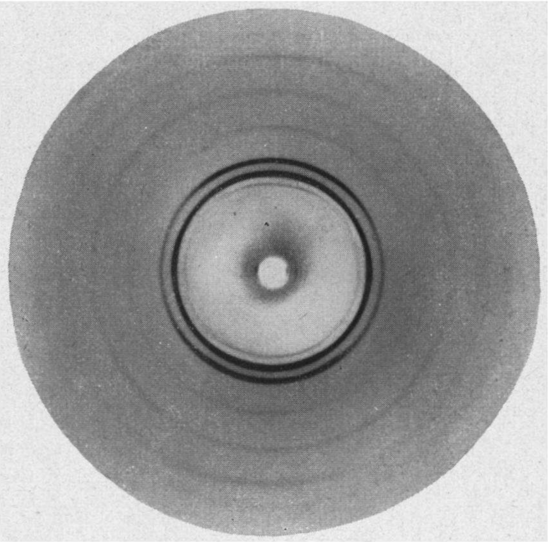

Fig. 16. Photograph of the wax layer obtained with the beam parallel to the cuticle surface.

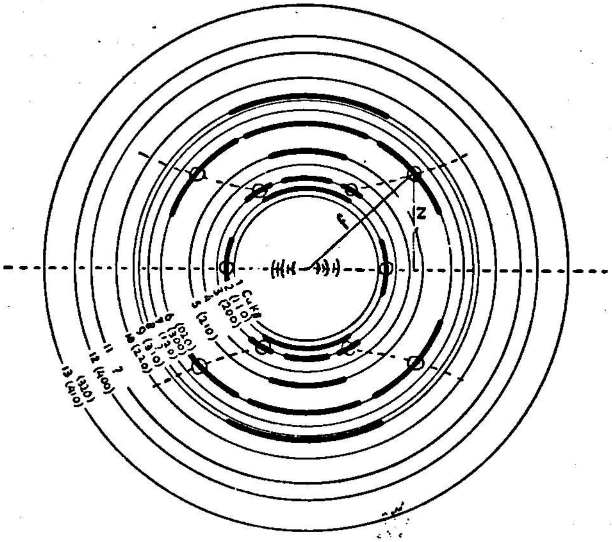

Fig. 18. Schematical representation of the photograph shown in fig. 16, somewhat reduced. Explanation is given in the text, cf. par. 3.

dispersion in the position of the crystallites is present. We shall return to this point in part *b* of this chapter.

In the centre of the diagram, though somewhat blurred by the homogeneous blackening, the l.c. refl. are perceptible. They show a maximum in the equator. This must be interpreted so as to assume the net-planes with long spacing to be orientated parallel to the fibre direction. As has been pointed out in part A, the chain molecules are perpendicular to these planes. When, therefore, the planes are parallel to the fibre direction, the chains must be perpendicular to this direction, i.e. perpendicular to the longitudinal axis of the rodlets. The above conclusion is in good agreement with WEBER's (1942) results for the direction of the long axis of the molecules in the wax rodlets of *Phragmites*, obtained from the study of birefringence.

By indicating the direction of the longitudinal axis of the molecules their position on rotation about this axis remains undetermined, however. Neither is, of course, the position of the crystallites with respect to the fibre direction determined by indicating the direction of the chains. The position of the crystallites is only defined when it is known which crystal axis of the crystallites is orientated in the direction of the fibre axis. If the crystal structure is sufficiently known, in the latter case the position of the molecules is also determined. The study of birefringence does not render further evidence as to these details.

The fibre diagram, however, enables one to determine the identity period of the crystallite axis which is orientated in the fibre direction. As the dimensions of the elementary cell of the crystal structure of the wax is known, the identity periods can be computed from the elementary cell in all axial directions of the crystallite. Subsequently, one can establish which of these identity periods tallies with the one computed from the fibre diagram. The crystal axis thus found is the one which in the crystallites is orientated parallel to the fibre direction. Naturally, in the wax layer this must be an axis which is at right angles to the longitudinal axis of the molecules, the latter being perpendicular to the fibre direction.

Summarising this section it can be stated that from the diagrams obtained certain conclusions can be drawn without any previous computations, viz. that (1) there is a preferred orientation of the crystallites in the wax layer, the direction of the orientation being parallel to the longitudinal axis of the rodlets, (2) the chain molecules lie perpendicular to the longitudinal axis of the rodlets and (3) there is a relatively great angular dispersion in the position of the crystallites with respect to the fibre direction.

In the next section it will be attempted to determine the position of the crystallites more precisely using the method described above.

3. *Determination of the Orientation of the Wax Crystallites in the Wax Cover*

It has been mentioned in the preceding section that the preferred position of the crystallites can be determined when the period in the fibre direction has been computed from the diagram. This period can be found by means of the distances from the intensity maxima in the rings to the equator. For details we refer again to the general textbooks on X-ray crystallography. It will be seen that these distances can be measured more accurately as the arcs of enhanced intensity are shorter. The arcs in our diagrams being strongly elongated it is difficult to measure the distances between the maxima and the equator accurately, and it is therefore not to be expected that the identity period in the direction of the fibre axis can be deduced from them with great precision. As the distance of a maximum to the equator the distance of the middle of the region of enhanced intensity to the equator was used. The middle was estimated visually.

Fig. 18 is a schematical representation of the fibre diagram shown in fig. 16 in which scheme the maxima have been indicated which can be distinguished on the photograph. In the reproduction of the photograph these maxima are less easily recognisable. In addition, in fig. 18, all rings of the powder diagram are indicated; their intensity has not been taken into consideration. In those maxima which will be involved in the computation, viz. those which do not lie along the meridian of the diagram, the middle has been indicated by a circle. The maxima visible along the meridian were not taken into consideration for computation of the fibre period, as they might be connected with the disorientation in the specimen and also might be a result of overlapping of different maxima. Also the numbers and indices of the side-spacing reflections have been indicated in the diagram; the indices only for those reflections which were assumed to belong to the lattice-planes parallel to the direction of the chain molecules, i.e. the lattice-planes (*hk*o), or the zone [001]. One should compare the appendix of this section for the indexing of the reflections.

On the basis of the maxima in the rings 3 and 6 it will be attempted to determine which crystallite axis is orientated in the fibre direction. For the computation of the identity period in this direction only the maxima of reflections 3 and 6 come into consideration, the maximum of reflection 2 lying in the equator. This maximum will be taken into consideration afterwards.

The identity period T can be calculated from the maxima on each ring separately by means of the equation:

$$T = n\lambda \frac{\sqrt{f^2 + r^2}}{z}$$

in which λ = wavelength of the X-rays (for CuK_α is $\lambda = 1.54$ Å), f = radius of the reflection circle, r = distance between specimen and film (40 mm), z = the distance of the middle of the maxima to the equator, n = order number of the layer line [1]) on which the maximum lies. The only layer line that can be distinguished on the photograph is indicated by a broken line in fig. 18.

For the determination of z the middles of the maxima of the two most distinct fibre diagrams obtained were estimated as accurately as possible. Subsequently z was found for each pair of opposite maxima (across the equator) by halving the distance measured between these maxima. This rendered 4 values of z for each of the two rings considered, because in the two diagrams a measurement on the left and on the right of the meridian was carried out. The mean of these 4 values of z for one ring was converted for $r = 40$ mm. By doing so for the maxima in reflection 3 we found: $z_{40} = 15.2$ mm and for those in reflection 6: $z_{40} = 19.6$ mm. The number n was put equal to 1, only one layer-line being perceptible. In this way, from reflection 3 a value of $T = 4.42$ Å and from reflection 6 a value of $T = 3.88$ Å was found. The relatively considerable difference between these values is not remarkable considering the elongation of the maxima from which they were computed.

Now these values of T, having a mean of 4.15 Å, will be compared with the identity periods of the principal axes in the crystal lattice perpendicular to the chain direction. These identity periods amount to: 7.43 Å along the a axis, 4.95 Å along the b axis (see fig. 2, A, I, 2) and 9.04 Å in the direction [110]. They are the shortest periods which can be measured in the lattice. The mean value of T found appears to be the nearest to the halved period in the direction [110]. If in the above-mentioned equation for T the value 2 had been chosen for n, in other words if it had been assumed that a layer line of the second order is present and that the one of the first order is absent, there would have been found for T a length

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[1]) For the concept "layer line" and the derivation of the above equation we refer again to the textbooks on X-ray crystallography.

of c. 8.3 Å. This value would be the closest to the whole period in the direction [110]. Accordingly, assuming the layer line of the first order to be absent, the most probable orientation of the crystallites is with the direction [110] parallel to the fibre axis. Additional evidence for this assumption is found in the fact that the reflection (110) shows a maximum in the equator. If, namely, the crystallites lie with the direction [110] parallel to the fibre axis, the plane (110), i.e. the diagonal plane of the unit cell parallel to the c-axis, will be vertical and must indeed reflect in the equator on rotation about [110].

We may conclude that, in spite of the considerable dispersion in the positions of the crystallites, the diagram yields just enough evidence to assume that the crystallites are preferentially orientated with the direction [110] parallel to the fibre axis.

Appendix.

The indexing is based on a rhombic unit cell with the axes $a = 7.45$ Å and $b = 4.97$ Å (cf. fig. 2 in A, I, 2.). The axis c although irrelevant as regards the indexing of the (hko) reflections, is the same as the long-chain spacing, which in the case of *Saccharum officinarum* wax amounts to 75.4 Å, cf. table I.

In the following table VII a survey of the side spacing reflections is given, in which the d-values (interplanar spacings) for these reflections as computed from the powder photograph of sugar cane wax ($d_{obs.}$) are compared with the theoretical d-values ($d_{theor.}$) calculated from the above mentioned cell dimensions for the planes (hko) mentioned in column 6. For this computation the quadratic equation:

$$\frac{1}{d^2} = \left(\frac{h}{a}\right)^2 + \left(\frac{k}{b}\right)^2 + \left(\frac{l}{c}\right)^2,$$

which is valid for the rhombic system, is used.

4. *The Layer Lines on which the Maxima of the Reflections* (hko) *are to be expected in the Case of Perfect Orientation of the Crystallites with the Direction* [110] *Perpendicular to the Cuticle Surface.*

As mentioned in the preceding section, only two rings in the diagrams obtained show maxima of which, in spite of their elongation, the middle could be indicated with sufficient accuracy to allow an estimation of the fibre period. If the orientation had been more perfect, these maxima would have been less elongated and other maxima might have been expected on the other reflection circles.

TABLE VII.

Table of side-spacing reflections (*hk*o) in the powder photograph of sugar cane wax.

Int.: Intensity. (See also fig. 1 in A, I, 2.)
$2f_{40}$: Reflection diameters for a specimen-film distance of 40 mm.
$d_{obs.}$: Lattice-plane distances calculated from the above diameters. (See BRAGG'S law in A, I, 2.)
$d_{theor.}$: Theoretical d-values calculated from the paraffin unit cell according to MÜLLER (1928), cf. fig. 2 in A, I, 2.
(*hk*o): Indices of net-planes the d-values of which are calculated and are listed in the preceding column.

No.	Int.	$2f_{40}$	$d_{obs.}$	$d_{theor.}$	(*hk*o)	Remarks
1	f	28.0	4.15		(110)	CuK_β
2	vs	31.3	4.15	4.14	(110)	radiation
3	s	35.5	3.71	3.72	(200)	
4	vf	41.0	3.28	3.28		(1, 1, 14)
5	f	46.1	2.97	2.98	(210)	
6	m	57.6	2.50	2.48	(020), (300)	
7	vf	62.1	2.37	2.36	(120)	
8	vf	66.1	2.27	?	?	
9	f	68.5	2.22	2.22	(310)	
10	f	75.6	2.08	2.07	(220)	
11	vf	85.2	1.93	?	?	
12	f	92.0	1.85	1.86	(400)	
13	f	104.0	1.74	1.79	(320), (410)	

Note to reflection 4:
The reflection 4 can be indexed (1, 1, 14) for which indices $d_{theor.}$ = 3.28 Å. As was discussed in A, III, 5, the wax of *S. officinarum* contains much C_{28} prim. alcohol. Since two chains per period occur, the period of the c-axis, accordingly, contains 56 C atoms. The distance c/14 in the chain direction can therefore embrace exactly 4 C atoms. For this reason the reflection 4 is probably associated with the C-C periodicity in the carbon chain.

This would permit a more accurate determination of the fibre period. Starting from the assumption that the crystallite axis with preferred orientation parallel to the fibre direction is known, we are now able to determine theoretically in which layer lines the other rings would show a maximum, given a sufficiently perfect orientation. If we could afterwards succeed in obtaining a diagram satisfying this theoretical prediction, from a specimen in which a more perfect orientation is present, this would confirm the indexing in the preceding section of the crystallite axis which is orientated parallel to the fibre direction. This indexing might be doubted on account of the fact that it is based on such vague and so few maxima.

It will be considered below on which layer lines the maxima of the reflection circles ($hk0$) must lie if the crystallites are orientated with the direction [110] perfectly parallel to the fibre axis. This position of the maxima will be the same as the position of the reflection spots obtained when a single crystallite is rotated about its direction [110] in the X-ray beam, with the beam perpendicular to the axis of rotation. For the more perfect the orientation in the specimen the more nearly will all cyrstallite positions present agree with the positions consecutively adopted by a single crystallite on rotation about the axis under discussion.

A survey of the position of the reflection spots with respect to the layer-lines in such a rotation photograph can be obtained with the aid of the reciprocal lattice, a device developed by EWALD (1914). In the case of a wax, this is a comparatively simple procedure, because a rectangular system of axes is present. For the essentials and merits of the reciprocal lattice and its application to the evaluation of rotating-crystal diagrams, we

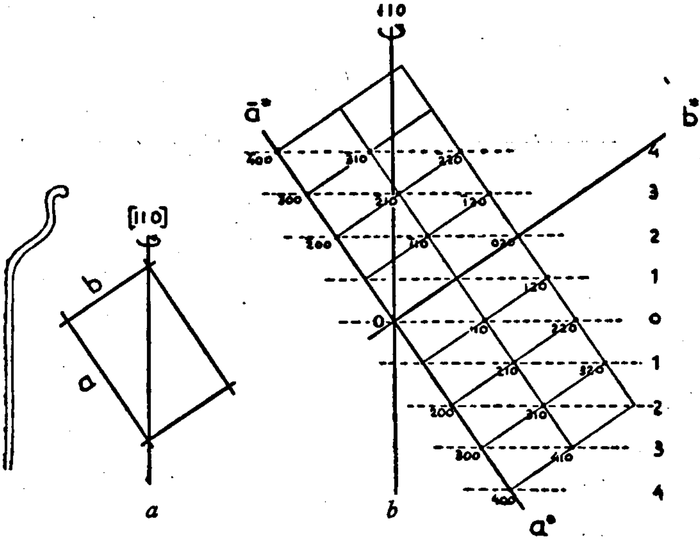


Fig. 19.

a. Supposed position of the axes *a*, *b* and [110] of the crystallites in a wax rod.

b. Reciprocal lattice-plane a^*b^* in which the layer line relationship of the reflections ($hk0$) on rotation of a crystallite about [110] with the beam perpendicular to that direction, is indicated.

must, however, refer to the literature on X-ray crystallography.

In fig. 19*b* the reciprocal lattice-plane *hk*o of the wax crystallites is represented. The directions of the principal axes a^* and b^* of the reciprocal lattice are the same as those of the axes *a* and *b* in the *ab* plane of the unit cell represented in fig. 19*a*, the systems of the axes of the reciprocal lattice and of the crystal lattice being parallel in the rhombic system. The points of the reciprocal lattice-plane are connected by broken lines representing the projection of circles formed by these points on rotation of the reciprocal lattice about the axis indicated in the plane and parallel to [110] of the crystal lattice. The points lying on such a line represent crystal lattice-planes which reflect in the same layer line. Thus the figure enables one to ascertain at a glance on which layer lines the maxima of the reflections (*hk*o) will be found on rotation of a wax crystallite about [110]. The lines have been numbered in accordance with the order number of the corresponding layer line.

No.	(*hk*o) ($\bar{h}$*k*o)	*n*
2	110	0
	$\bar{1}10$	2
3	200	2
	$\bar{2}00$	2
5	210	1
	$\bar{2}10$	3
6	020	2
	$0\bar{2}0$	2
	300	3
	$\bar{3}00$	3
7	120	1
	$\bar{1}20$	3
9	310	2
	$\bar{3}10$	4
10	220	0
	$\bar{2}20$	4
12	400	4
	$\bar{4}00$	4
13	320	1
	410	3

TABLE VIII.

Survey of the side spacing reflections indexed in table VII, and the order numbers *n* of the layer lines in which they can be expected to show a maximum on account of fig. 19, given perfect orientation of the crystallites in the wax layer.

In table VIII the side-spacing reflections in the powder diagram of the wax which can be indexed (*hk*o) are listed once more, the layer line(s) on which a reflection must be expected to show maxima according to fig. 19*b*, being mentioned after each reflection.

In chapt. II^B, sect. 2, a diagram will be discussed which in fact exhibits the maxima to be expected.

B. INVESTIGATION BY MEANS OF A NEWLY DEVELOPED MICRO-METHOD

1. *The Value of a Micro-Method for X-ray Diffraction Studies*

The considerable length of the arcs of enhanced intensity in the fibre diagram discussed above might be due to various circumstances: firstly, a considerable dispersion of the position of the crystallites with respect to the fibre direction in the straight part of every individual wax rodlet, secondly, insufficient mutual parallelism among the rodlets (in spite of the fact that the least disturbed part of the isolated chips of wax was chosen) and, finally, the disorientation in that part of the wax layer in which the rodlets are curled.

In order to obtain an idea about the degree of preferential orientation in every individual rodlet it would be necessary to take a diagram from a single rodlet, or at least from a packet of only a few rodlets in which the rodlets are exactly parallel and in which the curled parts of the longer rodlets do not contribute to the diffraction.

It turned out feasible to isolate small packets of rodlets (c. 50μ thick when measured perpendicular to the long axes of the rodlets) under the dissecting microscope by means of a fine needle. In these packets the rodlets were almost completely parallel, except the curled ends of the longer rodlets.

It goes without saying that the standard X-ray technique available, which involves the use of pinholes at least 5 times as wide as the dimensions of such a packet of rodlets, is not suited for the purpose of obtaining good diagrams of these objects. The specimen being very small, only a low intensity of diffracted radiation is produced and the inherent long exposure would cause a total blurring of the reflections by the blackening due to scattering at the glass edge and in the air. One might try to take certain precautions to eliminate this scattered radiation, such as the use of an additional diaphragm between specimen and capillary (in order to screen the scattered radiation from the glass edge) and more forward position of the lead cup (see part A, I, 3) or the use of a hydrogen atmosphere (in order to avoid scattering in the air). However, these measures do not exclude the difficulty that the bent rodlets contribute to the diffraction, and they involve additional technical difficulties themselves. When the lead cup is moved forward one is compelled to use a comparatively long distance between specimen and film, whereas it is desirable to reduce this distance as much as possible, as otherwise the exposure time would become too long. Making the photographs in a hydrogen atmosphere necessitates a gas-tight case, which would give rise to many difficulties with the type of camera used.

An effective solution of these difficulties can only be reached if a very narrow pinhole is used. It must be so narrow that it can be completely covered by that part of the packet in which the rodlets are not bent, so that it enables radiation to be diffracted in this part of the packet only. The X-ray beam passing through such a narrow pinhole would also cause considerably less scattered radiation in the air, so that the ratio between the intensities of this radiation and that from the specimen would be much more favourable. For that reason it would not be necessary to enlarge the distance between the lead cup and the film and, again on account of this, it would not be objectionable to reduce the distance between specimen and film.

It will be understood that the above mentioned problems will continually arise in X-ray investigations of objects of microscopic size (see e.g. also PRESTON (1934)) and that they are, accordingly, of a general nature. This applies especially to those objects of which one desires to take a diagram of a part of the object and when the substance surrounding this part must not contribute to the diffraction. Imagine, for instance, the cases that one wishes to obtain X-ray evidence about the structure of a particular part of a cell wall in a microtome section, about a part of a fibre section or of a starch grain. Many vegetable and animal microscopic units could be mentioned here. As regards starch it is called to mind that the crystal structure of this important product is very insufficiently known and that this, among other things, is due to the fact that a micro-method to obtain a fibre diagram of a fragment of a starch grain was not available. The diffraction of the grain as a whole, on account of the radial arrangement of the crystallites, yields a powder diagram from which the necessary conclusions cannot be drawn. A micro-method would also have advantages in obtaining powder diagrams of substances of which only a very small amount is available.

KRATKY (1931) described a micro-method for the study of X-ray diffraction of microscopic single crystals, his so-called "Mikrokonvergenzverfahren", in which a slit system is applied enabling a fan-shaped primary beam to be transmitted, with a convergency of c. 30°. The slit consists of a flat trapezial space between the flat sides of the two halves of a massive gold cylinder, and it has at its narrow end a slit width of 10μ and a length of c. 80μ.

This method, though suited to KRATKY's aims, is less suitable for our purposes because the fan-shaped beam involves equator blackening, which in many cases will prevent the observation of equator reflections. Furthermore, the slit aperture is badly adapted to the shape of many objects. This slit system is also costly and difficult to be made. As a rule a pinhole aperture is to be preferred. Also the design of KRATKY's camera has certain drawbacks for our purposes. Narrow pinholes made of WOOD's metal, cast around a hair, were used by ASTBURY and PRESTON in order to obtain diagrams of hairs and other biological objects, which technique has not been described in detail.

It seemed to us that the possibilities of micro-methods had not been sufficiently investigated. On account of its importance we intended to develop a generally applicable micro-method in order to examine the wax rodlets and, possibly, other objects of biological interest, if we were successful. As to the results of this work we only mention here that a micro-method for X-ray diffraction has been developed in which extremely thin X-ray beams, only a few microns in diameter, can be used. The minimum thickness of the X-ray beam which still yields diagrams with sufficient details is not a definite one and is dependent on the nature of the specimen and on the demands made upon the diagram to be obtained. It can be roughly estimated at 10μ for organic objects. At a distance of c. 1—2 mm between object and film and using a 2.5 kw X-ray tube, minimum exposure times of approximately 50 hrs must be expected when using a 10μ pinhole.

In an appendix we have given an idea of the design of the micro-camera developed, while in the next section some details are mentioned about the pinholes and the mounting of the specimens. For preliminary communications about the method and the results obtained by its aid in the investigation of a part of a starch grain we refer to KREGER (1945, 1946, 1947). Detailed publications concerning these subjects will follow elsewhere.

It may finally be mentioned that after the war it appeared that also in U.S.A. during this period attention had been paid to micro-methods for X-ray diffraction. FANKUCHEN and MARK (1943) used narrow glass capillaries in X-ray investigations of nylon threads. A description of the technique has not been given. "The American Philips Corporation" has brought out a micro-camera with pinholes of 50 and 100μ diam. and allowing a minimum distance of 10 mm between specimen and film to be used (CHESLEY 1947).

2. *The Diagram of the Wax Rods, Obtained by the Micro-Method*

A fibre photograph of the packet of wax rods, as obtained by means of the new micro-method and greatly enlarged, is shown in fig. 20*a* (Plate XXVI). The photograph at natural size is shown in fig. 20*b*. Fig. 21 represents a sketch of pinhole, secondary pinhole and object in which the proportions are to scale.

The photograph was obtained by means of a conic capillary (1) of heavy lead-glass (s.w. c. 5.2) 1 cm long and with terminal diameters of 320μ and 56μ. The secondary pinhole (2), serving to reduce the divergency of the scattered radiation from the glass rim to a sufficiently small angle, is of lead and has a length of 0.4 mm and a diameter of 105μ. The distance from specimen to film was 1.9 mm. The specimen was mounted in the opening of a micro-specimen holder (3), hanging in this opening between collodion films (5). This specimen holder consists of a thin copper sheet (10μ thick, c. 1 mm broad and 6 mm long) in which an opening of 0.5 mm diameter is punched out. The holder was fixed with small wads of mounting wax on the lead of the secondary pinhole.

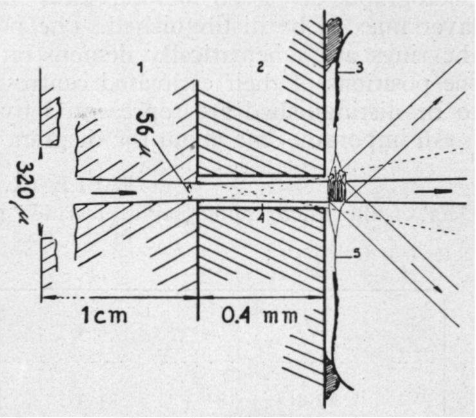


Fig. 21. Method of mounting the specimen when obtaining the photograph shown in fig. 20. Drawing to scale. Explanation is given in the text.

The exposure time when using the rotating target tube at 25 kv and 100 ma with CuK_α radiation (10μ Ni filter) amounted to 2.5 hrs on Agfa high-contrast X-ray film, coated on one side.

The long-chain reflections are not shown on the photograph. They are lost in the central blackening due to scattered radiation from the glass rim of the pinhole. By using a longer secondary pinhole it would be possible to reduce the divergency of this radiation and to have the long-chain reflections visible. They would lie in a small region with a diameter of c. 1 mm around the central bore in the film, but would be badly resolved owing to the coarse grain of the film and the comparatively high divergency of the beam passing through the capillary. In order to obtain these reflections clearly visible and well resolved it would be necessary to use a more nearly cylindrical and narrower pinhole and to enlarge the distance specimen-film considerably. This would involve a much longer exposure time as well as several other drawbacks. As these reflections are already sufficiently visible on the photograph taken by means of the standard apparatus we made no attempt to take a microphotograph on which they are shown.

All side spacing reflections exhibit, in contrast to those on the photograph discussed before, clear maxima, which enable four layer lines to be distinguished. The positions of these maxima on the rings are schematically demonstrated in fig. 22 by indicating the positions of their estimated centres. The layer lines they allow to be distinguished are represented by broken lines.

All important data about the diagram are summed up in table IX.

TABLE IX.

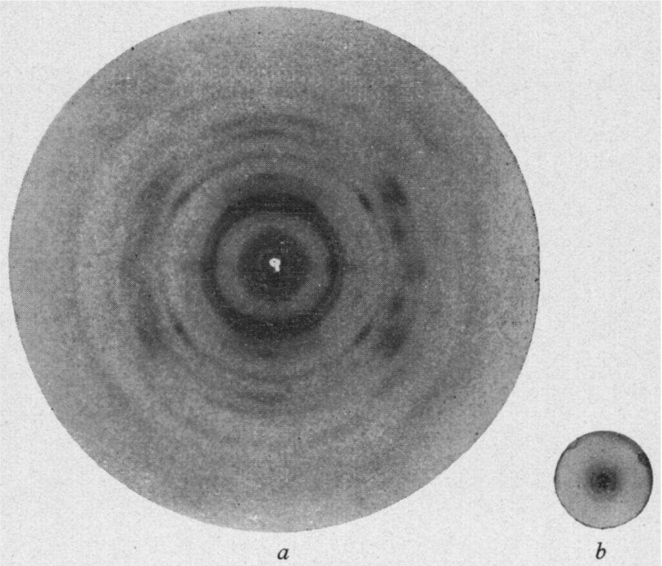
Data of the micro photograph presented in fig. 20. Explanation is given in the text.

1 No.	2 $2f_t$	3 $2f_{40\,\text{pred.}}$	4 $2f_{40\,\text{obs.}}$	5 n	6 $2z_{40}$	7 T (in Å)
2	17.4	31.3	31.3	0		
				2	28.6	9.20
3	20.2	35.5	35.4	2	30.2	8.92
4	23.0	41.0	40.8	0 —		
5	26.1	46.1	46.3	1	15.4	9.21
				3		
6	32.6	57.6	57.8	2	33.9	8.96
				3		
7	36.2	62.1	62.5	1	17.4	8.95
8	37.5	66.1	66.5	1 —	17.7	9.02
9	38.8	68.5	68.8	2	36.4	8.94
				4		
10	42.4	75.6	75.2	2 [1]	38.2	8.86
				4	73.2	9.24
11	48.1	85.2	85.5	1 —	19.6	9.20
				3 —	59.7	9.06
12	51.7	92.0	91.8	4	83.5	9.00
13	59.2	104.0	105.0	1	22.2	9.15
				3	66.6	9.15
					T (av.)$=9.06 \pm 0.11$	

---

[1] This maximum is not predicted by consideration of the reciprocal lattice-plane represented in fig. 19. It may probably be due to a reflection that cannot be indexed (*hk*0) and is superimposed on (220).

— Reflections which cannot be indexed (*hk*0), cf. table VII.



a *b*

Fig. 20. *a.* Enlarged micro-diagram of a packet of wax rods, obtained with the beam at right angles to the long axis of the rods.
b. The same diagram at natural size.

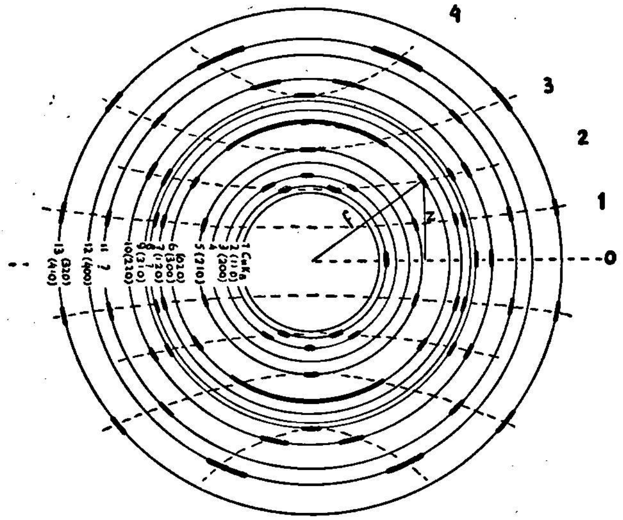


Fig. 22. Schematical representation of the photograph shown in fig. 20a, somewhat enlarged. Explanation in the text.

In the successive columns of this table are listed:

1. The reflection numbers.
2. The diameters of reflection circles measured on the micro-photograph after enlargement to an arbitrary convenient scale ($2f_?$).
3. The diameters of the same reflections as measured on a common powder photograph made at 40 mm distance from the specimen ($2f_{40\,powd.}$), cf. table VII, col. 4.
4. The diameters of col. 2 converted to the same scale as those mentioned in col. 3, by multiplying them by a factor obtained as the mean of the values $2f_{40\,powd.}/2f_?$ for the two strongest reflections. This indirect way of calculating $2f_{40}$ for the reflections of the micro-photograph ($2f_{40\,obs.}$) is necessary because the small distance of specimen to film at which this photograph was made, cannot be measured with sufficient accuracy.
   From comparison of the columns 4 and 3 it may be seen that the micro-diagram permits a comparatively accurate measurement of the diameters of the reflection rings.
5. The layer-line number(s) n of the layer line(s) at which every ring shows a maximum.
6. The z-values of the positions of the spots for a specimen-film distance of 40 mm, obtained by multiplying the z-values measured (compare II*a*, 3 for this measurement) on the enlarged print mentioned under 2 by the same factor as mentioned under 4.
7. The identity period T in the direction of the fibre-axis calculated with reference to each double pair of spots from the same formula as mentioned in II*a*, 3, in which $r = 40$, $\lambda = 1.54$ and f, n and z are given in the preceding columns 4, 5 and 6.

The average identity period T is found to be 9.06 Å ± 0.11, which practically corresponds to the theoretical identity period in the direction [110], being 9.04 Å (cf. chapt. II*a*, sect. 3).

It is seen from this result that the micro-diagram enables the fibre period to be determined with an accuracy of c. 1%, whereas the photograph discussed in the first part of this chapter only permits a rough estimate correct to c. 20%. The identity period found is in good agreement with the period to be expected on account of the suggestion put forward in chapt. II^A, 3 according to which the crystallites would be orientated with the direction [110] in the fibre direction. It also appears that the positions of the spots on the micro-diagram with regard to the layer-lines correspond to the positions predicted by means of the reciprocal lattice and mentioned in tab. VIII. Compare the columns for n in the tables VIII and IX.

To sum up: It is found that the assumptions for which the photographs by standard apparatus yield only just enough data, are confirmed by ample evidence from the micro-diagram. Finally, it is to be noted that the small length of the arcs of enhanced intensity in the rings of the micro-diagram indicates a much better orientation of the crystallites in the individual wax rods than could be suspected from the photographs obtained first. A small amount of desorientated crystallites, however, must also be present, since the more intense rings are visible along their entire circumference.

We may end this chapter with the following brief statement: The X-ray investigation has enabled the position of the crystallites to be determined with respect to the longitudinal axis of the wax rods; they were found to possess a comparatively strong preferential orientation with the direction [110] parallel to this axis of the rods, that is with [001], or the longitudinal direction of the molecules, perpendicular to the longitudinal direction of the rods, cf. fig. 23.

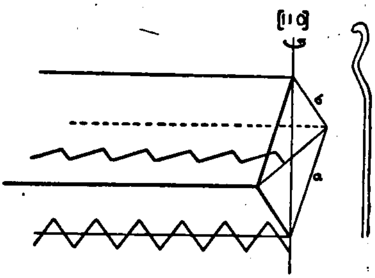


Fig. 23. Orientation of the crystallites in a wax rod according to X-ray evidence.

It is noticed that the above statement is not conclusive as to any crystallite position in the rods obtainable on rotation about the axis [110]; that is, the molecules may be orientated with their long axis radially as well as tangentially in the wax rods.

The point in the next chapter is whether any decision in the latter respect may be achieved.

CHAPTER III

EXAMINATION OF THE WAX RODS UNDER THE POLARISING AND THE ELECTRON MICROSCOPE

1. *Investigation of the Arrangement of the Wax Crystallites in Directions Perpendicular to the Longitudinal Axis of the Wax Rods, Based on Observations in Polarised Light*

We may imagine four essentially different conditions of arrangement of the wax crystallites with respect to directions perpendicular to the longitudinal direction of the wax rods. In fig. 24A, *a*, *b*, *c* and *d*, these arrangements are represented diagrammatically. In these diagrams the axes of the crystallites parallel to the longitudinal direction of the molecules are represented in projection on the cross-section of the wax rods. The rods are assumed to be cylindrical. a_1 and a_2 represent two possibilities of mutual arrangement of the rods when their crystallites are arranged according to *a*. It will now be ascertained whether it is possible to decide upon one of these arrangements as being the one occurring in the rods:

In the case of condition *a*, in which the crystallites in the rods are placed parallel to each other with respect to every one of their axes and the rods are also placed parallel to each other with all directions, the X-ray diagram obtained on irradiation of the wax layer in the longitudinal direction of the rods, should not show homogeneously blackened rings. Since, however, such rings do appear (see chapter II^A, 2) this possibility must be excluded.

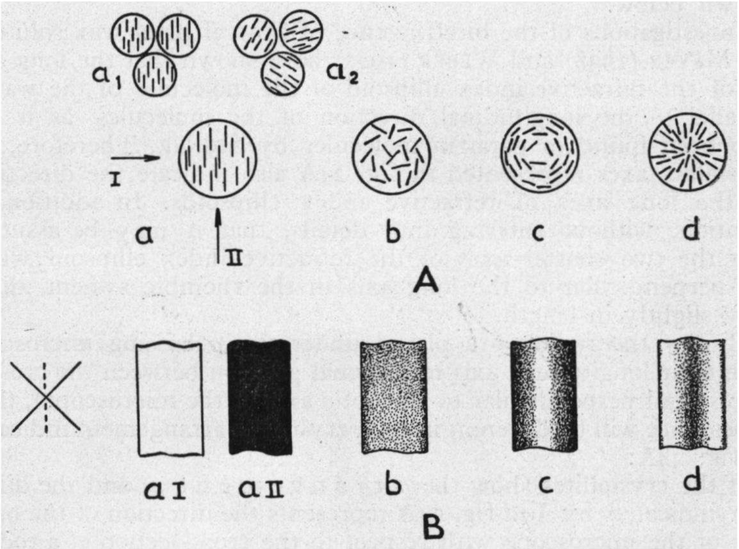


Fig. 24A. Theoretical possibilities of arrangement of the crystallites in the wax rods in directions perpendicular to the longitudinal direction of the rods, represented by indicating the direction of the crystallite axes with long period on the cross-section of the rod. Fig. 24B. The behaviour of a rod placed in diagonal position between crossed nicols for the various arrangements represented in fig. 24A.

The possibility that the rods would only show mutual parallelism at their bases and for the rest would possess a torsional a-structure, cannot be excluded referring to the above mentioned diagram, nor can the arrangements *b*, *c* and *d*. It will easily be seen that the crystallite axis under consideration in any one of these instances may have all possible directions perpendicular to the X-ray beam, and this will result in homogeneous rings. Provided that no torsion occurs in the rods the condition a_2 might be recognised by means of a micro X-ray diagram of a packet of only a few rods, because it would give "coarse-grained poly-crystalline" reflection rings. In order to identify the other cases it would be necessary to take photographs of parts of rods obtained by dissecting the rods in the longitudinal direction and irradiating those parts individually along their longitudinal direction. This, however, is impossible because the rods are too small to be treated in this way. It is the polarising microscope that might enable further evidence to be obtained in a comparatively simple way with regard to the various possibilities suggested. The way in which this might be accomplished theoretically will be shown below.

Investigations of the birefringence of flow of plant wax solutions by MEYER (1938) and WEBER (1942) have shown that the long axis n_γ of the refractive index ellipsoid of the molecules of the wax is parallel to the longitudinal direction of the molecules, as it had also been found in paraffin molecules by SIGNER. Therefore, the crystallite axes represented in fig. 24A also indicate the directions of the long axes of refractive index ellipsoids. In addition we mention, without entering into details, that it may be assumed that the two shorter axes of the refractive index ellipsoid, which are perpendicular to the long axis in the rhombic system, differ only slightly in length.

If now the rodlets are placed under the polarising microscope with their longitudinal axis in diagonal position between the crossed nicols (and perpendicular to the optic axis of the microscope), their appearance will be different in each crystallite arrangement indicated in fig. 24A:

If the crystallites show the arrangement *a*, and the direction indicated by I in fig. 24A represents the direction of the optic axis of the microscope with respect to the cross-section of a rodlet, the effective refractive index ellips of all crystallites contains the long axis of the index ellipsoid and the rodlet will appear bright, cf. fig. 24B, *a* I. If the rodlet has such a position that the optic axis of the microscope has the direction indicated by II with respect to the cross-section, the effective index ellips contains the short axes of the index ellipsoid and the rodlet will appear dark, or almost so (fig. 24B, *a* II). If the rodlet were rotated about its longitudinal axis, it would appear alternately bright and dark, and if it showed torsion it would contain brighter and darker areas. A great number of rodlets in the condition *a*, put on a microscope slide at random and, therefore, having intermediate positions between *a* I and *a* II with respect to the microscope axis, would show, provided they are not twisted, considerable differences in brightness when they are consecutively placed in diagonal position.

In the arrangement *b* of the crystallites all possible positions of the long axis of the index ellipsoid between those of *a* I and *a* II occur in the rodlet simultáneously, and these positions are distributed at random in the cross-sectional area of the rodlet. The rodlets, when placed in diagonal position between crossed nicols would therefore always show exactly the same brightness. They would, however, appear less bright than in the condition *a* I, as the long axis of the effective index ellips in most crystallites is not coincident with the long axis of the index ellipsoid, cf. fig. 24B, *b*.

In the arrangement *c* of the crystallites, an axial plane of the rodlet, parallel to the optic axis of the microscope, intersects crystallites the long axes of which are perpendicular to the optic axis and which, consequently, show a maximum brightness. On both sides of this plane, the angles at which the above-mentioned long crystal-axes (long axes of index ellipsoids) meet the direction of the microscope axis, decline towards the sides of the rodlet. This decline, and in addition the decreasing thickness of the rods towards their sides, causes a quickly decreasing brightness towards the sides. The rodlet would, therefore, show a bright stripe in the middle and decrease rapidly in brightness towards the sides, cf. fig. 24B, *c*.

In the arrangement *d*, the axial plane of the rodlet would only intersect crystallites which are placed with the long axes of their index ellipsoids parallel to the microscope axis and therefore, would appear dark. On both sides of this plane the angles between the long axes of the index ellipsoids and the axis of the microscope increase, and the rodlet would be increasingly brighter towards the sides. The rodlet would, accordingly, show a dark stripe in the middle and appear brighter towards the sides, cf. fig. 24B, *d*.

When examining the rodlets (mounted dry or in glycerine) in the diagonal position between crossed nicols under the microscope by means of an oil immersion objective at c. 900 × magnification, none of the above-mentioned conditions could be identified. Longitudinal, parallel, bright and dark, irregularly distributed and sometimes tapering lines are always present. The brightest stripes were mostly observed at the sides, the darker ones near the middle of the rods. Sometimes only one side of the rodlet shows a bright stripe, sometimes the brightest stripe is in the middle. Occasionally, one of the stripes is discontinued, a bright one changing into a darker one, or vice versa. De Bary reported already that under the polarising microscope the rodlets, especially the broader ones, show a longitudinal striation, and he wondered whether this could be associated with "Strukturverhältnisse" or with "Vorsprünge der Oberfläche".

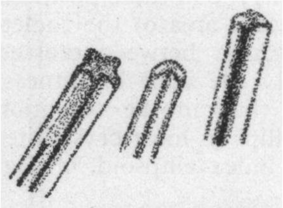


Fig. 25. Images of broken ends of wax rods observed at a magnification of c. 900 × under the polarising microscope.

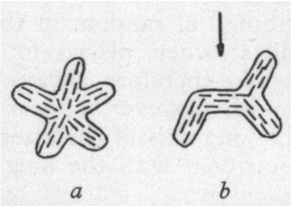


Fig. 26. Cross-sections of rods in which the direction of the crystallite axis with long period is indicated as supposed on account of the observations under the polarising microscope.

When the broken end of a rod is examined it becomes evident that the striation must be associated with the rodlets not being cylindrical as originally assumed, but coarsely ribbed. Instead of an elliptical cross section a more or less stellate image is observed. A riblet is not always continuous along the full length of the rodlet and this explains the above-mentioned discontinuation of a bright or dark stripe. Some images as observed are represented in fig. 25.

It appears that the riblets protruding laterally (when placed in the diagonal position between crossed nicols) appear bright as a rule. A riblet protruding in the direction of observation usually corresponds to a dark line. Although it is extremely difficult to distinguish diffraction images and birefringence phenomena in these observations, which were done near the limit of the resolving power of the microscope, the above-mentioned rule could be established.

Since the riblets appear bright if they protrude laterally and are, consequently, placed with their axial plane horizontally, i.e. perpendicular to the optic axis of the microscope, and appear dark if their axial plane is vertical and parallel to the optic axis, it appears that the position of the long axis of the refractive index ellipsoid corresponds to the arrangement *d* of the crystallites. In a cross-section of a ribbed rodlet this position is indicated in fig. 26*a*.

It will be seen that it is now also possible to regard the individual riblets as more or less flattened rodlets showing the arrangement *a* of fig. 24A, which have fused to form thicker stellate rodlets, the latter appearing as the wax rodlets proper. On account of this conception structures as represented in fig. 26*b* may also be expected. In the diagonal position between crossed nicols these would show a bright line in the middle when viewed in the direction indicated in the figure. As has been mentioned before, a bright stripe is occasionally observed in the middle of a rodlet. A broken end which would be suggestive of a similar shape was never found, however.

The rodlets can easily be split by gentle pression with a fine needle. This is in agreement with the above conception. The isolated riblets, or rather the flat partition rodlets constituting the wax rodlets, appear bright under the polarising microscope in diagonal position as a rule. Assuming that they usually adhere with their flat faces to the glass owing to the relatively great coherence between glass and wax, the brightness in this position is in accordance with the above indicated direction of the long axis of the refractive index ellipsoid, viz. parallel to the flat sides of the riblets.

To sum up: It can be stated that the optical observations suggest the wax rods to be aggregates of more or less flattened filaments of wax in which the crystallites are orientated more or less parallel in such a way that the long axis of the refractive index ellipsoid — and consequently the longitudinal axis of the chain molecules, or the direction [001] of the crystallites — is parallel to the flat side. The elementary ribbons are probably united lengthwise so as to cause a more or less radial arrangement of the chain molecules in the wax rodlet.

We recall here that it had been concluded from the X-ray data that the crystallites lie with the direction [110] parallel to the longitudinal axis of the rodlets. Combining this evidence with the abovementioned conclusions the crystallites are probably parallel with reference to all their axes in the elementary ribbons.

WEBER (1942) suggested for the wax rodlets of *Phragmites* a perpendicular position of the chain molecules with respect to the longitudinal axis of the rodlets and a radial arrangement of the longitudinal axes of the molecules. These conclusions were based upon the fact that the rodlets, when placed in diagonal position between crossed nicols and after insertion of a gypsum plate red I with n'_γ parallel to the long axis of the rodlets, show the yellow subtraction colour.

n_γ of the wax molecules being parallel to their longitudinal direction, as shown by the birefringence of flow, the first of WEBER's conclusions is sufficiently accounted for. That, in addition, the chains should have a radial direction in the rodlet, is a conclusion which, in our opinion, cannot be based upon the above-mentioned observation only. Further evidence for this statement is, however, not given by WEBER. The mere observation of the yellow subtraction colour is not conclusive as to any one of the possible orientations represented in our fig. 24A. Therefore, we object to the scheme presented in WEBER's fig. 36*b* in which the molecules are pictured orientated radially with their long axes, and of which WEBER says: „Der schematische Aufbau wie ihn die Fig. 36*b* und *c* zeigen, beruht deshalb zum groszen Teil auf einer Hypothese; nur die Richtung die die Moleküle einnehmen, kann mit Sicherheit bestimmt werden". It is not their direction, but only the plane to which they are parallel that is definitely established by WEBER's observation and, in our opinion, also the direction of the molecules belongs to the hypothetical features represented in that figure. One might, however, suppose a radial arrangement on account of the fact that, according to WEBER, the waxes separate as optically positive sphaerites from their solutions in CCl_4.

As regards the other hypothetical features represented in the figure of WEBER, i.e. the packing of the chains and the way in which chains of different nature are distributed, some further objections seem to be justified. In the first place, as to the packing, we would observe that the crystallinity of the wax has not been given expression in the figure: On the cross-section of the rods, which WEBER has assumed to be circular, individual molecules in radial arrangement are drawn instead of radially arranged groups of parallel molecules, in which latter way the crystallinity could easily have been indicated. Also elsewhere WEBER is unclear about his views regarding the crystallinity of other types of wax covers: On p. 165 we read: „Die Körnchenüberzüge sind in unversehrtem Zustande isotrop, über ihre submikroskopische Struktur lässt sich deshalb nichts aussagen. Es muss ein regellose Molekülgruppierung angenommen werden". A little further we read: „Es darf jedoch nicht angenommen werden, dass die Wachse der Körnchenüberzüge keinen kristallinen Charakter besitzen". Irregular arrangement of molecules and crystallinity are irreconcilable in our opinion.

In the second place, as to the distribution of different types of chains, we may now assume that *Phragmites* wax (cf. part A, III, 5) chiefly consists of primary alcohol as is the case with *Saccharum* wax. Therefore, in a hypothetical diagram representing the distribution of the chains, many more alcohol molecules should be drawn than other typical wax molecules.

2. *The Dimensions of the Crystallites in the Wax Rods*

It will be attempted below to obtain an idea of the dimensions of the crystallites in the rods in order to form a better picture of the submicroscopic structure of the observed ribs or suggested elementary flattened filaments.

As an introductory remark it is called to mind that a crystallite is to be understood as a particle in which the molecules are arranged to form an undisturbed lattice region. The ability of such a region to produce sharp X-ray diffraction phenomena is dependent on its size, that is, on the number of identity periods its axial directions contain. A given minimum number of parallel lattice-planes is required in order to produce a sharp reflection. If the number of parallel planes falls below a certain value this manifests itself as broadening of the reflections, the more so as the particles are smaller. For details we refer to the literature on the subject.

The fact that our preparations give sharp reflections, enables one to make a rough estimate of the minimum number of identity periods the wax crystallites must contain in different directions and, since the dimensions of the unit cell are known, of their minimum size.

Let us first consider the dimension in the direction of the carbon chains. According to TRILLAT (1930) p. 160, a film built-up from monomolecular fat layers needs 100—200 monolayers to produce with the X-ray beam incident at a small angle, a set of sharp long chain reflections. According to CLARK and LEPPLA (1936) a built-up film of 25 layers of calcium stearate produces a set of long-chain reflections which show no broadening, while the first interferences become already visible when the film is only 3 layers thick. It may be taken as well-known that in these films built up from monomolecular layers on a glass or metal background, the carbon chains are at right or oblique angles to the surface of the background. Therefore, the number of identity periods in the chain direction is the same as the number of layers, or the halved number if double-layers are formed. In fats and calcium stearate double-layers will be present. Hence, the above-mentioned data lead to the conclusion that minimum 12—50 periods are required to produce perfect sharpness of the long-chain reflections in substances similar to those of which waxes consist.

As to the calcium stearate, however, we suppose the extent of the reflecting planes in the above-mentioned experiments to be a factor acting strongly in favour of the appearance of sharp reflections. The increased charge of Ca ions may also improve parallelism in the film and, hence, sharpness of its reflections. The conditions prevailing in the wax rods are much less favourable, and we may expect that in the wax crystallites considerably more molecules placed in alignment in the chain direction are required in order to produce sharp long-chain reflections.

Though in the experiments of TRILLAT the reflecting surfaces were also of a much wider extent than they can be in the wax rods, a minimum number of 50 periods in the chain direction seems to be a better assumption. Considering, in addition, that in patterns for colloidal gold and silver, broadening of the reflections sets in at particles sizes of c. 200 Å (cf. CLARK (1940) fig. 225) and that the edges of the unit cell in these metals are c. 4 Å, so that in these cases c. 50 periods as a minimum appear to be required for producing sharp reflections, we assume as a rough estimate that in the wax crystallites at least c. 50 periods are present in the direction of the carbon chain. As the side-spacing reflections of the wax do not show a marked broadening either, the above minimum number of periods is also assumed for the axes *a* and *b*.

As the dimensions of the unit cell of the chief component of wax of *Saccharum officarum* (C_{28} prim. alcohol) amount to 75.2 Å along the *c* axis, 7.45 Å along the *a* axis and 4.93 Å along the *b* axis, we may estimate the minimum dimensions for the crystallites, on account of the above-mentioned assumption, at c. 3750 by 375 by 250 Å. There is no reason, however, to suppose that the crystallites will actually possess these dimensions and that they would not be bigger or have developed in some direction. Therefore we will now ascertain their maximum dimensions.

Along the *c* axis, which, according to the evidence from observation of the birefringence, must be orientated more or less radially in the ribs of the wax rods, the crystallites cannot be larger than c. 2μ, i.e. 20,000 Å. This will be seen when it is remembered that the rods have a maximum thickness of c. 4μ and that a rib regarded as an individual unit containing parallel crystallite axes, encounters other ribs in the centre of the rod. In those other ribs directions of the *c* axis occur that are different from the direction in the rib under discussion and, therefore, they cannot belong to a same crystallite.

In the tangential direction of a rod, i.e. normal to the axial plane of a rib or normal to the (110) planes of the crystallites, their dimensions are set bounds by the thickness of a rib.

It is likely that in the direction [110], i.e. in the longitudinal direction of the rods, the crystallites are strongly developed. This is based upon the fact that the rods are comparatively resistant. When it is endeavoured to separate a packet of rods from the wax layer by means of a fine needle, the curled ends of the rods often catch into those of other rods and some force is needed to separate the packet from the layer by breaking these links. This ability of the rods to resist a certain pull and their tendency already mentioned to split up in elongated fragments under gentle pressure, are interpreted difficultly except by assuming the crystallites to possess a large dimension in the direction [110]. The possibility that they have the length of a rod cannot be definitely excluded, provided lattice distortions are assumed to be present in the curled parts of the rods.

The above considerations lead us to the conclusion that the crystallites of the wax rods probably are strongly elongated in the direction of the rods, that they may vary between c. 3750 and 20,000 Å in the direction of the carbon chains and between c. 250 and 10,000 Å in directions perpendicular to the axial planes of the ribs.

If the crystallites possess the maximum dimensions, the ribs are to be regarded as single crystals. In this case, the cross-section of a rod should be represented as shown in fig. 27*a*. It would form an aggregate of a few filamentary, more or less flattened, single crystals, aggregated laterally with their sharp sides.

If the crystallites are thinner filaments then there are various possibilities of their packing together within the cross-section of a rod to form bundles of a coarse-ribbed habit. Anyhow, their

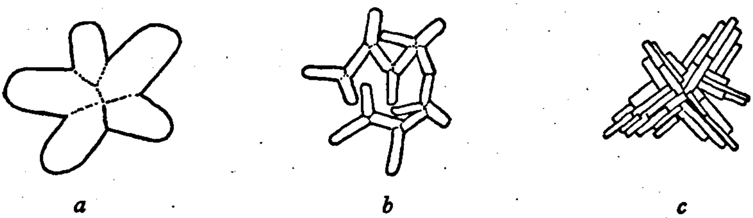


Fig. 27. Possible dimensions and arrangements of crystallites in the wax rods. In the direction perpendicular to the plane of drawing the crystallites are considered to be very elongated. Further explanation is given in the text.

position in the ribs must be with the *c* axis oriented more or less radially with reference to the rods, as otherwise the optical behaviour is not accounted for. We think of a possibility like that represented in fig. 27*b*. Such bundles would also possess a coarsely ribbed habit when examined at high magnification. They would easily be split up lengthwise and under the gentle pressure exerted in order to split the bundles, the filaments would tend to orientate with their flat sides facing the surface of the microscope slide. The details of this structure would fall below the resolving power of the optical microscope, however.

A different possibility of building up the rods from filamentary flattened crystallites is represented in fig. 27*c*. The figure needs no further explanation.

The usual optical methods do not enable one to decide upon one of the three possibilities of structure suggested. In the next section, evidence obtained by means of a more powerful method will be recorded.

3. *Electron-Microscopic Investigation of the Wax Rods*

When in 1942 we started this (subsequently interrupted) investigation on wax rods, there was no possibility of using an electron microscope. After the war, however, the electron microscope of the Delft Institute for Electron Microscopy constructed by J. B. LE POOLE in the Laboratory of Technical Physics at Delft (LE POOLE 1947), came at our disposal.

From an electron microscopic point of view the wax rods are very thick objects; in the microscope a heavy absorption of electrons in the rods was to be expected. Consequently, their temperature might rise above the melting point of the wax (70—80° C). Nevertheless, it seemed desirable to see if, under the electron microscope, further evidence could be obtained as to the possibilities of structure of the rods mentioned at the end of the foregoing section. Even if, owing to the low melting point of the waxes, no useful direct observation in the electron microscope might be feasible, there still was a possibility of investigating the surface structure of the rods by means of the replica technique. In this latter method thin films bearing the surface relief of the objects are studied in the electron microscope, instead of the objects themselves. Consequently, the difficulty involved in the thickness of the rods and the low melting point of the wax would not hinder the observation of surface structure. Before applying this replica technique, however, the rods themselves were brought into the field of the electron microscope. They were mounted on a specimen holder of collodion film. Against all expectations it appeared that the rods did not melt and that a good and detailed picture could be obtained on the fluorescent screen of the microscope. Therefore, it was not necessary to use the replica technique which, presumably, would have involved considerable technical difficulties.

On plate XXVII some electron microscope photographs of the rods are given in which especially the broken ends of the rods are represented, because they show the greatest amount of structural details. It is seen on these photographs that the rods consist of long filaments which give the impression of being very flat and, hence, ribbon-shaped.

This flat shape might explain the fact that the rods do not melt, for in such a structure the absorption of the electrons will be much less than in massive rods.

The photographs give the impression that the edges of the thin ribbons have grown together to form T-beams and similar, more complicated structures. It was difficult to decide whether all ribbons of one rod are cohering sideways. Also from the fact that the rods are relatively strong the conclusion might be drawn that there must be a lateral coherence between the ribbons. The non-coherent ribbons would not be solid enough to remain in a fixed position, perpendicular to the cuticle. The loose piece in fig. 29 gives a clear picture of the lateral coherence of the ribbons.

The breadth of the ribbons, as a rule, appears to be somewhat smaller than the minimum dimensions of the crystallites in the direction of the *c* axis, as given in the last paragraph. It appears to vary from c. 0.2—0.5μ, i.e. 2000—5000 Å.

Nowhere in the figures could a clear side-view of the ribbons be obtained. One gets the impression, however, that they are very thin and that the thickness does not amount to more than 1/10 of their breadth, so that, also in this direction, they do not have greater dimensions than those supposed to be necessary in wax crystallites in the directions of *a* and *b* axis to give sharp X-ray reflections.

A somewhat clearer impression of the thickness of the ribbons can be derived from fig. 30 representing a photograph of a preparation which has been lightly "shadowcast" by evaporation of a piece of gold at some distance from the object in a vacuum chamber. Note the piece of ribbon protruding from the rod which gives a good estimate of the thickness. The edge stands out more clearly because the sharp side of the ribbon has been more nearly perpendicular to the direction of the particles of evaporated gold than the flat surface, so that the edge has a thicker layer of gold-dust. The apparently granular surface of the ribbon has been caused by coagulation of the thin film of gold particles.

From the small thickness of the ribbons (c. 400 Å) the same conclusion may be drawn as that which has also been derived from our observations under the polarising microscope, i.e. that the long-chain molecules must lie parallel to the flat surface of the ribbons. If their direction had been perpendicular to this surface there could only have occurred about 5—10 identity periods in the direction of the chains, which number is to be regarded as insufficient to give rise to long-chain reflections in the X-ray diagram. As mentioned in chapt. II those reflections may distinctly be observed in the diagram of fig. 16.

Since the ribbons have, in two directions, the minimum dimensions of wax crystallites from which still sharp X-ray reflections may be expected, it cannot be assumed that they are built up from separate crystallites. Consequently, they must be regarded as single crystals. It might be, however, that in the longitudinal direction of the ribbons, here and there, the crystal lattice is discontinuous.

Because the (110) plane lies parallel to the longitudinal direction of the ribbons, as could be derived from the X-ray diagram, and the carbon-chains lie parallel to the flat surface, as is in agreement as well with the observations under the polarising microscope as with the thickness of the ribbons, the flat surface of the ribbons must be parallel to the (110) plane.

We want to draw attention to the fact that, from the way in which the edge of the piece of ribbon shown in fig. 30 has been crumbled off, the impression is given that the ribbons tend to split along the (110) planes. This may be understandable on consideration of fig. 2, chapt. A I, 2. It is seen from that figure that the interlock of the carbon chains along the planes (110) is less than along (100) and (010). Therefore, also the coherence between the first-named planes might be less than between the latter.

From the above it may be concluded that the electron-microscopic observations give the indication of the rods having a structure which, in principle, agrees with the possibility presented in fig. 27*b*, based on observations under the polarising microscope.

Fig. 28

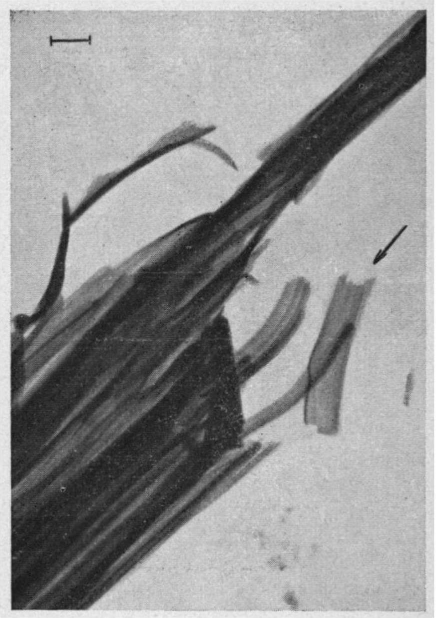


Fig. 29

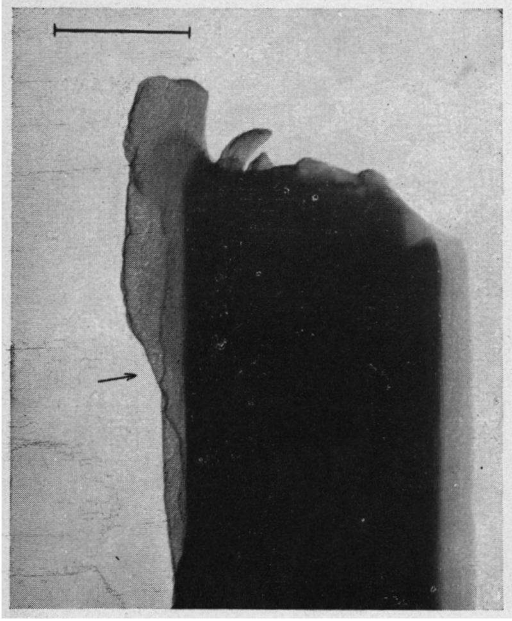


Fig. 30

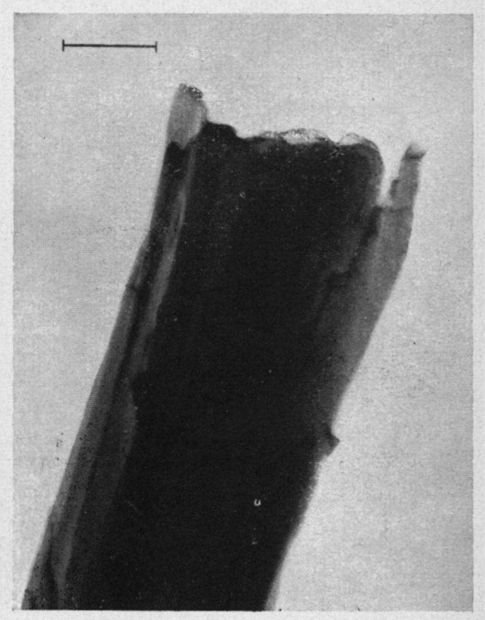


Fig. 31

Electron-microscopic photographs of broken ends of wax rods. The scale indicates 1μ. Rods of f. 30 and 31 lightly shadowcast. Further explanation is given in the text.

CONCLUSIONS FROM CHAPTER II AND III.

The wax rods of sugar cane stems are to be regarded as aggregates, or bundles of aggregates, of ribbon like crystallites lying parallel to the longitudinal direction of the rods.

The ribbons cohere along their lateral edges to form T-beam like structures. The ribbons are c. 2000—5000 Å broad and c. 200—500 Å thick.

As the flat faces of the ribbons are at angles with each other much open space is left between them.

The outer ribbons of a rod are more or less orientated with their flat surfaces in the radial direction of the rod.

The wax-molecules are lying with their longitudinal axes perpendicular to the longitudinal direction and parallel to the flat surfaces of the ribbons.

The molecules are arranged sidewise in the well-known paraffin packing according to MÜLLER (1928), while the ends bearing the alcohol groups are turned towards one another. The [110] direction is parallel to the longitudinal direction of the ribbons and the plane (110) is lying parallel to their flat surfaces.

Based on the results mentioned in part A, III, 5 it may be added, as to the chemical composition of the wax, that it mainly consists of n-octacosanol.

Finally, the remark must be made that a thin layer of the wax, lying against the cuticle, might have some other structure. This structure, however, has not been investigated.

CHAPTER IV

SOME GENERAL CONSIDERATIONS ON THE PLACE OF ORIGIN OF THE WAX AND THE FACTORS INFLUENCING THE MICROSCOPIC AND SUB-MICROSCOPIC STRUCTURE OF THE WAXY COATINGS

1. *The Place of Origin of the Wax*

The wax might originate either in the cell-wall or in the protoplast. DE BARY had already tried in 1871 to obtain conclusive evidence concerning the two above-mentioned possibilities. He found the wax to be present in the cell-wall, but he did not succeed in proving its presence in protoplasm.

As DE BARY detected wax in the cell-wall only, and as he observed, in addition, that in some plants it appears first in places on the cuticle as far removed from the protoplasm as possible, he supposed the wax to be formed exclusively in the cell-wall. Furthermore, wax being a C-H-O compound poor in oxygen, DE BARY supposed that it might be a product of reduction of the ubiquitous C-H-O compound cellulose. He proved that the formation from cell-wall material, however, could not be regarded as a change of cell-wall layers into wax layers, a so-called "metacrase", as was supposed by older investigators. The wax is excreted on the cuticle and wax deposits in the outer cell-wall layers of the epidermal cells are formed, according to DE BARY, by intussusception of wax molecules.

The modern conception is that in all probability the wax is formed in the protoplasm or, at least, that the carbon-chains of the wax are preformed in the protoplasm. Consequently, they pass through the cell-wall to the outside, during which process they might possibly undergo chemical changes. Considering the fact that protoplasm is capable of building up the carbon-chain occurring in fats and oils, it may be expected that it can also form the similar, though longer, chains of the wax. Theories on the metabolism of the formation of plant waxes are treated in part A, IV.

In connection with the above it may be recorded that CHANNON and CHIBNALL (1929) isolated wax from cabbage leaves, which they considered to come from the protoplasm and which contains the same components as the wax collected by us from the surface of those leaves, as mentioned here in part A, III, 5 for *Brassica*. Also from leaves of Lucerne (CHIBNALL, WILLIAMS et al., 1933), from meadow grasses, from leaves of young wheatplants (POLLARD, CHIBNALL, PIPER, 1931, 1933) and from several other plants, wax has been isolated in the same way as from the above-mentioned cabbage leaves. Furthermore, LEE and PRIESTLEY (1924) have made it probable that, in the formation of the cuticle, fatty substances pass from the inner tissue-cells through the cell-walls to the outside where, on the surface, oxidation and polymerisation take place.

It is difficult to get a clear picture of the transport of the waxes to the places of deposition. As to this problem WIJNBERG (1909) draws attention to the pores in the thickenings of the cell-walls of the so-called "long cells" in the epidermis of *Saccharum*. NIEUWENHUIS van UEXKÜLL-GULDENBAND (1914), BURGER (1920) and ZIEGENSPECK (1928) have observed microscopically very fine, vertical canals in certain cell-walls of some plants. DOUS (1927), by means of a surface-microscope, has detected very fine pores in the cuticle of several wax bearing plants, and these he supposed to be the ends of small canals in the cell-wall. DOUS is convinced of the probability that wax is excreted through these small canals, and has in fact shown this by a melting experiment. Only in a few of the cases he examined could DOUS not find any pores in the cuticle, indicating the presence of small canals.

FRITZ (1935) and MEYER (1938), however, in their research on the cutinised cell-wall layers of some plant species containing wax on or in the epidermis, have not observed any vertical small canals. It might also be that the wax does not pass the cell wall through special small canals, but that the carbon-chains diffuse through meshes inherent in the net-like structure of cellulose-chains and cutin, as has been assumed by LEE and PRIESTLEY (1924) and FRITZ (1935) for fatty substances which form the cuticle.

For the present we shall have to reckon with both possibilities of passing of the wax through the cell-wall. Both might also occur at the same time in one and the same species.

2. *Circumstances which might affect the Microscopic and Submicroscopic Structure of the Wax Covers*

In the following we shall consider the factors which theoretically might play a part in the formation of the diverse microscopic and submicroscopic structures of wax layers. As such factors may be distinguished:

1. the composition of the wax,
2. the distribution of the openings of small canals possibly present in the cuticle,
3. the submicroscopic structure of the cuticle,
4. the physico-chemical character of the surface of the cuticle,
5. organising influences from the protoplast.

The factors will be discussed below, beginning with number 3.

When wax is excreted on the epidermis it always has to pass the cuticle. Generally, the cuticle is described as being isotropic. It is supposed to consist of a network of irregularly branched chains of a fatty nature, about which, however, very little is known, cf. e.g. LÜSCHER (1936). Because of the supposed irregular structure of the cuticle it cannot be expected that the wax molecules after having passed the cuticle by diffusion, are excreted on the surface with a definite orientation. If the wax passes through small canals, it is imaginable that the molecules in the canals are united to form very small crystallites which might be given a certain orientation by movement through the small canals. This orientation might be maintained after the emergence of the wax on the cuticle. It is more probable, however, that the wax is either in the dissolved state when passing through capillaries, or in a liquid pre-phase; because, if it were solid, it would only be able to pass under high pressure. In this connection it may be mentioned that DE CANDOLLE (1833) already supposed that the wax is secreted as a liquid which solidifies afterwards, and that POHL (1928) has observed this phenomenon in young leaves of *Tulipa silvestris* and in seedlings of *Lupinus albus*. Thus it is, indeed, probable that crystallisation of the wax occurs on the surface of the cuticle and that an eventual preferential orientation of the crystallites only takes place here. No importance can then be attached to the factor mentioned under 3.

The fourth factor, the physico-chemical character of the surface of the cuticle, might have influence on the direction the carbon-chains preferentially adopt with respect to the surface. Consequently, it might cause a certain orientation in the first crystallisation-nuclei, which orientation might persist on further growth. We suppose that the above-mentioned character of the cuticle surface, generally, is the same in different species. Therefore, this character cannot, in our opinion, be regarded as a cause of important differences in the structures of wax layers.

The fifth factor, an organising influence from the protoplasm, such as is supposed to occur in the formation of deposits from protoplasm by apposition or intussusception, e.g. in the cell wall, is improbable in our opinion. The wax is separated from the protoplasm by the cell-wall and the cuticle, and continuation of protoplasmic branches into small canals, which are possibly present, does not seem to have a high degree of probability. It is, therefore, difficultly to be imagined how such an influence could assert itself.

An important role will probably have to be ascribed to the factor mentioned under 1. It goes without saying that the composition of a mixture of substances will have influence on its mode of crystallisation. Also the factor mentioned under 2 may be of importance. If small canals are present, one may expect that the distribution of the apertures of those canals in the cuticle causes a similar distribution of the emerging wax. DOUS (1927) observed such a connection in certain species, e.g. in *Kleinia articulata* and *K. repens*.

Thus, on account of theoretical considerations and certain data from the literature, we come to the conclusion that differences in microscopic and submicroscopic structure of wax layers might principally be caused by differences in composition and that a special distribution of the wax on the epidermal cells might be caused by a similar distribution of the ends of small canals in the cell wall. In the formation of the structure, a direct organising influence of the protoplasm does not seem probable. Nevertheless, an indirect influence has to be ascribed to the protoplasm, as the composition of the wax must, necessarily, be caused by the living material; the same holds true for the formation and distribution of the pores. Also the distribution of the wax on the plant surface as a whole will be a matter of organisation.

In the next sections we shall try to check those points of view with practice. We shall distinguish, for the sake of convenience, between the "passive" and the "organised" crystallisation of the wax: in the former the living matter does not play a direct part, whereas in the latter such an active part must be assumed.

3. *Correlation between the Composition (Powder Diagrams) and the Microscopic Structure of the Wax Covers*

If the microscopic and submicroscopic structure of the wax layers have to be considered as a result of passive crystallisation only, one might expect that waxes which have a similar composition also possess the same structure (irrespective of the distribution as a feature of structure). Consequently, it might be expected that wax layers having the same, or corresponding X-ray powder diagrams, should also have similar microscopic features. It will be tested below in how far this expectation holds true. The waxes of which the powder diagrams have been described in chapter I, have not been examined by us microscopically. Therefore, in comparing those waxes for the above purpose, we shall have to restrict ourselves to those among them of which the microscopical description has been given by De Bary.

According to De Bary the wax layers of *Elymus*, *Secale* and *Eucalyptus* consist of an accumulation of fine, randomly orientated needles or rodlets. The diagram of every one of those three species appears to belong to the type DG. (See table I, in A, II, 2, also for the following cases. Data on the composition are to be found in table III, of A, III, 6.)

The wax layers of *Kleinia* and *Ricinus* both consist of an accumulation of granules. Their diagrams both belong to group M. The wax layers of *Brassica* and *Allium* are considered to have a single granular layer. The diagrams both belong to type Ns.

The wax layers of *Saccharum*, *Coix*, *Eulalia*, *Andropogon* and *Phragmites* are considered by De Bary to belong to the rodlet layers, as well as those of *Musa* and *Strelitzia*. All those diagrams belong to the group Nl. The waxes of the five first-mentioned species consist for a high percentage of primary alcohol.

The wax layers of *Opuntia*, *Cereus*, *Copernicia* and *Myrica* are assigned to the crusty wax layers. Their diagrams are similar in that in *Copernicia* and *Myrica* the long-chain reflections are not very distinct, whereas in *Opuntia* and *Cereus* they could not be observed at all. This means that in all four species the composition of the wax must be rather complicated.

Those cases give support to the supposition that, generally speaking, waxes with similar structure produce similar X-ray diagrams and, hence, possess a similar composition. Therefore, it is likely that the different compositions of the waxes are cause of the diverse structures of the wax covers.

However, there are a few waxes which do not confirm the above view, viz. the wax layers of the fruits of *Benincasa* (type M) and those of the leaves of *Cotyledon orbiculata* (type Nsa). According to DE BARY, they belong to the rodlet layers. The X-ray diagrams, however, do not belong to the same type as those of the other rodlet layers. In addition, these wax layers show some structural peculiarities which will be mentioned later. *Canna* (type No) also has a rodlet layer, the diagram of which differs from those of other rodlet layers. Furthermore, according to DE BARY, *Iris germanica* has a single granular layer; the diagram, however, does not belong to type Ns like in the above-mentioned granular layers, but to type Nl.

With reference to these latter cases, attention must be paid to the fact that a very small amount of admixtures in a wax might be of great influence on its mode of crystallisation. This might cause waxes possessing a practically identical composition to adopt different microscopic structures. Therefore, these deviations from the above-mentioned rule cannot be used as arguments against the principle of passive crystallisation. On the contrary this principle is indeed strongly supported by that same rule. Other arguments, to be mentioned in the next section, ought to be brought up against the principle of passive crystallisation and, consequently, as a support of the alternative, the organising influence of protoplasm.

4. *The Possibility of "Organised crystallisation" in the Formation of the Diverse Structures of Wax Covers*

Wax layers of randomly orientated fine needles or grains, either in a single layer or stratified, do not seem to contradict the supposition that the microscopic structure of the layers is brought about by passive crystallisation. In the formation of those structures there is no reason whatever to ascribe an organising influence to protoplasm, as also has been mentioned by DE BARY (see chapt. I). We have observed similar layers of small crystals on the surface of non living material, e.g. on old lumps of sugar cane wax from manufacturing process and on old chocolate.

On the contrary, in the formation of rodlet layers and of stratified wax covers, DE BARY did consider the existence of an organising influence. However, after having acquired more information as to the structure of the wax rods of sugar cane — and we suppose that many other rodlet layers will have a similar structure — it does not seem necessary to think of organisation in connection with that structure. It could be possible that the alcohol molecules which form the wax, after excretion, are lying with their long axes parallel to the cuticle surface, caused by factors as meant under 4 (sect. 2). When crystallisation begins they might settle with their dipole alcohol-groups turned towards each other (see fig. 32), in which opposite charges are in juxtaposition. Similar nuclei might grow into the direction indicated by the carbon chains of the first molecules by parallel apposition of new molecules. If more and more molecules are deposited, parallel to those nuclei on their lower surface, ribbon like crystallites might be formed, the edges of which cohere. It stands to reason that this principle for the formation of rodlet layers cannot explain all their structural details, as e.g. the relatively constant thickness of the rods, the dimensions of the ribbons of which they consist and the fact that some of the rods are much longer than other ones.

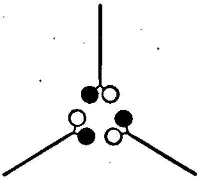


Fig. 32. Supposed stellate orientation of the alcohol molecules in the first crystallisation nuclei on the surface of the cuticle.

If wax is excreted by pores, there might exist an area around each pore where the wax coming from that pore, is crystallising. And if from some pores more wax is flowing than from other ones, there might take place increased growth of the ribbons in the surrounding areas of those former pores, resulting in the formation of longer rods. This would also imply that each rod is formed from wax coming from one pore only.

Supposing that the wax can spread only to a limited distance from the pore through which it is excreted, for example because of evaporation of a solvent, this might possibly explain the relatively constant thickness of the rods. If, however, the wax is excreted through the cuticle in a diffuse state, it is difficult to explain the difference in length of the rods.

Supposing that the wax is excreted through pores, an explanation is needed for the fact that the breadth of the crystallites is so much greater than their thickness and that they are orientated with [110], and not with e.g. [100] or [010] perpendicular to the cuticle. More fundamental theoretical considerations might indicate a possibility of explaining those circumstances as well, without necessitating the assumption of an organising influence of protoplasm.

In several wax layers, however, details occur which, at first sight, are much more difficult to explain by accepting the principle of passive crystallisation of the wax on the cuticle. As such we mention the following structures observed by DE BARY:

1. The remarkable globular thickenings formed in the already developed wax rods on the fruit of *Benincasa*. Two or three of those thickenings occur in each rod, situated at the same height in the several rods, which imparts a stratified appearance to the wax layer.
2. The cone-shaped bundles of rods occurring on a continuous wax layer on the leaves of *Cotyledon orbiculata* and situated on top of each epidermal cell. They are formed when the continuous wax layer is already present.
3. The ring of wax rods around the stomata of *Strelitzia ovata* and formed on the transverse walls between the companion cells and the adjacent cells of the epidermis.
4. The structure of stratified prisms in the wax layer of *Klopstockia cerifera*. Those prisms occur, one on top of each epidermal cell. They are separated by a wall of clear and transparent wax, probably of a different composition from that of the wax of the prisms.

In order to explain the growth of the above-mentioned structures the only possibility seems to be to accept an organised crystallisation. It is difficult, however, to imagine how an organising influence from the protoplasm can be realised through the different layers of the cell wall and the homogeneous cuticle. Therefore, we could only imagine this influence as to be an indirect one; this point of view may be supported by several possibilities:

It might be e.g. that the cell wall (by direct organisation) is formed in such a way that only in certain places can wax pass through it; for instance because small canals might occur only in definite parts of cell walls or in the walls of definite cells. This might lead to deposition and crystallisation of wax on the epidermis, in a definite place.

Another possibility is that the protoplasm produces waxes of different composition, periodically alternating, so that in crystallisation on the cuticle, this might give rise to a layered structure.

The fact that wax can emerge on the cuticle in a liquid state may also be of importance in explaining several structures. Liquid fatty substances often tend to creep, so that it would be possible that after crystallisation of certain compounds the still liquid ones move into and accumulate in open spaces remaining between the already crystallised wax. In those spaces they might solidify and, consequently, fill them up with wax of another composition.

However, we shall not endeavour to explain along the abovementioned lines the peculiar structures recorded by DE BARY. Without preceding investigations into the submicroscopic structure such explanations are, in our opinion, of too speculative a character. It might be feasible, however, by making use of the possibilities mentioned above in several combinations and by supposing, in addition, the occurrence of such processes as local solvation and recrystallisation, to project schemes for the physico-chemical formation also of the complicated structures of wax coatings which at first sight suggest their formation under the direct organising influence of the protoplasm.

Consequently, we share WIESNER's opinion (see chapt. I), though not based on exactly the same grounds.

CONCLUSIONS FROM CHAPTER IV

The conclusions from this chapter, based on data from the literature, on the results of our own investigations, and on theoretical considerations, may be summarised as follows:

1. It is probable that the long-chain compounds of the cuticular waxes in general are formed in the protoplasm, or that the chain molecules are preformed in the protoplasm and undergo chemical changes either on the cuticle, or on their way to the surface of the cuticle.
2. The wax reaches the surface of the cuticle either through fine canals, or by diffusion, or possibly in both ways. Possibly this process is different in different species or even in different stages of one and the same species.
3. The wax can be excreted on the cuticle in a liquid state, and it is probable that it is generally excreted in an unorientated state.
4. After emergence from the cuticle the wax crystallises.
5. It has been stated for heaped wax layers, for singular granulated layers and for rodlet layers that, generally, waxes with a similar microscopic structure, also possess a similar composition. This makes it probable that the microscopic structure is determined

by the mode of crystallisation characteristic of a certain composition.

6. The microscopic structure of heaped wax layers and singular granulated layers does not give rise to the supposition that it is brought about by other factors than those mentioned under 5, so that no organising influence of the protoplasm need be considered.
7. The submicroscopic structure of the wax rods of sugar cane, which is probably the same in other rodlet layers, is not indicative either of an organising action of the protoplasm in its formation.
8. It is unlikely that in the formation of the structure of wax layers with a less simple microscopic structure than the abovementioned ones the protoplasm has a direct organising inflence.
9. It is probable that it is not the structure, but only the composition of the wax layers of the cuticle that may be regarded as a feature directly affected by the organising influence from the protoplasm.

Summary of Part B

As an introduction, in chapter I, a survey is given of the microscopic structures of wax cuticles on plants distinguished by De Bary (1871) and of the older opinions concerning their submicroscopic structure and their formation. The importance of obtaining a clear picture of their still very insufficiently known submicroscopic structure in order to gain insight into the problems concerning their formation, has been pointed out. Then some more recent investigations into the submicroscopic structure of the wax on, and in the outer wall of the epidermis have been mentioned and the desirability of carrying out X-ray investigations has been argued.

In chapter II*a*, the X-ray investigation of a wax cuticle by means of a standard equipment for the study of X-ray diffraction in use in the Laboratory of Technical Physics, Delft, has been described. As an object to study, the wax layer of the sugar cane stem was chosen in places where it consists of rods growing nearly perpendicularly to the cuticle. In sect. 1, this choice has been justified, a description of the object has been given as well as an account of technical details concerning the preparation, the method of taking X-ray photographs and some difficulties connected with the small size of the object.

The X-ray diagrams obtained have been described in sect. 2, and it is ascertained what conclusions about the structure of the wax layer they allow to be drawn without carrying out special calculations. It appeared that the crystallites in the wax layer are preferentially orientated with one of their axes perpendicular to the cuticle, whereas they can occupy all possible positions by turning around that axis. In this orientation, however, a great angular dispersion of the positions of the crystallites occurs, which might be due equally to lack of parallism between the rods of the layer as to imperfect orientation of the crystallites in the individual rods. In addition, the diagrams show that the longitudinal direction of the chain molecules is perpendicular to the axis of orientation. This implies that the chain molecules of the wax are lying with their longitudinal direction parallel to the cuticle and perpendicular to the longitudinal direction of the rods. In sect. 3 we have tried to calculate the identity period of the axial direction of the crystallites which is orientated perpendicular to the surface of the cuticle, in order to be able to index this axis. The calculation is greatly handicapped by the considerable angular dispersion in the positions of the axis under discussion. It is found that the axial direction is probably [110].

In sect. 4 it is ascertained, by means of the reciprocal lattice, in which places on the photograph maxima of blackening must be expected if the crystallities are perfectly orientated with the above-mentioned axial direction perpendicular to the cuticle. The desirability is argued of obtaining a photograph actually showing those maxima as a confirmation of the more or less doubtful conclusions from the first-mentioned photographs with regard to the indexing of the axis of orientation of the crystallites.

In chapter II*a*, the X-ray investigation of the wax rods by means of a newly developed micro-method has been recorded. In sect. 1 it is stated that the desired photograph mentioned above might be obtained from a small packet of perfectly parallel wax rods. The impossibility of obtaining such a diagram by means of the available standard equipment, and the necessity of a special micro-method for that purpose have been argued. Then the general importance which a micro-method might have for X-ray studies of microscopic and, especially, biological objects, has been discussed. Since no description of such a method in a design suitable for most biological objects was found in the literature, investigations were carried out in order to develop a generally applicable micro-method. These investigations, however, have not been described here. The results were briefly mentioned. The next section describes how, by means of the newly developed micro-method, a micro-diagram could be obtained from a small bundle of parallel wax rods. This diagram shows maxima in the places predicted before in sect. 4 and thus confirms that the axial direction with which the crystallites are orientated perpendicular to the cuticle, has been correctly indexed on the ground of the diagrams obtained earlier. The diagram enables the identity period of this axis to be determined with much more accuracy than the diagrams obtained first. It also confirms that in the individual wax rods, a comparatively perfect orientation of the crystallites occurs.

Chapter III describes the examination of the wax rods by means of the polarising microscope and the electron-microscope in order to elucidate their submicroscopic structure further. This concerns in particular the orientation of the crystallites with respect to the directions in the wax rods normal to their longitudinal direction, those details not being attainable by means of X-ray diffraction on account of the small diameter of the rods. When using high magnifications under the polarising microscope it appeared that the rodlets were coarsely ribbed. The orientation of the crystallites in the ribs could be indicated on account of the observed birefringence phenomena combined with data from the literature about the birefringence of waxes. They are orientated with the longitudinal direction of the molecules in radial directions of the rodlet. We have disputed WEBER's claim (1942) to having demonstrated the same direction of the molecules in wax rods of *Phragmites*. Sect. 2 gives some considerations on the limits of the dimensions of the crystallites and on the possibilities for the structure of the rods within the range of those limits. In sect. 3 it has been described how the picture of the submicroscopic structure obtained in the preceding sections, could be confirmed and supplemented by making use of the electron-microscope.

The conclusions concerning the submicroscopic structure of the wax rodlets to which this investigation has given rise, are summarised after chapt. III.

Finally, in chapter IV, some general view-points on the place of origin of the cuticular plant waxes and on the formation of their submicroscopic structure have been put forward. Sect. 1 deals with the place of formation of the wax on the basis of data from the literature. In sect. 2 the factors which might possibly affect the structure of the wax coatings are briefly discussed. The composition is suggested to be the most important factor. Therefore, in sect. 3, it is ascertained whether waxes giving similar X-ray powder diagrams also tend to exhibit similar microscopic structures; this has been found, in general, to be true. In sect. 4 it is attempted to give an explanation of the structure of the wax rods. Furthermore it is discussed in particular whether it is necessary to ascribe a direct organising influence to the protoplasm in the formation of the structures of wax cuticles strongly suggesting such an influence. We have advanced possibilities of formation of those structures as an indirect effect of chemical activity of the protoplasm. Finally the conclusions from this chapter are summarised.

APPENDIX

SHORT DESCRIPTION OF THE MICRO-APPARATUS DEVELOPED FOR X-RAY DIFFRACTION

From figs. 33, 34 and 35 (plate XXVIII) some idea may be obtained of the micro-camera developed; fig. 33 presents a drawing of the camera in its adjusting ring, fig. 34 shows the single camera and fig. 35 the dismounted oamera. The adjusting ring is screwed into the window of the X-ray tube and enables the camera axis to be brought to bear on the focal spot. This ring will not be discussed further here. In fig. 35 some important parts of the camera have been numbered in correspondence with the numbers indicating those parts in fig. 33.

The conic capillaries used as pinholes (6), the way of screening the scattered radiation from the glass rim by means of a narrow bore in a lead plate and the way of mounting the specimen have already briefly been described in part A, II*b*, 2. Special techniques of manufacturing pinholes and narrow screening cups have been developed and much attention has been paid to theoretical considerations on the most favourable proportions (with regard to the exposure time) of the wide and narrow apertures of the capillary and the length and diameter of the screening cup as functions of the size of the specimen and the smallest diffraction angle to be observed.

The capillaries are mounted in a brass cylinder (4) in which they can be centred by means of small screws (5). A 0.5 mm thick Pb disc fitting closely around the end of the capillary protruding from the cylinder, is mounted on the cylinder (black in fig. 33 and not shown in fig. 35). It serves to prevent radiation from passing sideways of the capillary, and on this disc the lead plate with the secondary pinhole (screening cup) is mounted. Coaxial adjustment of the screening cup is done under the microscope. In cases of long and narrow screening cups this is not easily feasible and the adjustment must be checked by means of X-ray tests. If the primary beam is insufficiently centred in the screening cup, the radiation will strike the lead. Centring of the capillary in its cylinder by means of the small screws precedes the adjustment of the screening cup. It is also done under the microscope, and for that purpose a cap with a small central bore is slipped on the cylinder on both ends in turn. The pinhole is centred in that bore. This cap is pictured beneath the cylinder in fig. 35. The cap pictured above the cylinder is put on to protect the mounted specimen until it is brought into the camera.

We shall now give an account of how the dismounted camera as represented in fig. 35 is arranged to make a photograph.

Before bringing the pinhole-cylinder with the mounted specimen into the camera, the distance of the film to the specimen must be adjusted and the film must be put in and provided with a small, exactly centred perforation to let pass the primary beam. In order to adjust the above-mentioned distance, a small ring of the required thickness (15) is brought into the wide opening of ring (14). Then a round piece of aluminum foil and a small film (13 mm diam.) exactly fitting in the same opening, are put on the small ring. Next, the ring (14) is screwed into the central body (17) of the camera, and the tunnelled cylinder (18) is brought into the central body on the opposite side, after which the camera is closed on that side with the cover (22). The cover is fitted with a springed plate that presses cylinder (18) on to the film. The plate forms part of a cylindrical tunnelled piece that may slide to and fro through an opening in the cover. The piece at its end opposite the plate, bears a fluorescent screen on lead glass to observe and stop the primary beam, and a gas outlet.

In order to make the perforation in the film, a tunnelled cylinder with a needle (pictured on the far left at the bottom of fig. 35) is put into the opening of ring (14) which serves to take up the pinhole cylinder. By carefully turning the needle, possessing a tip with sharp edges, a perforation of 0.3 mm diam. is drilled into the centre of film and aluminium sheet. Then the cylinder with the needle is removed and the pinhole cylinder with the specimen is put in. Finally the camera is closed with the cap (1).

The camera also allows larger films (29 mm diam.) and increased distances between film and specimen (c. 6—16 mm) to be applied. For that purpose cylinder (18) is removed and the film is put on larger rings which are placed into the wide end of the central body.

PLATE XXVIII

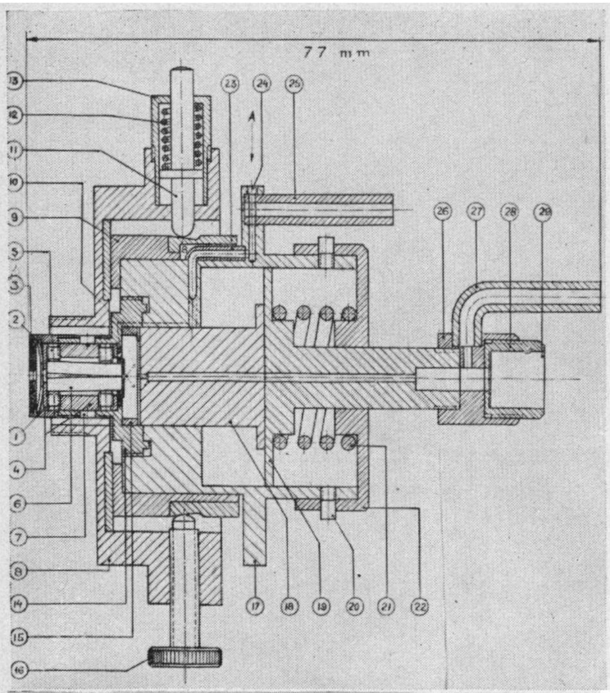


Fig. 33. Drawing of the micro-camera in its adjusting-ring. Explanation is given in the text.

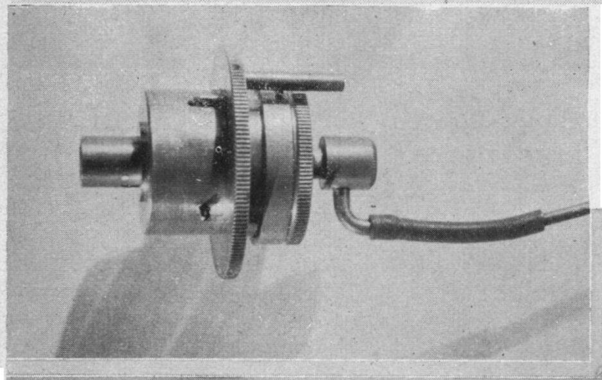


Fig. 34. Single micro-camera.

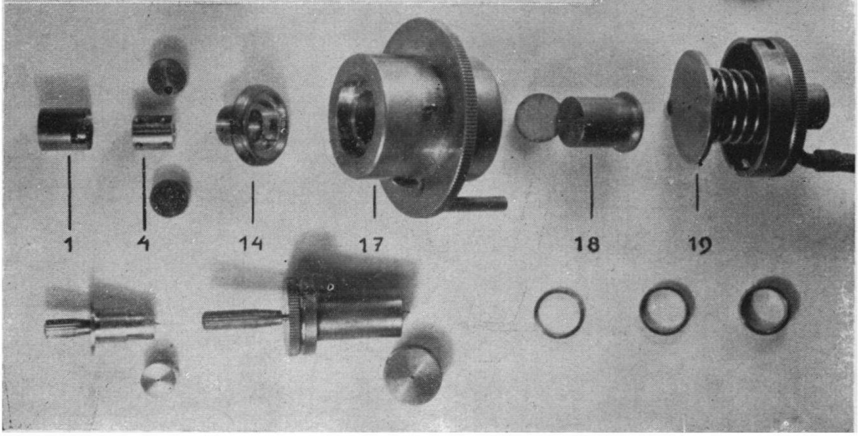


Fig. 35. Dismounted micro-camera

The perforation in the film is then made before screwing ring (19) in the central body, by using the thicker cylinder and needle shown on the left at the bottom of fig. 35.

The camera is fitted to let hydrogen or damp air pass through the chamber between specimen and film. The gas inlet is indicated by (25), the gas outlet by (27). From fig. 33 it will be easy to follow the way of the gas. In certain cases the use of a hydrogen atmosphere is desirable to obtain pictures of sufficient clarity around the central perforation in the film. It goes without saying that in connection with the flowing through of hydrogen, but also for other reasons, the manufacture of the camera is precision work.

The adjustment of the camera in front of the X-ray tube by means of the adjusting ring in order to bring the camera axis to bear on the focal spot, offers difficulties when pinholes narrower than c. 15 μ (and a 2.5 kw X-ray tube) are applied, as the bright spot on the fluorescent screen becomes very faint below those dimensions of the pinhole. In those cases, preceding adjustment by means of a wider capillary is necessary. However, when very narrow pinholes are applied, it is desirable to use a Geiger-counter equipment to bring the axis exactly to bear on the intensity maximum of the focal spot. For that purposes, the lead-glass beam-stop (29) with fluorescent screen can be screwed out.

Up to now, no practice in applying narrower capillaries than 20 μ to the study of organic specimens has been obtained. A 6 μ capillary has been used in obtaining a photograph from a 12 μ tungsten thread, cf. KREGER (1945).

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