I accept with pleasure the invitation with which the Royal Swedish Academy of Sciences has honoured me to speak about my investigations on plant pigments. My task is lightened by the fact that several years have elapsed since the completion of this work. Consequently, it is as if I were able today to conduct this chosen audience to a height from which a survey can be made of the main lines of research without having to weary you by wandering through the twisted and tortuous paths which I have trod. The intention behind my work was to establish the constitutional characteristics of the most widely distributed plant pigments, of chlorophyll in particular, and to gain some criteria with regard to its chemical function.

Although a considerable literature was already in existence on the pigments of green leaves, my work is actually linked with that of Berzelius, who in 1837 and 1838 conducted the first chemical investigation of leaf pigments. The method used by Berzelius was to treat the chlorophyll with acids and alkalis, and in this method lies also the basis of my work. From the innumerable investigations by Berzelius there is actually only one result which is outstanding, which Hoppe-Seyler found and which Schunck and Marchlewski have stressed, viz. the recognition of a certain relationship between the pigments of the blood and of leaves, or more correctly, between the structural materials of their molecules. But the first questions with regard to chlorophyll were still unanswered: what features in the composition and structure distinguish chlorophyll from haemin analogous to the basically different functions of the leaf pigment and the blood pigment? Is there only one chlorophyll, or are there several or many? Gautier had laid down the principle that chlorophyll was different in monocotyledons and dicotyledons, and in a much admired work which appeared in 1906, Etard claimed to show that in a single plant there exists a large series of quite different chlorophylls and in the entire plant world an unlimited number of the most different leaf pigments. In this work every green-coloured wax or fat was regarded as a chlorophyll, whereas Berzelius had already clearly distinguished the leaf green from the resins, waxes, and fats.
In one point, however, my first experiments have already led me to take up a different view from that of Berzelius. According to him, chlorophyll should not be decomposable either by strong acids or by alkalis. This was an error, caused by the chlorophyll-green colour which is precisely special to the salts of the decomposition products formed with strong acids and alkalis. But chlorophyll itself does not produce a salt; it is inert in its intact state towards both acids and bases. It is extremely easily transformable, and it can be decomposed hydrolytically by acids and bases alike. Even in its neutral solutions, e.g. in alcohol or ether, it undergoes extraordinarily easily a substantial transformation - I designated it allomerization - which is not, it is true, revealed in the colour.

Chemical indifference, ease of change, and ease of solubility of the leaf pigment, which is mixed with yellow and a huge number of colourless accompanying substances - all these were obstacles to its isolation. Our method of work was, first of all, without isolating and investigating the chlorophyll itself, to deduce the peculiarities of its constitution by two methods of decomposition, i.e. from the examination of the two series of derivatives which are produced from the reactions with acid and with alkali.

Even a test-tube experiment shows that as a result of the action of alkali hydroxide on the neutral chlorophyll an acid is produced which forms salts soluble in water; the colour of these salts is still chlorophyll-green. This means that by this reaction, without a significant optical change, a component which was bound to an acid group must have been hydrolytically liberated.

It is evidently another part of the molecule onto which is directed the action of the acid, and the mildest action at that. The chlorophyll colour changes during this process into olive and at the same time the fluorescence is diminished. Whilst this is taking place there is not as yet a salt-forming group formed; this means that saponification is avoided here. Consequently it is possible when splitting by acid to spare that component of the chlorophyll, and to trace it in the decomposition product, which is split off by alkalis and which is lost in the mother liquors, that is, in the substances accompanying the extracts. Conversely, the alkali derivatives of the pigment must still present a characteristic atom group, which is destroyed so easily by acid and with such striking alteration of colour. This was the guiding thought of our work, from which it was possible, before the chlorophyll itself was known, to combine its features from the analysis and from the reactions of the derivatives formed by acid and alkali, and to do this so completely that
the analysis produced nothing new at all when it was finally possible to prepare the natural pigment in pure state.

With the mild action of oxalic acid on the alcoholic extract of leaves, a chlorophyll derivative - phaeophytin - difficultly soluble in alcohol, is almost quantitatively precipitated, free from colourless and yellow accompanying substances. It is a waxlike nitrogenous substance which leaves no ash residue, shows a very weak alkaline reaction and has no acid properties. This substance can be saponified like an ordinary wax; the result of this proves it to be an ester, it yields a mixture of high-molecular nitrogenous carbonic acids which have a pigment character, and a colourless and nitrogen-free alcohol, phytol, which corresponds to the formula $\text{C}_{20}\text{H}_{39}\text{OH}$. This alcohol, which has the appearance of glycerine, but is of course insoluble in water, is primary, unsaturated and aliphatic; its carbon chain has several branches, it is oxidized by ozone, with the loss of three carbon atoms, to a ketone.

This phytol occurs constantly as a component of chlorophyll and forms a third of its molecule. One of the first changes produced by the alkalies on the chlorophyll molecule is, therefore, the saponification of the phytol ester group. The phaeophytin, and therefore the chlorophyll itself, contains also a COOCH$_3$ group, which is subsequently hydrolysed. Furthermore, there is still a characteristic change resulting from the action of the alkalis, which is revealed by a striking change of colour, by the so-called brown phase. When an alcoholic solution of potassium hydroxide is added, the green solution immediately turns into an intense brown, then a few minutes later the original colour of the fluid returns. This behaviour, which the chlorophyll loses by allomerization when its solutions are merely left to stand, is to be attributed to the solution of an easily hydrolysable atom group and the formation of a similar group; it can probably be explained as a re-lactamization, i.e. as the opening of an existing lactam ring and the closing of a new ring, similar indeed but resistant to alkalies.

When chlorophyll is saponified by potassium hydroxide, very easily decomposable chlorophyll-green carboxylic acids are produced - the chlorophyllins. They can be separated from the accompanying substances by the solubility in water of their salts and by transfer into ether upon acidification, and can be purified by careful transfer from the ether into disodium phosphate as an alkali, and liberated with monosodium phosphate as an acid. Analysis showed that the chlorophyllins are magnesium compounds. They contain the metal in an electrically non-dissociable state, as haemoglobin contains iron, it is bound in them to nitrogen with a complex bond. This
magnesium-containing group is very sensitive to acids, but is stable in an alkaline medium. Consequently, it remains intact during fundamental changes of the molecule, by which even the carboxyls of the chlorophyllins are split off, one after the other. The assumed type of bonding of the magnesium was confirmed during the prolonged action of the alkalies, i.e. by the decomposition of the chlorophyll with concentrated alcoholic alkalies at temperatures up to 250°. The series so produced consists of decomposition products which crystallize well, have splendid colours and fluoresce intensely; acids with three, two and finally with only one carboxyl group, are the so-called phyllins, e.g. glaucophyllin, rhodophyllin, pyrrophyllin. All contain magnesium and are moreover free acids; they have one atom of magnesium to four atoms of nitrogen. The atom group (i) with the basic metal

\[
\begin{align*}
\text{N} & \quad \text{C} & \quad \text{N} \\
\text{C} & \quad \text{Mg} & \quad \text{C} \\
\text{N} & \quad \text{C} & \quad \text{C}
\end{align*}
\]

is the essential feature of chlorophyll and is to be regarded as the actual carrier of the chlorophyll function in the synthesizing life of the green plant.

The magnesium content of the chlorophyll is constant, close to 3%. It has been found to be similar in land and water plants of the most varied classes. To the complete comparison of the leaf pigments of different origin there belongs also the analysis of the intricately composed nitrogenous carboxylic acids which appear upon decomposition, whether upon the hydrolysis of the phaeophytin by alkalies or upon the splitting of the chlorophyllins and other phyllins by acids. This was at first always a very complicated mixture of pigments of an acid nature. Its disentanglement and then the simplification of the results of the decomposition were only possible by a method of analysis, the development of which was initiated by my investigations. The method of determining and separating chlorophyll derivatives is based upon the alkaline properties of these amino acids, which are extremely varied, and upon the different distribution between ether and diluted hydrochloric acid. The more cautious and uniform were the conditions during hydrolysis, but also during the preparation of the extracts containing the chlorophyll, the more simple in its composition became the mixture of the alkaline cleavage products. Finally it was possible to conduct the production of the phaeophytin
and to arrange its splitting in such a way that there always appeared only two magnesium-free carboxylic acids,

Phytochlorin e with the composition $C_{34}H_{34}O_5N_4$
Phytorhodin g with the composition $C_{34}H_{34}O_7N_4$

the former being olive-green and the latter a splendid red in neutral solutions.

The determining factor for the identity of the chlorophyll when comparing preparations from over 200 plants of numerous classes of cryptogamia and phanerogamia, was therefore the content of magnesium and of phytol and the elucidation of the two characteristic cleavage products, phytochlorin and phytorhodin. With regard to the obstacles which had to be overcome in this comparison, it is noteworthy that the phytol content of the phaeophytin preparations at first showed great fluctuations; it often, indeed, reached one third of the molecule, but sank considerably in other cases, even to a small percentages and still smaller figures. For these differences, however, only one transformation was to blame which the pigment underwent during the extraction from the fresh or dried leaves with alcohol under the action of an enzyme. This enzyme, chlorophyllase, which belongs to the esterases, accompanies the chlorophyll in the green parts of the plant, often occurring very sparsely, but often very richly. Under its effect, which once recognized could be easily avoided, the phytol is replaced by ethyl or methyl alcohol or in an aqueous medium is removed by saponification. The action of this chlorophyllase also explained the appearance of the so-called crystallized chlorophyll, the remarkable and magnificent green crystals which Borodin had first observed in 1881 in microscopic leaf sections. The crystallized chlorophyll is ethylchlorophyllide; it originates through ethanolysis from the phytol compound. The formation of the crystallized chlorophyll was now no longer accidental; on the contrary, the effect of the chlorophyllase was utilized to an extensive degree for the purposes of preparation, also for partial synthesis of the chlorophyll from chlorophyllide and phytol.

The same characteristics which serve for comparing the leaf pigments from whichever starting material allow us also to decide whether, in the course of the preparatory work, the chlorophyll remains unchanged in the finer details of its molecule. Hence this characterization was the basis for preparing the chlorophyll itself in an uninjured and pure state and for confirming, by analysis, the conclusions which had first been reached from the
investigation of its derivatives. The isolation of the chlorophyll relied upon calorimetric determination of the degree of purity of its solutions, and depended upon the systematic increase of this purity by methods of distribution between several immiscible solvents, such as petroleum ether and aqueous alcohol. By this method, colourless admixtures and the yellow pigments of the leaves were separated. The simultaneous occurrence of these yellow pigments, the carotenoids, with the green pigments, which seemed to indicate a special physiological role of the substances because of their great affinity to oxygen, gave rise to the preparation of the yellow substances in the pure state and their analysis. Two well-crystallized and nitrogen-free pigments occur in every green part of the plant and in many yellow parts. One of these, identical with the long-known carotene of carrots, is an unsaturated hydrocarbon of the formula $C_{40}H_{56}$. Its partner, xanthophyll, was still unknown in structure, although it predominates in leaves; according to composition and properties it is a carotene oxide ($C_{40}H_{56}O_2$). Only in the Phaeophycae are carotene and xanthophyll accompanied and repressed in quantity by a third carotenoid, richer in oxygen, fucoxanthin, which can be isolated in crystal form and has the composition $C_{40}H_{56}O_6$.

Whilst chlorophyll in the plant extracts is accompanied by other substances, as much as fifteen to eight times in quantity, solutions of about 70% pure chlorophyll can be obtained by separation methods. From this point on, a surprising observation helped to solve the problem. When the substance has reached a certain degree of purity, it reveals its true solubility characteristics, which were distorted before by the admixtures. Pure chlorophyll is not soluble in petroleum ether and is precipitated from the alcoholic solvent when the alcohol is washed away. The procedure permits, with good yields, the isolation of pure chlorophyll from dried or fresh leaves just as easily as that of any other plant substance, alkaloid or sugar.

However, this pure chlorophyll is not as yet a simple substance. The occurrence of phytochlorin and phytorhodin in the mixture upon its decomposition could also have been attributed to a cleavage of the phaeophytin into the two products or to successive decomposition to these. But in the course of the demixing operations which were used for the isolation it appeared - as had already been observed by the English physicist Stokes in 1864 in the course of small-scale experiments - that the chlorophyll consists of two differently coloured and differently soluble components, which are distributed unequally, between methyl alcohol and petroleum ether. By means of systematic fractionation with these solvents the two pure chlorophyll com-
Components were finally obtained from the mixture. One of them, chlorophyll a, is blue-green; and the other, chlorophyll b, is yellow-green; component a predominates; to almost three molecules of chlorophyll a in the leaves there is only one molecule of chlorophyll b. Despite the optical difference, the composition is similar. The difference is due to a different degree of oxidation. What the formulae of phytochlorin and phytorhodin allowed us to foresee is confirmed by the chlorophyll: the compounds of series b are derived probably from a by the fact that two atoms of hydrogen are replaced by one atom of oxygen, corresponding to the formulae:

Chlorophyll a: $C_{35}H_{72}O_{5}N_{4}Mg$ i.e. $[C_{32}H_{30}ON_{4}Mg](CO_{2}CH_{3})$

\[
(CO_{2}C_{20}H_{30})
\]

Chlorophyll b: $C_{35}H_{70}O_{6}N_{4}Mg$ i.e. $[C_{32}H_{28}O_{2}N_{4}Mg](CO_{2}CH_{3})$

\[
(CO_{2}C_{20}H_{30})
\]

With a specific reducing agent, magnesium alkylhaloid, the compound which is richer in oxygen can be converted to derivatives of the u-series. The decomposition of the chlorophyll by an alkali has also led, by way of different intermediate products, to the same end-products, and finally to one and the same carboxyl-free and altogether oxygen-free parent substance, aetiochlorin, of the formula $C_{31}H_{56}N_{4}$, from which is derived the magnesium-free aetioporphyrin of the formula $C_{31}H_{56}N_{4}$- just as the carboxylic acids of the series, when acted upon by acid, are converted into analogous magnesium-free compounds, the porphyrins.
This parent substance became even more important because we were also able to decompose the blood pigment to the very same aetioporphyrin; thus for the first time a common transformation product has been obtained from haemin and chlorophyll, with its molecule still in close relationship to the pigments. From oxidation and reduction experiments it is clear that the molecule of the simplest porphyrin is built up from four multi-substituted pyrrole nuclei. Although there are as yet no definite details about the way in which these pyrrole nuclei are bound, nor any exact statements with regard to certain details of their substitution, we may be allowed nevertheless to draw a provisional picture, Formula (II), of the structure of aetioporphyrin, based on considerations of probability.

As with the proteins and the nucleic acids, so with haemoglobin and with chlorophyll there are still many difficult problems to solve regarding the detailed determination of their constitution. Nevertheless, the broad features of chemical knowledge of the leaf pigments already gained open new paths for the treatment of biological questions and for the investigation of the function of chlorophyll in the process of assimilation.

The English botanist Wager had submitted the hypothesis that chlorophyll is consumed in the assimilation process, that it builds up its molecule with carbon dioxide and forms its reduction product by its complete disintegration. The quantitative determination of the chlorophyll content allows us to make a decision on the correctness of this concept. It has been shown, however, that the chlorophyll content of a leaf remains absolutely constant throughout a long-continued and maximal assimilatory performance. We could therefore relate the assimilatory performance of the plant at a favourable temperature and moistening and with an excess supply of carbon dioxide and light to the amount of chlorophyll and check the proportion of the assimilated carbon dioxide to the amount of chlorophyll. This is subject to
great fluctuations according to the chlorophyll concentration in the leaves, and further to the growth and to the season of the year. From an examination of the cases in which this quotient differs very widely from the norm, we must conclude that in the assimilation process the chlorophyll works in collaboration with another internal factor, i.e. one of an enzymatic nature, an enzyme which is probably engaged with the decomposition of an intermediate product formed from chlorophyll and carbon dioxide. Chlorophyll, that is to say, each of the two components, indeed combines in a colloid state with carbon dioxide to form an addition product, which can be dissociated. This observation can form the basis of a theory of assimilation which assumes that the light which is absorbed does its chemical work in the chlorophyll molecule itself, of which carbon dioxide has become a part by attachment to the magnesium complex, in that by a regrouping of the valencies the carbon dioxide molecule is rearranged into a form which is suitable for spontaneous decomposition, which occurs in such a manner that the whole of the oxygen of the carbon dioxide is liberated.

It is therefore the knowledge of the complex nitrogen-magnesium group of the chlorophyll which contributes to the determination of its physiological function. In investigating the lesser or non-vital natural pigments, such as indigo blue and madder red, the analytical work was rewarded by the suggestions which were allotted to the organic synthesis by combinations which the imagination of the scientist would certainly not easily have invented. The easily crystallizing and stable plant pigments were the ones which the earlier generation of chemists succeeded in elucidating. What remained enigmatical were the wonderful but easily disappearing decorative and enticing colours of the vegetable kingdom which surround us in the flowers with their gay colours, in fruits and roots, in the barks and the red leaves. The first experiments led us to expect that in those cases we might be dealing with a class of pigments with many members. But here, as in the case of the assimilatory pigments, the laboratory of the plant cell works sparingly with chemical combinations and to some extent disappoints the chemist by the simplicity which is the basis of the natural wealth of colour. A fascinating problem of constitution was presented to me by the flower pigment in the test-tube when, during a holiday, I took a rose from my Zurich garden into the laboratory. The aqueous solution of the pigment prepared from this rose changed with sodium carbonate from red to emerald green; if, however, the anthocyanin solution was first acidified with a drop of mineral acid, then the colour changed with alkali suddenly from red to deep blue. Between the red...
of the acid solution and the blue of the alkali solution there appeared a violet neutral colour, a puzzling phenomenon with a nitrogen-free pigment, but soon explained in the course of our work by the observation that the anthocyanins are quinoid compounds with tetravalent oxygen, that is to say, in Nature widely distributed representatives of the class of oxonium salts, the first examples of which were recognized by Collie in synthetically obtainable compounds of the pyrone series. The isolation of the anthocyanins in a pure state and their analysis are therefore founded upon their basic nature and through the preparation of well-crystallized salts with hydrochloric or picric acid. These acidic compounds are red, the alkali salts are blue, and the violet neutral forms are to be regarded as inner salts, as phenol betaines. Many variations of flower colours are caused solely by the occurrence of these three forms of compound. The neutral and alkaline solutions fade rapidly; this fading is caused by the conversion of the oxonium bases to carbinols, whose alkali salts are yellow and mix with the blue of the colour salt to form green.

The anthocyanins proved to be glucosides, in which the actual pigments, compounds with phenolic hydroxyl groups, are paired with one or two (or even more) molecules of sugars, with glucose, galactose, and rhamnose. The constitution of the sugar-free substances, the anthocyanidins, is elucidated by decomposition through fusion with alkali; the molecule is split into two pieces, into phloroglucin or one of its methyl ethers and an aromatic hydroxy-acid, p-hydroxybenzoic acid, protocatechuic acid or bile acid or a methyl ether of these phenolcarboxylic acids. If the variants are disregarded which are caused by the occurrence of the different methyl ethers, by the variety of the sugar components and their different ways of bonding, most of the anthocyanins can be traced back to only three closely related anthocyanidins, viz. pelargonidin, cyanidin and delphinidin, which are hydroxylated phenyl compounds of the benzopyrilium synthesized by Decker and von Fellenberg—cf. Formulae (III), (IV), (V), and (VI).

The assumptions of these constitutions were confirmed in several ways—by synthesis of cyanidin and pelargonidin from phloroglucinaldehyde as the starting-material, and by the reduction of quercetin with magnesium amalgam to the cyanidin, thus bridging a gap between the flavone pigments occurring throughout Nature and the anthocyanins.

The cyanidin appears in combination with two molecules of glucose, e.g. as pigment of the rose, the cornflower and the poppy; with one molecule of glucose in the anthocyanin of the aster and the chrysanthemum; and with galactose as the pigment of the cranberry. Pelargonidin is the base of the
anthocyanins of the pelargonium, the red sages and of certain dalias; delphinitin gives the deep colours of the larkspur, the woodmallow, the petunia, the bilberry and the grape.

My students were actually planning to extend and complete the investigations of the series of the basic pigments when the World War broke out and destroyed the leisure time of the scientific workshops. The cultures in the flower-beds at Dahlem were neglected, and soon we were carrying baskets full of purple-red asters to the hospitals for the wounded soldiers.

The combination of the synthetic methods with analytical checks was also of decisive importance for the isolation of the anthocyanins. It was of two-fold dependency; at every step from the vegetable starting material to the chemical compound it consisted in quantitative determination of yield and quantitative pursuit of the degree of purity and concentration of the substance. This method of working, and in other respects also the described work in this field will, I hope, contribute still more to the solution of further problems of biochemistry. It seems to me that the most important future
problem of physiological chemistry is that of enzyme research, which has been successfully pioneered in this Laboratory of the Stockholm University by von Euler and his students. Will efforts be successful in making accessible as chemical individuals these most remarkable and active agencies of the animal and plant cell, whom we know only from their effects, and the nature of whose substance is not known at all? May the Swedish Academy of Sciences which has shown such goodwill towards the investigation of vegetable pigments devote its stimulating interest also to the chemical investigation of the enzymes, and may a successful and fortunate professional colleague soon be able on this spot to unveil the secret of the chemical nature of the enzymes!