CAROTENOIDS OF THE UREDIOSPORES OF RUST FUNGI (UREDINALES)

by

Moishe Golubchuk

Being a thesis submitted to the Faculty of Graduate Studies and Research, in partial fulfilment of the requirements for the degree of

Master of Science

University of Manitoba

September, 1952

ACKNOWLEDGMENT

The writer wishes to extend grateful acknowledgement to Dr. J.A. Anderson, Chief Chemist of the Board of Grain Commissioners' Research Laboratory, who made available the facilities of the laboratory for the investigation on which this thesis is based.

He is especially indebted to Dr. G. N. Irvine, Chemist at the Grain Research Laboratory, for his guidance and constructive criticism in the preparation of the thesis.

Dr. T. Johnson, Principal Plant Pathologist of the Dominion Laboratory of Plant Pathology at Winnipeg, provided the urediospore samples of most of the species of rust used in this study. Special thanks are due to him both for the provision of these samples and for the information on the nomenclature and classification of Rust Fungi.

The author expresses his deepest gratitude to Dr. A.D. Robinson, Professor of Agricultural Biochemistry at the University of Manitoba, for his encouragement during the course of this study and for his suggestions on writing up the thesis.

Financial assistance was provided by the Associate Committee on Grain Research of the National Research Council in the form of a grant for the year 1951-1952.

TABLE OF CONTENTS

CAROTENOIDS OF THE UREDIOSPORES OF RUST FUNGI (UREDINALES)

INTRODUCTION

The suggestion for this study was made by members of the Dominion Laboratory of Plant Pathology at Winnipeg. They believed that some insight into the physiology and genetic relationship in wheat stem rust (*Puccinia graminis tritici*) might be gained by further studies of the pigments of the urediospores of normal rust and of its colored mutants.

The majority of scientists will agree that the investigation of a research problem, which may lead to the extension of fundamental scientific knowledge, does not have to be defended on the basis of its potential value as applied to any particular industry. Nevertheless because of the enormous monetary savings that have accrued to the farmers of Western Canada in general and of the "rust area" of Manitoba and Eastern Saskatchewan in particular, as a result of the knowledge gained by the rust research laboratories, it is appropriate to consider the applications of rust research to agriculture.

Plant rust has been known since very early times and there are at least half a dozen references to it in the Bible which designated it by the Hebrew word "yerogown". Most English translations of the Old Testament have used the word mildew for plant rust. In Manitoba wheat stem rust was known as early as 1891. Stem rust epidemics causing heavy destruction have occurred until the late 1930's. In 1916 the loss in wheat production in Western Canada due to rust infection amounted to one hundred million bushels. This loss was estimated at ninety million bushels in 1927 and over eighty-seven million bushels in 1935. The average annual loss in Manitoba and Saskatchewan for the eleven year period 1925-1935 was calculated to be over thirty-five million bushels. The 1916 monetary loss to the farmers of Western Canada was estimated

at over 234 million dollars based on a price of $1.70 per bushel for wheat and on a lowering in quality of one to four grades on the wheat which was produced. The rust resistant varieties of wheat developed by the rust research laboratories were made available to limited areas in 1937 and were available for general distribution throughout Western Canada in 1938. The growing of rust resistant wheats in the so-called "rust area" of Western Canada up to 1943 was estimated to have increased the annual wheat production by over forty-one million bushels and the farm income by over twenty-seven million dollars. This annual monetary saving was over thirteen times the total expenditure on wheat stem rust research by the Canadian Government. The figures quoted in this paragraph are based on a report by Craigie (9) published in 1944. If these figures were extended to 1952, it would be seen that the monetary saving by the farmers of Western Canada due to the introduction of rust resistant varieties of wheat, has amounted to over one billion dollars in the past fourteen years.

Despite the fact that the work of the rust research laboratories had saved millions of dollars in farm income, not a great deal had been determined on the fundamental nature of rust. Craigie (10) in a 116 page article reviewed the findings of the research laboratories on the epidemiology of stem rust. They had found that the urediospores rarely survive the winter and that the initial inoculum consist of wind-borne spores originating in the Northern Mississippi Valley. The rust research laboratories had temporarily overcome the rust menace to wheat by the empirical method of developing, by cross breeding, certain varieties of wheat which were resistant to all the then known races of stem rust. However, in 1938 a new stem rust, Race 15B, appeared as a hybrid on barberry bushes in Pennsylvania. It was first found in Manitoba in Killarney in 1946 although damage was negligible. In 1950 Race 15B first caused serious damage to rust resistant varieties such as Thatcher. In 1951 the Mexican wheat

crops suffered a very destructive epidemic of this new rust and only a severe winter and drought in the United States southern winter wheat belt prevented rust from drifting northward through the Mississippi Valley into Manitoba and Eastern Saskatchewan. At the time of this writing in 1952 reports have come in of stem rust infection in Southern Manitoba and the rust has been tentatively identified as Race 15B. Newer rust resistant varieties of wheat have been developed and they will probably be available for general distribution by 1954. They will be resistant to the new Race 15B rust. However new rust races will probably evolve in time which will attack even the newer rust resistant grains and the process of finding even better resistant varieties, with suitable milling and baking properties, will have to keep pace.

This study on the pigments in rust fungi was envisioned as a small part of the elaborate plan of research, designed to learn as much as possible about the basic nature of cereal rusts. Previous work on the pigments present in wheat stem rust urediospores by Newton and Johnson (75) indicated that normal rust spores contain two classes of pigments, a carotenoid pigment located in the cytoplasm of the spore and a red pigment believed located in the spore wall. These investigators were unable to extract the spore wall pigment but were able to extract the carotenoid pigment with carbon disulphide as well as with acetone. On the basis of this study Johnson (42) postulated that normal rust contains both an orange carotenoid pigment and a red spore wall pigment; while the orange mutant contains only the orange pigment, the greyish-brown mutant contains only the red pigment and the white mutant contains neither pigment. Newton, Johannson and Johnson (76) later reported that they were able to extract varying amounts of carotenoid pigments from the urediospores of normal rust and of each of its color mutants.

It was thought that the present study of the same subject would be more informative because of the development of the modern photoelectric

spectrophotometer and the use of chromatographic adsorption analysis techniques. However, the Dominion Laboratory of Plant Pathology at Winnipeg was unable to produce any of the stem rust mutants except a small amount of the greyish-brown. It was thus decided to extend the study by investigating the color of the urediospores of other rust species, particularly with reference to their carotenoid content. The pigments of the urediospores of the following rust species were investigated: stem rust of wheat (Puccinia graminis tritici) and its greyish-brown mutant, stem rust of oats (Puccinia graminis avenae), stem rust of barley (Puccinia graminis hordei), crown rust of oats (Puccinia coronata avenae), leaf rust of wheat (Puccinia triticina), and flax rust (Melampsora lini).

The plant rusts belong to the order Uredinales of the basidia fungi class (Basidiomycetes) of the Thallophyta division of the plant kingdom. The six species investigated are members of the Pucciniaceae family and the Melampsoraceae family. Moreover the first five species listed belong to the genus Puccinia which is the most important of all the genera in the order Uredinales. The last species listed, falls into the Melampsora genus. This is in an entirely different family than that in which the species of the Puccinia genus lie. Some species of plant rusts are known to produce five different kinds of spores in their life history. However the urediospores or "summer spores" give the bright rust red or orange specks which one notices on plants infected with rust. It is therefore logical to begin an investigation of the color of rusts by studying their urediospores.

LITERATURE REVIEW

PIGMENTS IN RUST FUNGI

Although it has been known for some time now that the urediospores of the rust fungi contain carotenoid pigments, the pigments of only one species of cereal rust have been partially identified. As early as 1886 the German botanist, Bachmann (2), reported on a general investigation of fungal pigments, using a microscope and a spectroscope as his principal research instruments. He investigated five species of rust fungi including Puccinia coronata or crown rust. It was known in his day that the pigments of the individual fungi usually completely predominate either formed as an inner constituent or imbedded in the cell wall. In his investigation of fungi other than the Uredinales, he found that the cell wall imbedded pigments were in several cases a dihydroxyquinone and in other cases probably anthracene derivatives. He also reported that three species of the same genus contained one and the same cell wall imbedded pigment, as identified by its absorption in the spectroscope, despite their outward color differences. In the case of rust spores Bachmann believed that the pigments were bound in fat or lipid material within the cells. He observed under the microscope that these urediospores contain yellowish-red globules which appear to be pigments dissolved in lipid material. Moreover these yellowish droplets could be easily dissolved in ethyl ether and a yellowish red pigment could be taken up in petroleum ether after saponification with caustic soda. Bachmann's description of these rust spore pigments indicated that they were hydrocarbons such as carotene.

Two French chemists, Bertrand and Poirault (3) before the end of the last century investigated the coloring matter of spores. Included in this study were two species of the Coleosporiaceae family and one of the Melampsoraceae

family of the rust fungi. They observed under microscopic examination that the spores contained droplets of a "carotene oil" which dried up after several days to form intense red orange crystals. They stated that these crystals were not carotene but probably a supersaturated solution of a sterol in the oil. On the basis of their experiments, they concluded that the pigments of these rust spores were probably carotene. For comparison of absorption spectra, a carotene extract of carrots, obtained from Professor Arnaud, was used. Although Professor Arnaud was the first to establish that carotene was a hydrocarbon, it was not until the 1930's that the carotene fraction from carrots was shown to be a mixture of isomers. Therefore the statement by Bertrand and Poirault that their absorption spectra, obtained with spore pigments, was exactly superposable with the absorption spectra of pure carotene, must be considered together with the state of knowledge of the carotenoid pigments at that time.

The two papers previously mentioned by Newton and Johnson (75) and Newton, Johannson, and Johnson (76) are the only ones recorded in the literature which deal with pigments of the urediospores of the rust fungi species Puccinia graminis tritici or wheat stem rust. In the 1927 paper they reported that the carotenoid pigment, extracted with carbon disulphide or acetone, was probably carotene. They used a Hilger constant deviation spectrometer and obtained a band with the red end being 537 mu for normal rust and 536 mu for the orange mutant. They were unable to obtain the other end accurately. On the basis of this band they excluded lycopin from being in the rust spores. Xanthophyll was excluded as the possible carotenoid pigment, since 80% methyl alcohol failed to extract any pigment. Rhodoxanthin was also eliminated as a possibility on the basis of lack of pink color in alcohol or acetone. In their 1935 paper Newton et al (76) merely stated that

the spectral distribution curves of the urediospores of normal wheat stem rust and its mutants indicated that the pigments were carotenoid in nature and in most instances apparently consisted chiefly of carotene.

Lederer, the French organic chemist, (64, 65) was the only investigator of the rust spore pigments who used the chromatographic adsorption analysis technique and a spectrometer with monochromatic light. He extracted with chloroform the pigments from 200 mg. of the urediospores of Puccinia coronifera obtained from the leaves of oats. No trace of xanthophyll could be found but, after saponification with alcoholic potash, the alcoholic potash solution was reddish while the petroleum ether solution was yellow. After filtering the petroleum ether solution over lime, a red zone was formed containing 80 γ of γ-carotene with absorption maxima of 491, 458 and 430 mu in petroleum ether. The filtrate was yellow and gave absorption maxima 481 and 450 mu in petroleum ether. Lederer inferred from the maxima that this yellow filtrate was a mixture of α and β-carotene with a total concentration calculated to be 50γ. The pigment in the alcoholic alkali precipitated as rose flakes on the addition of water. These flakes were insoluble in petroleum ether. However after acidification with dilute acetic acid, the pigment became soluble in petroleum ether. The color in petroleum ether was red-orange while in carbon disulphide it was violet. Lederer was unable to obtain the absorption maxima of this third pigment but he believed it to be an acid pigment similar to the acid pigment of Torula rubra.

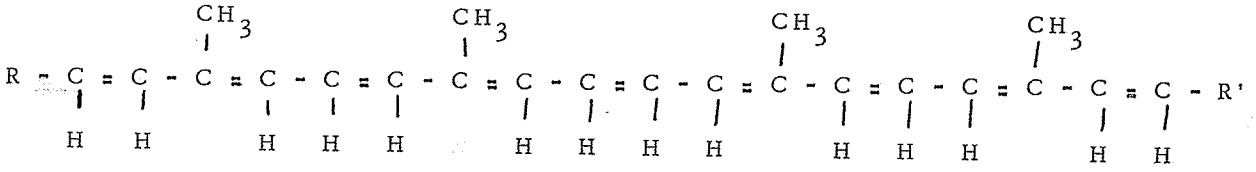
There is some disagreement between plant pathologists on the nomenclature of rust fungi. Plowright (31) divided the crown rusts into two species, one called Puccinia coronata and the other Puccinia lolii or Puccinia coronifera. However present day plant pathologists including those at the Dominion Laboratory of Plant Pathology at Winnipeg dispute this division and continue to call crown rust Puccinia coronata. Thus Puccinia coronifera

investigated by Lederer is actually crown rust of oats, the same species as the crown rust obtained from the Winnipeg rust research laboratory labelled as Puccinia coronata avenae. Accordingly from a review of the literature it is seen that of the six species of rusts investigated by us, only two species have been previously investigated. Furthermore only Lederer's work with crown rust has been performed with the knowledge of the modern methods of carotenoid research.

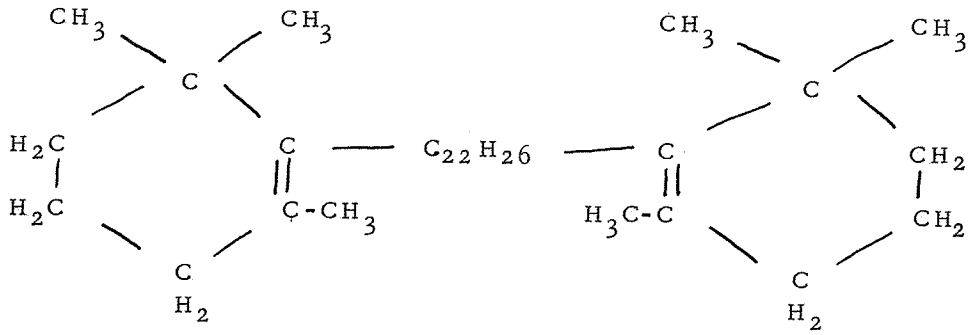
THE NATURE OF CAROTENOID PIGMENTS

Carotenoid pigments have been known ever since Wackenroder in 1831 isolated carotene from the roots of the carrot (Daucus Carota). Yet for practically one hundred years research in the field of carotenoid pigments made relatively slow progress. When Palmer's monogram on carotenoid pigments was published in 1922 (78) only seven natural carotenoids had been isolated and their structural formulae were still largely undetermined. However, research on carotenoid pigments has been greatly accelerated during the last two decades. At present over eighty naturally occurring carotenoids have been isolated from both plant and animal sources (35). Two factors were largely responsible for the impetus given to carotenoid research. The first was the elucidation of the constitution of β-carotene by Karrer and his co-workers in the period 1929 to 1931 (45, 48, 52) and its establishment as a pro-vitamin A. The second factor was the re-introduction of Tswett's chromatographic adsorption analysis technique (90) in 1931 by Kuhn, by Karrer, and by Zechmeister, for the separation of carotenoid mixtures. While the discovery of the relationship of β-carotene to vitamin A stimulated renewed interest in the study of carotenoids, the stratographic method of separation enabled rapid progress to be made in this field.

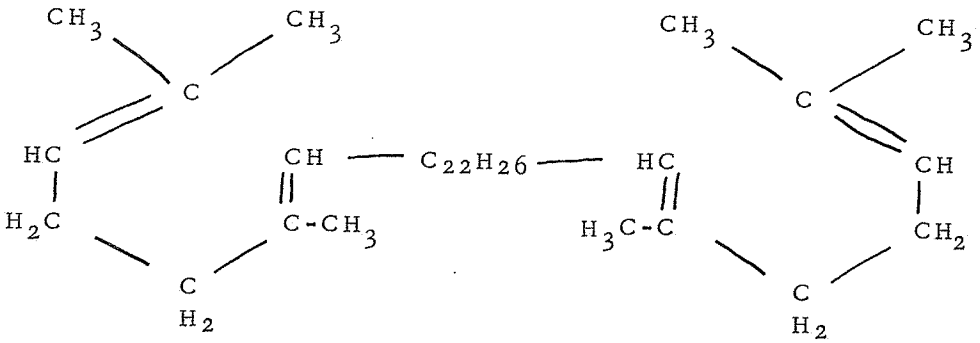
The carotenoids may be defined as a class of fat soluble, nitrogen free polyene pigments consisting wholly or chiefly of long acylic chains of carbon atoms, united in an uninterrupted system of conjugated double bonds. These pigments vary in color from light yellow to deep red or even purple. The central structure of the majority of the carotenoids is the methylated 18 carbon conjugated polyene radical $C_{22}H_{26}$.



The various carotenoids differ according to the nature of the R and R' groups. This can be illustrated by glancing at the structure of β-carotene, the most important carotenoid, and lycopene, the pigment to which all other carotenoids may be formally related.

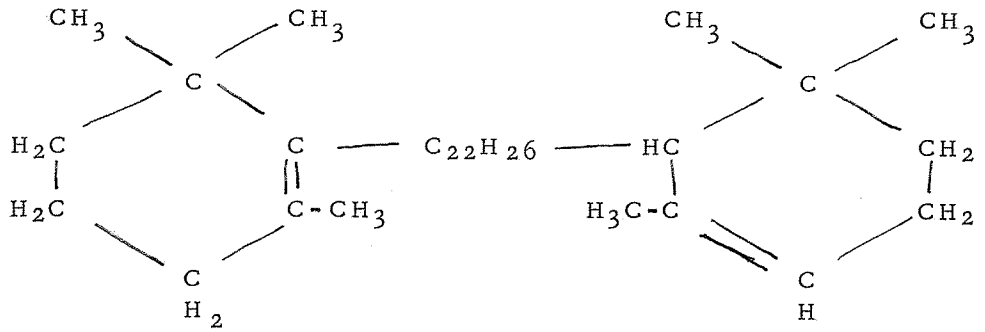


β-carotene (48, 52)

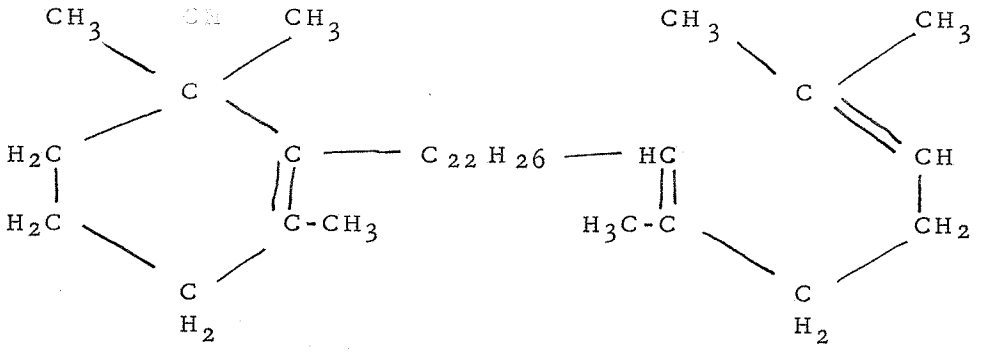


lycopene (53, 63)

In the case of β-carotene both R's consist of β-ionone residues while in lycopene the two R's are so-called lycopene residues. However both R's are not necessarily identical in the carotenoids. This is illustrated by α-carotene and by γ-carotene.

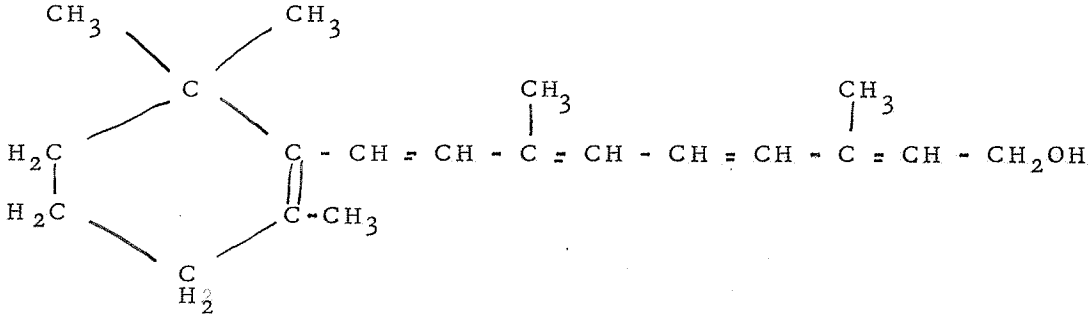


α-carotene (47, 50)



γ-carotene (57)

γ-carotene, as seen in the above formula, consists of one half molecule of β-carotene and one half molecule of lycopene. The β-carotene half of the molecule makes γ-carotene pro-vitamin A since it can theoretically break down to give a molecule of vitamin A of the following formula (38, 49):



Lycopene and the carotenes whose structural formulae were given above all have the empirical formula $C_{40}H_{56}$ and are structural isomers.

Cis - Trans Isomerism of the Carotenoids

In addition to structural isomers the carotenoids also contain geometric or cis-trans isomers. Since the phenomenon of cis-trans isomerism has been found to be of considerable value in interpreting investigations on carotenoid pigments, it would be worthwhile to review some of the literature on this subject. Due to pioneering investigations of J. Wislicenus in 1887 (44) and the numerous observations in organic chemistry since then, we know that any ethylene derivative because of the double bond, may exist in two stereoisomers. The majority of carotenoids contain the central methylated 18-carbon conjugated radical $C_{22}H_{26}$ and thus have at least nine double bonds. From simple theoretical considerations they could exist in at least 512 different cis-trans isomeric forms. However Zechmeister, Pauling and their collaborators (79, 108) concluded that owing to steric hindrance only four to seven of the double bonds are available for isomerization, with the number of possible stereoisomers ranging from 10 to 128. β-carotene itself, has twenty possible stereoisomers.

On the basis of x-ray analysis (72) it was first thought that all natural carotenoids possess an all trans-configuration. An all trans-configuration would possess the smallest free energy and would therefore have the greatest stability. Herzig and Faltis in 1923 (40) observed the rearrangement of bixin to give two isomers. Karrer and his associates in 1929 (56) interpreted this as a cis-trans isomerization. In 1935 Gillam and El Ridi (19) reported that on re-chromatographing homogeneous β-carotene on alumina two zones formed, the upper zone being β-carotene and the lower zone a new pigment pseudo-α-carotene. Gillam and his associates studied this phenomenon over a three year period (20, 21) and

attributed the change to a re-arrangement which took place in the Tswett column under the influence of the adsorbent. They showed complete reversibility of the reaction: β-carotene ⇌ pseudo-α-carotene. They considered the rearrangement to be possibly due to migration of double bonds or to cis-trans isomerization. However Zechmeister and his collaborators (81, 99, 100, 105, 106) believe that this phenomenon is due not to the influence of the column itself but to a spontaneous isomerization in the solution of the pigment. They stated that their work indicated a partial trans-cis shift and explained the above reversible conversion, which had nothing to do with the chromatographic method. Their experiments showed that polyene pigments could be isomerized by dissolution, by melting the crystals, by treatment with iodine or with acids and other operations. These isomers with a partial cis-configuration were called neo-isomers. Zechmeister (81) obtained nine neo-isomers as well as trans-β-carotene from an isomerized solution of β-carotene. These neo-isomers had absorption maxima in petroleum ether ranging from 433 and 465.5 mu to 450 and 481 mu. Only one of these neo-isomers was obtained in a crystalline form. Zechmeister and Polgar's investigations (99, 100) showed that the isomerization products often contain new maxima in the ultra violet spectra which they termed "cis peaks".

Although it was first assumed that the neo-isomers with partial cis-configurations do not occur in nature, Le Rosen and Zechmeister (69) have discovered that pro-lycopene is the main pigment of a certain type of tangarine tomato. They believe pro-lycopene is a natural occurring stereoisomer of lycopene. The chromophoric system of pro-lycopene is said to probably contain 5 to 7 cis double bonds. About the same time Zechmeister and Schroeder (103) reported the isolation of pro-γ-carotene, a natural occurring stereoisomer of γ-carotene. They suggested that the chromophoric grouping of pro-γ-carotene

has 6 or 7 double bonds of trans and 5 or 4 of cis configuration. The prefix "pro" has been attached to several natural occurring isomers having a partial cis-configuration while the prefix "neo" has generally been used for the artificial isomers. The absorption maxima of the two pro-carotenoids discussed above differ considerably from that of the starting material. On the other hand the absorption maxima of the neo-isomers generally are only moderately lower than that of the all trans carotenoid.

Since very few of the neo-isomers have been isolated in a crystalline form much of the work on cis-trans isomerization requires further confirmation. Nevertheless as long as this concept is of value in interpreting some of the phenomena encountered in carotenoid research, it is probably correct to think in these terms.

Relative Solubilities of the Carotenoids

The carotenoid pigments are soluble in fats or lipoids and for this reason they are also called lipochromes. They are all soluble in varying degrees in the different organic solvents such as carbon disulphide, chloroform, benzene, ethyl ether, petroleum ether, acetone, methanol, and ethanol. The carotenoids which are entirely hydrocarbon in nature and those which contain one hydroxyl group, are more soluble in the first three solvents. The carotenoids with two or more hydroxyl groups are more soluble in the last two solvents. The carotenoids also show characteristic solubilities when placed in a solution of two immiscible solvents such as petroleum ether and 90% methanol. Those pigments which are found predominately in the upper petroleum ether layer are called epiphasic pigments, while those which are found in the lower aqueous methanolic layer are called hypophasic pigments. The partitioning of the carotenoids between two immiscible solvents has become one of the major steps in the isolation of these pigments from plant or animal sources. The use of this method is largely due to the pioneering efforts of Willstatter and Stoll (95).

According to Lederer (66), on partition between 90% methanol and petroleum ether, the epiphase may contain carotenoid hydrocarbons, carotenoids having a single atom of oxygen either as hydroxyl or carboxyl, esters of xanthophylls and carotenoids with methoxyl groups. The hypophase may contain the free xanthophylls having more than one hydroxyl group, astacine and several of the esters of the acids. The separation of hydrocarbons and xanthophyll esters can be accomplished by saponification. The monohydroxy carotenoids can also be separated from the hydrocarbons by increasing the concentration of the methanol.

According to the literature there are at present only three carotenoids of totally determined structure that contain single hydroxyl groups. They are lycoxanthin (97), rubixanthin (59), and cryptoxanthin (60). On partition between 90% methanol and petroleum ether, they are found in the epiphase. However, using 95% methanol, they are all reported to be hypophasic. There is not complete information available on the relative solubilities of other carotenoids believed to contain a single hydroxyl functional group. Celaxanthin (70) is reported to be entirely epiphasic on partitioning between 85% methanol and petroleum ether. However with 95% methanol, the lower layer is also colored. Myxoxanthol (36) is reported to be entirely epiphasic on partition between 90% methanol and petroleum ether but no information is given for 95% methanol. Unlike celaxanthin, gazaniaxanthin (84) is reported to be entirely similar to the three known monohydroxy carotenoids in the partition tests.

The only mono-carbonyl carotenoid whose constitution is known with some degree of certainty is myxoxanthin (37). It is entirely epiphasic on partitioning between 90% methanol and petroleum ether. On the other hand the dicarbonyl rhodoxanthin (58) colors both layers with the same solvents.

Chromatographic Adsorption Analysis as Applied to Carotenoids

The methods of separating the carotenoid pigments based on the differences in solubility between petroleum ether and methanol were not adequate for the complete separation of carotenoid pigments. The introduction of methods for separating substances based on the phenomenon of adsorption has proved of immense value in the field of carotenoid pigments. Adsorption has been defined as the concentration of a gas, liquid, solid or the solute or solvent of a solution on the surface of a liquid or solid. Column chromatography is based on the adsorption of a solute on a solid adsorbent. The adsorptive affinities of such substances as polyene pigments are greatly influenced by small differences in molecular structure. By choosing the proper adsorbent the various groups of pigments separated on the basis of their relative solubility, can be spatially separated on an adsorption column. The pigments with the strongest adsorptive capacity will be held at the top of the column. The less strongly adsorbed pigments will be held at various lower levels. The adsorptive process however is not allowed to remain a static situation. A dynamic equilibrium is set up between the solid adsorbent and the liquid phase. By washing with further solvent the strongly adsorbed pigments will displace those that do not have as great an adsorption affinity. Using the proper amount of development one can obtain a clear separation of the pigments into colored bands with colorless zones between each pigment layer.

It is unnecessary to give a detailed account of the chromatographic method since many excellent works have been written about it. The first problem encountered in applying column chromatography to carotenoids is the determination of the best possible combination of solvent and adsorbent. For chromatographing epiphasic pigments Karrer reports (46) that petroleum ether or a mixture of petroleum ether with benzene is commonly used. In a review

article Zechmeister (107) states that petroleum ether-acetone mixtures have been frequently used for epiphasic pigments. However the use of petroleum ether-acetone mixtures is generally restricted to separating out single epiphasic pigments from chlorophyll or xanthophyll mixtures such as in the determination of the pro-vitamin A content of vegetables (1). Calcium hydroxide was first used for the separation of carotenoid hydrocarbons by Karrer and Walker in 1933 (46). Calcium hydroxide and alumina are the two main adsorbents reported by Karrer and Jucker (46) for the separation of the hydrocarbon pigments. Strain (87) obtained very good results in the separation of carotenes by the use of a specially prepared brand of magnesium oxide. This micron brand magnesium oxide was also found to be excellent in the separation of the epiphasic rust spore pigments, in the present work.

The actual position of the carotenoid pigments on the adsorption column can give relative information as to identity of the pigments. The adsorptive affinities of the different carotenoids bear a definite relationship to their chemical structure. Thus for two pigments having the same structure otherwise, that containing the largest number of hydroxyl groups, carbonyl groups, esterified hydroxyl groups or double bands is adsorbed more strongly. The effect of the functional groups on the strength of adsorption decreases in the order just mentioned. α-carotene with eleven double bonds, only ten of which are conjugated, is adsorbed less strongly than β-carotene which contains eleven conjugated bonds (51). However β-carotene is found below γ-carotene on the chromatographic column (51). γ-carotene with twelve double bonds was reported by Kuhn and Brockmann (57) to be found above β-carotene but below lycopene in the chromatogram on calcium hydroxide or alumina. A colored photograph of the carotenes and lycopene on a chromatogram is shown by Karrer (43). According to the summary in Table 6

of Karrer and Jucker (46), the mono-hydroxy compounds such as cryptoxanthin, rubixanthin and lycoxanthin are adsorbed more strongly than lycopene. However there are exceptions to this general scheme. Cryptoxanthin is reported by Le Rosen (68) to be adsorbed above lycopene on calcium carbonate or alumina but below it on calcium hydroxide. Zechmeister and Schroeder (104) stated that cryptoxanthin was adsorbed below lycopene but above γ-carotene on calcium hydroxide. Gazaniaxanthin was however more strongly adsorbed than lycopene on both calcium hydroxide and calcium carbonate. This mono-hydroxy-γ-carotene was adsorbed much above cryptoxanthin. Thus in determining the relative position of an unknown pigment on the chromatogram with reference to known pigments one would do well to make these determinations on a number of suitable adsorbents.

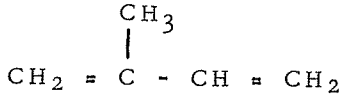
Workers in carotenoid research are in universal agreement that the use of a mixed chromatogram is the best method of identifying small amounts of carotenoid pigments. This method of three tubes or "mischchromatogram" is adequately discussed by Zechmeister and Cholonoky (98). A mixed chromatogram is used when the pigment under investigation appears to be similar in absorption maxima to a given pigment described in the literature. Solutions of the unknown pigment and of the known pigment in a given solvent are adjusted to the same intensity. Three identical adsorption columns are filled with a suitable adsorbent. On two tubes solutions of the two pigments to be compared are adsorbed. On the third column a mixture of equal parts of the unknown and the known pigment are adsorbed. The three tubes are then connected to the same source of vacuum and the same volume of liquid is allowed to pass through the columns. The three columns are developed with equal volumes of fresh solvent and any separation in the "mixed" tube is noticed. If there is exactly the same number and same appearance of the zones,

then the pigments are said to be identical. If the "mixed" chromatogram shows two distinct zones while the others have only one zone each, then the pigments are evidently dissimilar.

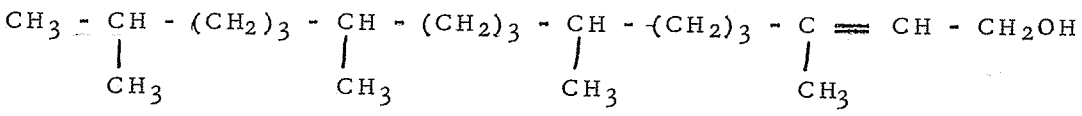
Phytochemical Synthesis of Carotenoids

The majority of textbooks on plant biochemistry usually contain a section on the biogenesis of carotenoids in plants. Gisvold and Rogers (22) text on the "Chemistry of Plant Constituents" and the newer one by Bonner (4) both gave detailed accounts of the possible mode of formation of carotenoids in the plant kingdom. According to Karrer (46) no experimental evidence exists with regard to the phytochemical synthesis of carotenoids. Karrer and his associates (52) considered the possibility that the pigment lycopene may be formed (in tomatoes) from phytyl aldehyde by a benzoin condensation or by a pinacol reduction followed by dehydrogenation. Although very little has been determined with regard to carotenoid synthesis in the phanerograms, still less information was deduced about carotenogenesis in the cryptograms. Recently however Goodwin and his associates at the University of Liverpool have published a series of papers (17, 18, 23, 25, 26, 27, 28) on Carotenogenesis in the fungus species Phycomyces blakesleeanus. The following discussion on the phytochemical synthesis of carotenoids will review the literature and summarize present hypotheses on this subject as well as point out that any individual cryptogram or phanerogram synthesizing a carotenoid such as γ-carotene could very easily synthesize both lycopene and β-carotene by the very same mechanism.

The carotenoids are conveneintly classified as terpene derivatives. The terpenes and their derivatives are considered to be a group of naturally occuring plant materials which may be conceived as derivatives of the five carbon branch chain compound isoprene.



While the simplest terpenes may be considered as condensation products of two isoprene groups, the forty carbon carotenoids are thought of as containing eight of these isoprene type units. For this reason the carotenoids have been classed as tetraterpenes. Hall (32) reviewed some of the theories advanced to explain the synthesis of compounds containing isoprene units. In the case of the forty carbon carotenoids some of the theories are based on the fact that they all (except Rhodoxanthin) have a double bond in the exact centre of the molecule. If it is assumed that the carotenoids are built up from a condensation of isoprene type units, then the direction of polymerization is apparently reversed in the centre of the molecule. This symmetry in the polyene chain is said to be an indication that the final carotenoid molecule is formed from a condensation of two isoprene containing groups of twenty carbon atoms each. The existence of two such twenty carbon groups in nature viz. vitamin A and phytol, lend support to this hypothesis. Since animals can form up to two molecules of vitamin A from β-carotene it is concluded that the first point of physiological attack is the centre of the molecule. The formula for vitamin A and some of the pro-vitamin A carotenes has been illustrated previously. The formula for phytol is given below:



No one has suggested seriously the possibility that the carotenoids may be formed in plants from vitamin A by a reverse process to that in animals. However Willstatter and Mieg (94) were the first to suggest the possibility that phytol might be a precursor in carotenoid synthesis. This was based on the fact

that carotenoid synthesis is generally active in green leaves in which considerable amounts of phytol are being formed as part of the chlorophyll molecule. Since the carotenoids were believed to be synthesized in the organs in which they occur and since such non-chlorophyll containing parts as carrot roots contain carotenoids, the carotenogenesis from phytol appeared to necessarily have an independent mechanism than the chlorophyll synthesis. This view ignored the possible translocation of carotenoids in plants. The fact that phytol could be synthesized from isoprene in vitro has been established by the work of three different investigating teams. By the use of the Barbier and Bouveault Synthesis citral has been formed from isoprene. Hexahydropseudoionone has been formed from citral by the Tiemann and Krieger Synthesis. In 1928 Fischer and Löwenberg (14) synthesized phytol from hexahydropseudoionone to complete the synthesis of a twenty carbon molecule from a five carbon precursor. Bonner (4) suggested that phytol represents a reduction product of some common precursor of both phytol and the carotenoids rather than the precursor itself. He stated that the final carbon skeleton of the carotenoids could be produced by the combination of two twenty-carbon precursors. The condensation of two appropriate vitamin-A-like structures would form β-carotene while the condensation of the appropriate twenty carbon open chain molecule would form lycopene. γ-carotene could be formed by the condensation of one molecule of the β-carotene precursor combining with one molecule of the lycopene precursor. On the basis of this hypotheses if γ-carotene was formed by a certain plant family for a definite physiological purpose, one would be very likely to find smaller amounts of both β-carotene and lycopene present.

The work by Goodwin and his associates may cast some light on the biogenesis of carotenoids in the cryptograms including the rust fungi. For

their experiments they used the fungal species Phycomyces blakesleeanus since it could be easily cultured on artificial media in contrast to the strictly parasitic rust fungi. As they had previously determined that β-carotene was the predominant pigment they endeavored to determine the conditions governing its synthesis in this fungus. They found (18) that the β-carotene production of cultures grown in the dark was half that of cultures grown in the light. When the cultures grown in the light were placed in the dark they continued to produce β-carotene at the "light" rate. In this paper they also stated that β-carotene was not formed in the absence of nitrogen. In a further paper (25) they reported that in the presence of sub-optimal amounts of glucose, the naturally occurring amino acids valine and leucine stimulated carotene synthesis. They thought it was very probable that both valine and leucine could provide the repeating unit for carotenogenesis. They believed that the reason this effect was not observed in the presence of large amounts of glucose was because glucose itself could provide adequate amounts of the building units. They postulated that since valine and leucine contain terminal dimethyl groups these could theoretically give rise to five-carbon building units such as β-methylcrotonaldehyde. In a further note (63) they reported that their experiments showed that acetate is probably not a specific carotene precursor. Their latest experiments (28) led them to re-interpret some of their previous results. They believed that their earlier inability to observe carotenogenesis in Phycomyces mats, dissimulating glucose in the absence of nitrogen, was not due to the latter factor but to the pH of the media. They stated that the reason valine and leucine stimulated carotene synthesis in the presence of sub optimal amounts of glucose, was because they yielded on deamination a carbon residue readily incorporated into the molecules. However in their more recent experiments they found that mats of Phycomyces

dissimilating glucose could synthesize more carotene in the absence of assimiliable nitrogen than in its presence. It would thus appear possible that carotenoids are formed in fungi from simple plant sugars such as glucose. In this process the host plant would be losing much of its own food supply. However more work will have to be done before one can state the basic five carbon branch chain units are derived from glucose.

The colorless polyene phytofluene described by Zechmeister (102) is known to accompany carotenoids in parts of higher plants which contain little if any chlorophyll. It has also been shown to occur in three classes of fungi (27). Phytofluene is a colorless tetraterpene which has a bright bluish green fluorescence in ultraviolet light. It contains seven double bonds and gives absorption maxima in light petroleum ether at 367, 348 and 331 mu. Bonner et al (5) in 1946 first suggested that phytofluene is an intermediate in the biogenesis of carotenoids. They worked with mutant strains of the carotenoid producing yeast, Rhodotorula rubra, using the genetic block method. They postulated the course of carotenoid synthesis in Rhodotorula as following a pathway in which colorless precursors are transformed first to the true tetraterpene phytofluene and from there to the carotenes and finally to the xanthophylls.

Possible Function of Carotenoids in the Rust Fungi

Very little is known about the functions of the carotenoid pigments in rust fungi but some of the ideas on the possible function of these pigments in the plant kingdom as a whole may be applicable to the Uredinales. Despite numerous investigations on the significance of carotenoids in plants no general agreement as to their functions exists. The earliest investigators such as Willstatter and Stoll (96) tried to demonstrate a possible influence of carotenoids on the processes of respiration and assimilation but without success.

More recent investigations have attempted to show the influence of carotenoids on sexual reproduction especially in the cryptograms. Craigie (8) proved that both the sunflower rust fungus (Puccinia helianthi) and the stem rust fungus of wheat (Puccinia graminis tritici) resemble the simpler mushrooms and toadstools in having spores of two sexes, (+) and (-). The carotenoids may therefore have some function relating to sex in the rust fungi as in other cryptograms.

Since the carotenoids occur in the chloroplasts in association with chlorophyll it was assumed that they play some role in photosynthesis. Actually no clear indication of the role of carotenoids in photosynthesis is known. Recently Dutton and Manning (12) working with the diatom Nitzchia and Emerson and Lewis (13) working with the green algae Chlorella have stated that some of the light absorbed by the carotenoids appeared to be available for photosynthesis. Noack (77) believed that both carotene and xanthophyll fulfilled the role of light filters for chlorophyll. Went (91) suggested that they were more likely to function as protectors for labile cell enzymes. On the other hand Frey-Wyssling (16) regarded the carotenoids, expecially the xanthophylls, as being functionless metabolic waste products. Spoer (86) has stated that plant material containing only yellow plastids lack photosynthetic activity but show phototropic responses. Although the carotenoids may have no physiological function, on account of their color they are very likely to play a definite role in attracting insects and other animals, which are factors in the fertilizing of the plant and in the dispersal of the seeds. According to Buller (6), in the rust fungi it is primarily in the pycnidial pustules, if at all, that these polyene pigments are useful in attracting insects. He further stated that the presence of the carotenoid pigments in the various spores and in the mycelium generally indicated that they are of importance in some unknown way for the general metabolism of

rust fungi. He did not overlook the possibility that they may merely be waste products. Hanna (33) studying the nature of rust resistance in wheat suggested that photosynthetic processes take place more rapidly in cells of wheat varieties having a high content of the chloroplast pigments (chlorophyll, carotene, xanthophyll). This was believed to furnish conditions suitable for the growth of rust mycelium. If this was true it would indicate that the carotenoids had a definite physiological function in the rust spores. The work of Karrer and his associates (54) has shown that the epoxides of carotenoids which contain a β-ionone ring are converted to isomeric furanoid oxides by dilute acid and at the same time a small amount of the epoxide loses its oxygen and reverts to the original carotenoid. Since the epoxides have been shown (46) to be widely distributed in plants and can revert to carotenoids by loss of oxygen it was suggested by Karrer that they may act as oxygen carriers in plants. The possible participation of the carotenoid pigments of Rhodotorula rubra in the respiration of the cells has also been reported (71).

There are a number of recent papers on the possible relationship between the carotenoids and the "befruchtungstoffe" or conception factors which deal with the motility of the gametes and conjugation. Fox and Emerson (15) observed in the phycomycete Allomyces that the sexual plants bore orange male and colorless female gametangia. The orange pigment in the male cells consisted almost exclusively of γ-carotene. Fox and Emerson suggested that γ-carotene played a part in the reproduction of these fungi. However Karrer and his co-workers (55) claimed that there was no certain foundation for this supposition. Kuhn, Moewus and Jerchel (62) reported that they determined the part played by carotenoids in the reproduction of the unicellular algae Chlamydomonas eugamentos f. simplex. When the algae of this species were

irradiated with red light the gametes became motile but became capable of copulation only on further irradiation with blue or violet light. After filtering a suspension of motile gametes the cell free aqueous filtrate was found capable of motilizing non-irradiated quiescent gametes. A chemical agent secreted by the motile gametes was believed to be contained in this filtrate. This solution was observed to have an absorption spectrum very similar to crocin. Crocin itself was found to be capable of motilizing the non-irradiated gametes by inducing the formation of flagella (minute tails for propulsion). Mixtures of trans- and cis-crocetin methyl ester were said to be required to convert the motile but still infertile gametes into male and female gametes. The ratio of cis- to trans-crocetin methyl ester were said to determine whether sperm cells or egg cells were formed.

The Use of the Spectrophotomer and Absorption Data in Carotenoid Studies

The spectrophotomer is a combination of a photometer with a monochromator. A photometer may be a visual photographic or photoelectric instrument for measuring absolute or relative light intensities. The monochromator is an instrument for the isolation of light of a single wave length. The older instruments could not do this but the modern photoelectric spectrophotometers such as the Beckmann DU come very close to isolating the light of a single wave length.

The spectrophotomer is extremely useful in carotenoid study both in identifying and quantitatively estimating pigments in amounts too small to be crystallized (e.g. to the order of several micrograms). Each carotenoid pigment in solution in solvents such as carbon disulphide or petroleum ether usually exhibits two or three characteristic absorption bands. Their position can be determined by the modern photoelectric spectrophotomer to within 0.5 mu and represent the wave lengths of the absorption maxima. There has been quite good

agreement by numerous investigators on the absorption maxima of the crystalline individual pigments of all trans-configuration. However one has to use caution in comparing the absorption maxima for crystalline pigments with those obtained from non-crystalline pigments separated by chromatography. Carotenoids with otherwise the same structure but differing by one hydroxyl group have closely similar absorption maxima. The fact that the cis-trans isomers of the same carotenoid pigment differ slightly but significantly from the all trans-form makes it impossible to determine from the absorption data alone whether one is dealing with stereoisomers or with carotenoids differing by one hydroxyl group. The statement by Heilbron and Lythgoe (36) that myxoxanthol is spectroscopically identical with both γ-carotene and rubixanthin, although they list absorption maxima differing from one to six mu, might be regarded as emphasizing this fact.

Solvent	γ-Carotene	Rubixanthin	Myxoxanthol
Carbon disulphide	533.5, 496.0, 463.0	533.0, 494.0, 461.0	529.0, 494.0, 464.0
Chloroform	508.5, 475.0, 446.0	509.0, 474.0, 439.0	508.0, 474.0, 441.0
Light petroleum (b.p. 70°-80°)	495.0, 462.0, 431.0	494.0, 464.0, 432.0	495.0, 465.0, 431.0

Besides the natural occurring rubixanthin and the synthetic myxoxanthol there is another mono-hydroxy-γ-carotene, gazaniaxanthin. Gazaniaxanthin was first isolated in Portugal from Gazania rigens flowers by Schon (84) in 1938. Five years later Zechmeister and Schroeder (104) isolated it from California Gazania rigens flowers and also determined the absorption maxima. A comparison of Schon's and Zechmeister and Schroeder's data is given below:

	Schon	Zechmeister and Schroeder
Carbon disulphide	530, 495.5, 463 mu	531, 494.5, 461 mu
Benzene	508, 476.5	509, 476, 447.5
Ethanol	494.5, 462.5	494.5, 462, 434.5
Pet. Ether	494, 461, 433.5	494.5, 462.5, 434.5

Zechmeister and Schroeder converted gazaniaxanthin into trans-cis isomers by the usual methods. They found that the longest wave length maxima in petroleum ether was located in the range 489.5 to 484 mu for all the isomers whereas it was 494.5 for the natural occurring gaxaniaxanthin.

Rubixanthin was first discovered in 1934 by Kuhn and Grundmann (59). Both Schon and Zechmeister and Schroeder reported its presence in Gazania rigens. A comparison of Kuhn and Grundmann's absorption maxima with those of Schon's is given below:

	Kuhn and Grundmann	Schon
Carbon disulphide	533, 494, 461 mu	533.5, 498, 465 mu
Pet. Ether	495.5, 463, 432	496, 461

γ-carotene itself was first discovered in 1933 by Kuhn and Brockmann (57) who isolated it from crude carotene where it forms 1/10 of 1%. It was also isolated by Schon and by Zechmeister and Schroeder from Gazania rigens along with the monohydroxy-γ-carotenes. Schroeder (85) isolated it from Mimulus longiflorus flowers which is said to be its best known source. A comparison of Kuhn and Brockmann's absorption maxima with Zechmeister and Schroeder's data is recorded below:

	Kuhn and Brockmann	Zechmeister and Schroeder
Carbon disulphide	533.5, 496, 463 mu	533, 495.5, 462 mu
Benzene	510, 477, 447	509.5, 477, 447.5
Pet. Ether	495, 462, 431	495, 462.5, 434

Hunter and Scott (41) found γ-carotene in palm oil and determined the following absorption maxima in carbon disulphide for three pigment zones containing γ-carotene.

530, 495, (466) mu

529, 496, 463

530, 496, 463

These maxima were evidently determined on non-crystalline material. White, Zscheile and Brunson (92) reported γ-carotene in yellow corn grain and gave maxima in light petroleum ether as 490, 460 and 430 mu. These disagreements in absorption maxima may be explained by reference to stereoisomerism. According to Zechmeister and Polgar (101) the stereoisomers of γ-carotene have maxima in petroleum ether ranging from 483 and 452 mu to 489 and 457.5 mu as compared with 494 and 461.5 mu for the natural γ-carotene.

It is also difficult to obtain exact agreement between absorption maxima for non-crystalline material and crystalline β-carotene. Munsey (74) has stated that it was impossible to obtain absorption maxima for the β-carotene of carrots and spinach which agree with the data for crystalline material. The absorption maxima given by Karrer and Jucker (46) for crystalline β-carotene are listed below:

Solvent	Absorption Maxima (mu)
Carbon disulphide	520, 485, 450
Chloroform	497, 466
Petrol	483.5, 452, 426
Hexane	477, 450, 425

Chanda and Owen (7) recently isolated a pigment from goat liver which they proved by a mixed chromatogram to be β-carotene. Their absorption spectra

in n-hexane showed maxima at 475, 451 and 423 for both this pigment and pure β-carotene. They stated that these maxima were concordant with those obtained by Karrer and Jucker despite the difference of from 1 to 2 mu. Strain (88) listed absorption maxima for β-carotene in carbon disulphide at 511 and 482 although Kuhn and Lederer (61) obtained maxima at 521 and 485.5 in close agreement to Karrer. The new isomers of β-carotene have absorption maxima in petroleum ether ranging from 469 and 437.5 mu to 481 and 450 mu as compared to 483.5 and 452 mu for natural β-carotene (46). Two of these stereoisomers, neo-β-carotene U and neo-β-carotene-B, are often present in the pro-vitamin A portion of vegetable pigments (1).

The tomato pigment lycopene has been reported to exhibit the following absorption maxima in various solvents (46).

Carbon disulphide	548 mu	507.5 mu	477 mu
Chloroform	517	480	453
Benzene	522	487	455
Pet. Ether	506	475.5	447
Ethanol	503	472	443
Hexane	504	472	443

Hunter and Scott (41) found lycopene in palm oil and reported the following maxima in carbon disulphide:

lycopene	544, 505, (481) mu
lycopene + neolycopene	543.5, 505, (478)
neo-lycopene	535, 496, (469)

However Zechmeister and Tuzson (105) stated that neo-lycopene had maxima in carbon disulphide at 536, 498, and 466 mu.

Although the investigator must exercise caution in comparing the absorption maxima of pigments he has isolated with those of pigments of

known structure, he must use even greater discretion when comparing them with the absorption maxima of pigments whose structure has not been definitely ascertained. In 1935 Heilbron et al (37) reported the isolation of a new epiphasic pigment myxoxanthin, from the blue green algae Rivularia nitida. It had a single absorption band in carbon disulphide solution at 488-490 mu. Heilbron and Lythgoe (36) found it occurred in large quantities in another algae Oscillatoria rubrescens. In 1935 Lederer (67) also isolated a new carotenoid, echinenone, from sea urchin. It gave absorption maxima in carbon disulphide at 520, 488 and 450 mu. Lederer suggested the possibility that the maxima 520 and 450 were extraneous and that echinenone was identical with Heilbron's myxoxanthin. However Heilbron in a private communication to Lederer did not consider this likely, although a mixed chromatogram was not made in order to prove this. Recently (1951) Goodwin (24) reported that his experiments showed that myxoxanthin and echinenone were identical. Moreover Goodwin believed that aphanin is also identical with both myxoxanthin and echinenone but was not prepared to state this definitely at that time. Aphanin an epiphasic pigment, was first isolated in 1938 by Tischer (89) from blue algae Aphanizomenon flos-aquae. He listed absorption maxima in carbon disulphide as 533.5 and 494 mu but stated "The absorption spectrum exhibits two maxima separated by a more weakly absorbing zone, which give the appearance of a single wide absorption band with two maxima. The wide absorption range has no sharp limits and although the two maxima are clearly recognized their centres can only be approximately determined".

With this information as a background it seems quite logical for the following experimental work to have this sequence: isolation of the crude pigments from the rust spores, chromatographic separation of the pigments using the best combination of adsorbents and solvents, determination of the absorption maxima of the separated pigments and finally proving their structure using a mixed chromatogram.

MATERIALS AND METHODS

The samples of rust urediospores used in this study were largely obtained from the Dominion Laboratory of Plant Pathology at Winnipeg. A complete list of the samples used in the course of this investigation is given in Table I.

The first seven samples were obtained from the Dominion Laboratory of Plant Pathology before this investigator was assigned to the project. Samples 8 to 13 inclusive were personally obtained from Dr. T. Johnson of the above laboratory during the course of this study. Samples 14 to 17 were obtained from Dr. W.O.S. Meredith of the Grain Research Laboratory. These 1941 samples had been stored in Dr. Meredith's Laboratory and were originally obtained from the Winnipeg plant pathology laboratory. The rust samples 18 to 21 were sent in by Dr. Ledingham of the Prairie Regional Laboratory of the National Research Council at Saskatoon.

Sample 1 was the largest of the original seven samples and was used for most of the initial experimental work. The other six samples gave normal carotenoid pigments at first. On re-examining them after numerous tests on sample 1 were performed, they appeared to have lost their normal carotenoid pigments. This was believed to have been caused largely by improper storing conditions. The newer samples obtained had been stored in a refrigerator immediately after collection and were also kept refrigerated until required for extraction. The four samples sent in by the Prairie Regional Laboratory were as old or older than the original seven samples. They appeared to have been properly stored since despite their age, a small amount of normal carotenoid pigment could be obtained in an initial sand grinding treatment.

Table I. Rust Urediospore Samples Investigated

Sample No.	Species or Variety of Rust	Weight	Date Collected
		g.	
1	Wheat stem rust, mixed races	15.0	Spring 1950
2	Wheat stem rust, mixed races	3.0	Spring 1951
3	Oat stem rust, mixed races	2.0	Spring 1950
4	Oat stem rust, mixed races	3.0	Spring 1951
5	Mixture crown rust and oat stem rust	8.0	Spring 1951
6	Crown rust of oats, mixed races	10.0	Spring 1950
7	Leaf rust of wheat, mixed races	2.0	Spring 1951
8	Wheat stem rust, race 15B	30.0	Winter 1951-52
9	Oat stem rust, mixed races	2.5	March-April 1952
10	Crown rust of oats, mixed races	1.35	May 13, 1952
11	Greyish-brown stem rust mutant, race 121	0.75	Jan.-Mar. 1952
12	Leaf rust of wheat, mixed races	1.40	March-April 1952
13	Flax rust	0.74	March-April 1952
14	Crown rust of oats, mixed races	10.0	1941
15	Wheat stem rust, mixed races	10.0	1941
16	Oat stem rust, mixed races	10.0	1941
17	Leaf rust of wheat, mixed races	8.0	1941
18	Barley stem rust, mixed races	25.0	August 18, 1949
19	Barley stem rust, mixed races	25.0	August 30, 1950
20	Wheat stem rust, mixed races	30.0	1948 - 1951
21	Oat stem rust, mixed races	25.0	1948 - 1950

The vegetable pigment extract from which α- and β-carotene were first isolated was obtained from Miss Margaret Thomson of the Nutritional Research Institute of the University of Manitoba. A 100 mg. sample of carotene (90% β - 10% α) was later purchased from General Biochemicals, Inc. of Chagrin Falls, Ohio. The tomatoes used for the isolation of lycopene and lycoxanthin were the ordinary variety Solanum Lycopersicum.

Microscopic examination of the rust spores was made with an E. Leitz microscope. The low power had a total magnification of 82, the high dry gave a total magnification of 384 while the oil immersion objective provided a total magnification of 840.

The spectral curves including absorption maxima were determined with a photoelectric spectrophotometer, the Beckman quartz spectrophotometer Model DU. The absorption by the pigment solutions was determined in one centimeter Corex cells over a range of 300 to 600 mu. The absorption maxima could be determined to within 0.5 mu with this spectrophotometer wherever they were sharply defined. However it was more difficult in the case of broad maxima to determine the exact wave length which best defined the maxima. Where uncertainties of this kind exist the wave lengths are listed in brackets.

Due to the difficulty of obtaining appreciable amounts of pigments from the first rust samples used, micro chromatographic methods were employed in the beginning of this study. Micro adsorption columns (3 mm. x 20 mm.) tapered at the bottom and glass plates with a circular hole in the upper plate, were used. The latter idea was obtained from Williams (93) text on chromatography. Later adsorption columns made from glass tubes one centimeter in diameter were prepared as described in page 27 of Karrer and Jucker (46). One large chromatographic column of 19 millimeter bore which was slightly tapered towards the bottom and had a removal ground glass bottom joint was used with

larger amounts of pigment material.

The method of extracting the pigments used in the majority of cases was to grind from 0.1 to 1.0 g. of the rust spores with sand in a porcelain mortar in the presence of ethyl ether. The colored ethyl ether was decanted through a filter paper. The residual spores were removed from most of the powdered sand by stirring with ether and decanting the mixture through the same filter paper. Most of the sand remained behind while the spore residue was found on the filter paper. These ground spores were then placed in a 50 ml. centrifuge tube together with 30 ml. of normal hydrochloric acid. The centrifuge tube was placed in a water bath at 100°C. and kept there for 30 minutes. After cooling and centrifuging the straw colored supernatant liquid which formed was poured off and the residue was stirred with ethyl ether. Various modifications of this standard procedure were used when required and they will be discussed in the experimental part.

The carotenoid pigments obtained in the above extraction were separated by the chromatographic method. The excellent condensed description of chromatographic methods given by Williams (93) was used as a guide for the determination of the best methods of separating the rust spore pigments. The dry method of gradually building up the adsorbent column was used since most of the adsorbents employed were powdery. The wet methods such as making a slurry of the adsorbent and pouring it in the column or filling the column with solvent and sprinkling in adsorbent, were tried with the coarse alumina. Even here the dry method of packing the column was found preferable. However, no suction was used when the columns were packed with granular alumina while moderate suction was used with the powdery adsorbents. The pigment zones were first extruded, cut into appropriate sections and placed in separate flasks containing the eluting solution. The extrusion method was employed, rather than the method of direct elution from the column, because

it afforded greater control over the separation of the pigment zones. The adsorbents and solvents used in the chromatographic separation were those which other investigators had found suitable for epiphasic pigments. One of the difficulties of the adsorption method was the obtaining of adsorbents of standard and unvarying activities. An adsorbent of a given chemical name had different adsorption affinities for the pigments depending on the manufacturer and method of preparation. A list of the adsorbents and solvents used is given in Tables II and III respectively. The determination of the best combinations of adsorbents and solvents will be elaborated on in the experimental part.

Quantitative estimations of the pigment content of the spores of the various rust species were performed with the aid of the Beckman spectrophotometer. The theory of colorimetry and spectrophotometry based on a combination of Lambert's and Beer's laws can be found in most textbooks on physical or analytical chemistry and need not be discussed here. The concentrations of the various pigments were determined from the following equation where C is the

$$C = \frac{\log I_o / I_t}{E^{1\%}_{1\,cm}}$$

concentration in grams per 100 cc%, the logarithm of the ratio intensity of the incident light to the intensity of the transmitted light is the optical density and $E^{1\%}_{1\,cm}$ is the extinction coefficient based on a 1% pigment solution in a one centimeter absorption cell.

The wavelength and $E^{1\%}_{1\,cm}$ values used for the quantitative determination of the rust spore pigments were obtained from Table IV of Goodwin's latest paper (30). The following values were used:

Table II. Adsorbents Employed in Chromatographic Separations

No.	Adsorbent	Manufacturer
1	Alumina for Chromatographic Adsorption	Brickman and Company Montreal, P.Q.
2	Aluminum Oxide, ignited powder	J.T. Baker Chemical Co. Phillipsburg, N.J.
3	Aluminum Oxide, Baker and Adamson brand	General Chemical Co. New York, N.Y.
4	Magnesium Oxide, heavy powder	J.T. Baker Chemical Co. Phillipsburg, N.J.
5	Magnesium Oxide, (fluffy powder)	J.T. Baker Chemical Co. Phillipsburg, N.J.
6	Magnesium Oxide, micron brand	California Chemical Co. Newark, California
7	Zinc Carbonate, precipitated	Merck and Company Rahway, N.J.
8	Calcium Hydroxide, U.S.P. grade	J.T. Baker Chemical Co. Phillipsburg, N.J.

Table III. Solvents Used in Chromatographic Separations

No.	Solvent	Supplier	Address
1	Skellysolve F, (light petroleum ether fraction b.p. ~40°)	Skelly Oil Co.	Kansas City, Mo.
2	Benzene, analytical reagent	British Drug Houses	London, N.1.
3	Carbon disulphide, B.A. reagent grade	General Chemical Co.	New York, N.Y.
4	Carbon tetrachloride, analytical reagent	British Drug Houses	London, N.1.
5	Methanol, pure	Dominion Chemical Laboratories	Winnipeg, Man.

β-carotene	λ = 450 mu	$E^{1\%}_{1cm}$ = 2580
γ-carotene	λ = 459 mu	$E^{1\%}_{1cm}$ = 2760
Lycopene	λ = 469 mu	$E^{1\%}_{1cm}$ = 3460

The solvent used for Goodwin's determinations was light petroleum ether. In this study the 2% methanol in petroleum ether eluant was used since it gave similar absorption spectra.

RESULTS AND DISCUSSION

1. EXTRACTION OF RUST UREDIOSPORE PIGMENTS

It was found possible to extract normal or degraded carotenoid pigments from the twenty-one samples of rust urediospores, with ethyl ether at room temperature after subjecting the spores to a thirty minute treatment with normal hydrochloric acid at 100°C. With fresh rust urediospores or spores which had been continuously stored with refrigeration, it was also possible to extract a certain amount of pigment by grinding with sand in the presence of ethyl ether prior to the acid treatment. The amount of color obtained with this sand grinding treatment depended on the age of the spores, with the fresher spores giving a larger amount of color for a given weight of rust. There was no sample, however, in which the amount of color extracted by this treatment was more than about 40% of the total quantity of color extracted from that particular sample. In every case with those spores which released part of their pigment content during the sand grinding treatment, a further solvent extraction after the thirty minute acid treatment, released the bulk of the pigment contained in these urediospores.

Carotenoid pigments can usually be extracted from most plant materials by solvent extraction at room temperature. Newton and Johnson (75) stated that they were able to extract carotenoid pigments from wheat stem rust spores with carbon disulphide as well as with acetone, while Newton, Johannson and Johnson (76) reported they were able to extract the carotenoids with naphtha (a technical grade of petroleum ether). At the beginning of this project samples 1 to 7 of rust spores were available, and colored solutions could not be obtained by extraction at room temperature with any of the three solvents previously used. In addition ethyl ether, methanol, chloroform, carbon tetrachloride, benzene and ethanol were used but extraction at room temperature

for varying lengths of time failed to remove any color. When the fresh urediospores were used, this method was still inadequate in extracting any pigment.

Strain (88) described the technique of grinding carotenoid containing material with sand in the presence of solvent. A personal communication from Johnson revealed that his group had used this sand grinding treatment, as well as the technique of shaking the spores with glass beads, in their extraction of the wheat stem rust pigments. Sand grinding treatment failed to extract any pigment with samples 1 to 7. However this method was satisfactory in extracting various amounts of color from the fresher spores.

Various acid and alkali treatments were tried and a dilute hydrochloric acid digestion was found to be satisfactory in making the pigments accessible to solvents. Various concentrations of dilute acid were used and 1 N. hydrochloric acid and a thirty minute digestion period at 100°C. were found to be the optimum conditions. The result of boiling with dilute aqueous sodium hydroxide and with alcoholic potassium hydroxide was to give a straw color to the solution. After decanting the liquid, treatment of the residue with ether and other organic solvents at room temperature failed to effect the extraction of further color. Dilute acetic acid digestion for two hours at 100°C. was ineffective for the extraction of any color. Boiling with dilute hydrochloric acid for 30 minutes at 100°C. resulted in a brown colored solution being obtained. After centrifuging and decanting the brown supernatant liquid, treatment of the residue with ether produced a bright yellow solution. This bright yellow ether extract was found to contain carotenoid pigments.

In addition to ethyl ether, carbon disulphide and petroleum ether were used to extract the carotenoid pigments. Ethyl ether and carbon disulphide were found to be equally efficient. The ethyl ether extract gave absorption maxima at 455, 430 and 315 mu while the carbon disulphide extract gave maxima at

485-490, (465), and 380 mu for rust sample I. Using equal quantities of rust spores, the petroleum ether was found to extract less pigment than the ethyl ether. Optical density for the petroleum ether extract was 0.493 and for the ethyl ether extract 0.822, both measurements being made at 490 mu using equal volumes of carbon disulphide. Due to the higher toxicity of carbon disulphide, ethyl ether was preferentially used in the majority of pigment extractions.

At first there was some doubt as to whether the pigments had been isolated as they existed in the urediospores, or whether the acid treatment had effected some alteration in them. The information in the literature on the effect of strong acids on carotenoids is not very conclusive. Very little work has been done on determining the effect of strong chemicals on carotenoids since, as Polgar and Zechmeister (82) stated, "it has been more or less tacitly assumed that the sensitive polyene chain is destroyed by such treatment". These investigators found that on shaking a petroleum ether solution of β-carotene with concentrated hydroiodic acid for thirty minutes not more than one-half of the total pigment content was converted into colorless irreversible products. They found that commercial concentrated hydrochloric acid had a milder effect, resulting in the formation of a number of stereoisomers. Quackenbush and his co-workers (83) reported that the effect of dilute hydrochloric acid on carotenoids was to produce new pigments rather than merely stereoisomers. They found that the effect of dilute hydrochloric acid on alfalfa during the ensilage process was the production of several new carotene like pigments in addition to the usual pigments obtained from alfalfa. These new pigments were predominately epiphasic and appeared to be produced at the expense of the xanthophylls. When the first supply of fresh stem rust (sample 8) was obtained, which released part of the pigment content prior to

acid treatment, it was possible to determine the effect of the acid by comparing both extracts. The pigment extract obtained in the sand grinding treatment, gave absorption maxima at 528.5 and 495.5 mu with minima at 518 and 395 mu in carbon disulphide. This absorption curve was very similar to that obtained by spectral analysis of the major rust pigment obtained after chromatographic separation. The ethyl ether extract obtained from the acid digested spore residue was rapidly filtered through anhydrous sodium sulphate. Part of this filtrate, evaporated to dryness and taken up in carbon disulphide, gave absorption maxima at 525, 495-500 and 380 mu. The spectral curve given by the "acid" extract was not as distinct as that given by the "sand" extract. However on chromatographic adsorption analysis both extracts gave at least three similar zones. Corresponding pigment zones in both cases agreed in spectral and solubility properties. It was concluded that on the whole the acid treatment did not substantially affect the original distribution of the carotenoid pigments in the urediospores and that the main pigments isolated by this method would represent the original pigments present in rust.

2. THE PARTITION OF THE RUST SPORE PIGMENTS BETWEEN IMMISCIBLE SOLVENTS

Partitioning of the rust spore pigments between aqueous methanol and pure petroleum ether has shown that rust fungi, as fungi in general, do not contain xanthophylls. Goodwin (29) in a recent review on fungal carotenoids reported that xanthophylls were rarely detected in fungi. Lederer (65) working with urediospores of crown rust of oats concluded that xanthophylls were absent. This conclusion is in agreement with the results of the present study on the various rust species. On partitioning the unchromatographed pigment extracts between 90% methanol and pure petroleum ether, all of the pigment went into the upper petroleum ether layer leaving the bottom methanolic layer colorless. Since the dilute acid digestion was believed to be sufficient to hydrolyse any xanthophyll esters, and as both "sand" and "acid" pigment extracts give similar

chromatograms, it was concluded that xanthophyll esters and xanthophylls containing two or more hydroxyl groups were absent. The major rust spore pigment was also entirely epiphasic on partitioning between 90% methanol and petroleum ether. Using 95% methanol and petroleum ether the hypophase was slightly colored but the bulk of the pigment still remained in the epiphase. The upper red pigment of the rust spores was entirely epiphasic on partitioning between 97% methanol and petroleum ether. This would definitely indicate it to be a simple hydrocarbon. The lowest rust spore pigment zone was entirely epiphasic on partitioning between 95% methanol and petroleum ether.

3. DETERMINATION OF THE BEST COMBINATION OF ADSORBENTS AND SOLVENTS FOR CHROMATOGRAPHIC SEPARATION OF THE RUST PIGMENTS

Micron brand magnesium oxide as the adsorbent, petroleum ether as the original solvent, 20% benzene in petroleum ether as the developing agent, and 2% methanol in petroleum ether as the eluting agent were found to be the best combination of adsorbent and solvents for the chromatographic separation of the rust spore pigments. This optimum combination was not used exclusively in these studies. Due to the initial difficulty in obtaining appreciably colored solutions, the first adsorbent-solvent combination capable of effecting a separation was employed originally. Thus Baker and Adamson aluminum oxide as the adsorbent, carbon tetrachloride as the solvent and methanol as the eluant, was the combination used in much of the earlier work. Various combinations of the adsorbents and solvents listed in Tables II and III, respectively, were tried with the rust pigments before the first mentioned combination was found to give the optimum results.

Only adsorbents and solvents used with success by other investigators for epiphasic pigments were tested, because of the results of the partition tests. The Baker and Adamson alumina was composed of fine granules which could be

packed easily and quickly in an adsorption column without the use of suction. On allowing the pigment containing carbon tetrachloride solution to flow through the column with the aid of gravity, a separation into at least three zones was noticed. The chromatogram could be easily extruded with moderate pressure while slightly damp and the colored zones could be eluted with pure methanol. Brickman chromatographic alumina (adsorbent No. 1) was composed of fine, not easily compressible, granules similar to the Baker and Adamson brand. However, a separation of the rust pigments could not be effected using petroleum ether, carbon tetrachloride and benzene as the original solvents. When the B and A alumina was exhausted and an identical replacement could not be obtained, an attempt was made to duplicate the granular size by sieving five pounds of powdery alumina (adsorbent No. 2) between sieves No. 60 and No. 30. This alumina obtained appeared to be granular but it could be easily compressed. It gave a fairly good separation on the column but the chromatogram tended to crack without suction. When the suction was used the chromatogram remained in one piece but greater pressure was needed to extrude the column. The difficulty of obtaining separate pigments after the separation had occurred on the column led to a search for better adsorbents.

Magnesium oxide powder (adsorbent No. 5) was tried next as the adsorbent. This magnesium oxide was very fluffy and required great care in preparing the columns properly even with suction. Three pigment zones were observed on adding the pigments in benzene and developing with the same solvent. The rate of filtration was quite slow, even though strong suction was applied. The chromatogram also had a tendency to crack and strong pressure was needed to extrude the column. The magnesium oxide heavy powder (adsorbent No. 4) gave a similar separation of the rust pigments. However it was easier to pack the

columns with it and extrusion was less difficult. The micron brand magnesium oxide (adsorbent No. 6) was found to give the clearest separation especially when larger and more concentrated pigment solutions were used. The relative distance between the colored zones as compared to the width of the pigment bands was much greater than with the other adsorbents. The column could be fairly easily extruded by inverting and tapping gently followed by moderate pressure.

Since Karrer and his associates had used calcium hydroxide as the adsorbent in most of their work with epiphasic pigments, as well as zinc carbonate for both epiphasic and hypophasic pigments, it was decided to investigate the use of these adsorbents. After sieving and grading U.S.P. grade calcium hydroxide (adsorbent No. 8) some preparations of good activity were obtained. However the separation was not as good as on the micron brand magnesium oxide. Moreover the column had a tendency to crack during extrusion. The zinc carbonate was found too weak an adsorbent to give a satisfactory fractionation of the rust spore pigments.

The complete chromatographic method finally adopted for the majority of rust pigment separations is given in this paragraph. The glass column was packed with micron brand magnesium oxide under moderate suction. The adsorbent was added a little at a time and was packed down with a ramrod composed of a cork, of slightly smaller bore than the column, attached to a handle. The column was then topped with a quarter inch of "celite". This non-adsorbing topping material was used to protect the magnesia column from getting ruffled on the addition of the colored solution. A concentrated petroleum ether solution of the pigments was added to the top of the adsorption column with suction being maintained at the lower end of the column. The adsorbent was not allowed to become dry once the pigment solution was added and before development was stopped. The separation of the pigments was hastened and the

distance between each colored zone was increased by developing with 20% benzene in petroleum ether. After sufficient development the chromatogram was extruded and cut into appropriate zones. Despite care in packing the columns, the zones were not always planes perpendicular to the axis of the column but convex, concave and even more irregular three dimensional patterns were formed. Cautious scraping was required wherever part of one zone came close to part of another zone. The separated zones were placed in flasks which contained 2% methanol in petroleum ether. The eluted pigment was separated from the adsorbent by pouring through filter paper. The colored solutions were evaporated to dryness under vacuum. The pigment residues were then taken up in carbon disulphide or some other pure solvent and the spectral curves were determined with the Beckman spectrophotometer.

Where variations of this procedure were used, they will be described in the appropriate experimental section.

4. VISUAL AND MICROSCOPIC EXAMINATION OF THE RUST UREDIOSPORES

Visual and microscopic examination of the rust spores, in conjunction with the isolation of their carotenoid pigment content, has shown that these pigments are not entirely responsible for the outward appearance of the spores. Spores of wheat, oats and barley stem rust and wheat leaf rust which have lost all their normal carotenoid pigments still have a pale brown color which is probably due to the reflection of light from the spore walls of numerous cells clustered together. Crown rust which has lost its normal carotenoid pigments still has a buff color. In this case the spore walls are either thinner or not as opaque. The similar straw color obtained in the acid digestion of each of these species (the color was dark brown when 5 g. of spores were used with the same 30 ml. digestion mixture) and their apparent phenolic properties point to a similar chemical constitution of the compound(s) responsible for the pale brown

or buff color of old rust spores.

The first stem rust samples examined had a subdued rust brown color. Microscopic examination of sample No. 1 showed that 25% of the cells contained a yellow blotch of color in the cytoplasm. The fresh wheat stem rust, sample No. 8, had a very intense brick red color which was much more brilliant than that of the stem rusts previously examined. Practically all the spores contained a bright yellow interior coloring matter. The fresh oat stem rust sample No. 9 was similar in outward appearance to fresh wheat stem rust although its shade of red was slightly less brilliant. The majority of the oat stem rust spores contained irregular yellow blotches of color. These yellow spots tended to be well within the spore walls although in some cases the yellow blotches filled almost the entire cell. The 1941 collection of wheat and oat stem rust spores were also quite similar. Both had a pale brown outward color. When viewed under the microscope no yellow color was visible in any of the spores. The cells showed considerable evidence of aging with both the cell wall and the cell nuclei being quite distinct. The cell nuclei appeared much darker than the cytoplasm and in many cases the nuclei were seen to have been divided into two segments. The barley, oats, and wheat stem rust spores received from the Prairie Regional Laboratory, samples No. 18 to 21, had a rusty brown outward appearance. They were darker brown than samples No. 1 to No. 14 but they lacked the intense red hue noticed with the fresh Race 15B spores and to a lesser degree in the fresh oats stem rust spores. Microscopic examination of the 1949 barley rust showed a large number of spores to be colorless, a few cells appeared completely colored while the majority had varying amounts of yellow color concentrated at opposite ends of the cells. The 1950 barley rust was quite similar except that there was a greater number of fully colored cells. The 1948-1950 collection of oat stem rust spores was

similar to the 1950 barley rust collection. The 1948-1951 collection of wheat stem rust contained the largest proportion of fully colored spores, probably due to the inclusion of more recent material. Microscopic examination of the fresh greyish brown mutant of wheat stem rust (Race 121) revealed that the vast majority of these spores contained no yellow color. However 1 to 2% of the spores contained a bright yellow color extending throughout the cell. From the quantity of carotenoid pigment isolated from these spores it is quite possible that all the color came from the few highly colored spores. A private communication from Dr. Johnson, Senior Plant Pathologist at the Dominion Laboratory of Plant Pathology, revealed that the few spores with an inner yellow pigment might possibly be normal wheat stem rust contaminants. Other less likely possibilities were that some of the mutant had reverted to normal type or that the pure mutant actually does contain one or two colored cells per hundred spores. All the stem rust spores examined had an oval or ellipsoidal shape.

The fresh crown rust sample No. 10 had a deep orange color in contrast to the buff color of the 1950 rust (sample No. 6) which had been stored without refrigeration. The 1941 crown rust examined (sample No. 14) was similar in outward appearance to the 1950 crown rust. The circular shaped 1941 spores appeared colorless when viewed singly under the microscope but a pale tan color could be seen when the spores overlapped. The fresh 1952 spores appeared to be completely colored. The general bright golden yellow color extended throughout the cell. Some fresh spores left in a drop of water on a slide for one to two hours showed evidence of plasmolysis. In some cells the yellow pigment was seen to be bunched in the centre. In others, there were breaks in the cell wall and yellow droplets exuded into the aqueous medium. In the 1950 spores, a microscopic examination revealed that the vast majority of the spores were colorless. However the few colored spores seen, in a number

of fields, appeared completely colored with a bright yellow pigment.

The flax rust sample No. 13 had an even brighter appearance than the fresh crown rust. It had an outward brilliant yellow orange color similar to the color of the carotenoid droplets inside the spores. Microscopic examination of the flax rust showed the spores to range from circular to oval shape. In contrast to the fresh crown rust, the brilliant pigment in the flax rust appeared in spots close to the spore wall, leaving the centre of the cell colorless. Old flax rust spores were not available for study but one would expect them to be practically colorless or at least lighter than the old crown rust.

The leaf rust urediospores were quite similar in appearance to the stem rust spores of equivalent age. They could be distinguished microscopically since the leaf rust spores have a uniform circular shape in contrast to the oval shape of the stem rust spores. The 1941 spores, sample No. 17, was light brown in color while the fresh spores, sample No. 12, had a reddish brown color. The 1951 leaf rust sample, No. 7, which had been improperly stored appeared microscopically similar in color to the 1941 spores. Microscopical examination revealed that all the 1941 spores were colorless, a few 1951 spores contained color while all the 1952 spores had a yellow orange color extending throughout the cells.

5. STUDIES ON THE PIGMENT CONTENT OF RUST UREDIOSPORES

(a) Carotenoid Pigments of Wheat Stem Rust (Puccinia graminis tritici)

The investigation of the pigments of wheat stem rust spores revealed that there are very probably only three normal carotenoid pigments present. The major rust spore pigment was later identified as γ-carotene. It was adsorbed in the middle of the chromatogram and accounted for most of the total carotenoid content extracted from the rust spores. Two other pigments were adsorbed above

and below the main pigment. The top pigment zone was shown to contain lycopene while the bottom pigment zone was shown to contain β-carotene. The experiments with the wheat stem rust species were performed on samples of mixed races as well as with samples of two pure races, Race 15B, and Race 121, the greyish brown mutant.

As the rust sample No. 1 was the largest of the seven samples initially available for this study, most of the first two months' work was done with it. The carotenoid pigments could be extracted from this sample only after an initial acid digestion period. Using Baker and Adamson alumina as the adsorbent and benzene, carbon tetrachloride and petroleum ether, respectively, as the original solvent, a number of chromatograms were developed from individual extractions. Although as many as nine different lines could be seen it seemed apparent that there were only three individual zones with the bulk of the pigment being found in the middle red-orange zone. The absorption maxima for this main zone in a number of trials is given in Table IV.

In these first experiments only one maximum in the ultra-violet zone was determined for the top zone. This was at a wavelength of 380 mu. The bottom pigment appeared to contain only one maximum in the visible range between 485 and 490 mu. It also had one maximum in the ultra-violet range near 380 mu in the majority of these trials.

When the fresh Race 15B spores (sample No. 8) were used, the pigment content could be extracted both by grinding with sand in the presence of ethyl ether and by solvent extraction, after treating either fresh spores or sand ground extracted spores with dilute hydrochloric acid for thirty minutes at 100°C. The division of the extracted pigments into three zones on the adsorption column was more distinct than with the older spores. As before the middle orange-red zone was the predominant zone. The lower orange yellow zone and the upper pink zone

Table IV. Absorption Maxima for Major Rust Pigment

	Adsorbent-Solvent Combination	Absorption Maxima in Carbon Disulphide (mu)
1.	Alumina plus Benzene	529, 494.5, 380
2.	Alumina plus Benzene	524, 494.5, (466)
3.	Alumina plus Benzene	528.5, 494.5, 466
4.	Alumina plus Benzene	(525), 495, 378
5.	Alumina plus Carbon Tetrachloride	528.5, 495
6.	Alumina plus Carbon Tetrachloride	528.5, 495, (470), 380
7.	Alumina plus Carbon Tetrachloride	(525), 494
8.	Alumina plus Carbon Tetrachloride	528, 494, 468, 380
9.	Alumina plus Petroleum Ether	526, 494.5, 380
10.	Alumina plus Petroleum Ether	529, 495, 468
11.	Alumina plus Petroleum Ether	527.5, 494.5, (463)
12.	Alumina plus Petroleum Ether	(523), 493, 380
13.	Alumina plus Petroleum Ether	528.5, 495.5, (471)

Table V. Absorption Maxima of Major Race 15B Pigment

	Description	Absorption Maxima (mu)
A	"Sand" Extraction	
	1st trial in carbon disulphide	530.5, 495.5
	2nd trial in carbon disulphide	529.5, 496
	3rd trial in carbon disulphide	531, 497
B	"Acid" Extraction	
	1st trial in carbon disulphide	528, 495.5
	2nd trial in carbon disulphide	529.5, 497.5, (471)
	3rd trial in carbon disulphide	(524), 493
C	Rechromatographed A-3	
	1. in carbon disulphide	532, 498.5, (467)
	2. in benzene	505, 475
	3. in chloroform	507, 476
	4. in petroleum ether	489.5, 460, 433, 350
	5. in ethanol	490.5, 459.5, 345

ranked second and third in the percentage of the total extract. Two extractions were made with 0.5 g. of the Race 15B spores while one extraction was made with 5.0 g. of these spores. The absorption data for the main orange red zone isolated in these three trials is given in Table V.

The chromatographic separation of the extracted pigments in these three trials was accomplished with Baker and Adamson alumina and petroleum ether as the adsorbent solvent combination. The bottom pigment, isolated from chromatograms of the "sand" extracted material for these three experiments, also gave one flat maximum in the visible zone at 488-490 mu. The absorption maxima for the pink zone isolated in the 5 g. experiment were (535), 505 and 475 mu. for "acid" extraction and 538, 505 and (480) mu for the "sand" extraction.

An attempt was made to obtain crystals from the pigment zones obtained in chromatographing the "acid" extract from the 5 g. sample. The colored zones were eluted, concentrated, and placed in a methanol-ether mixture. These solutions were concentrated further and placed in the refrigerator. Some colorless impurities separated and were removed by rapid filtration. The remaining solutions were concentrated further and a deep reddish orange resinous material appeared in several tubes. However crystals could not be obtained although benzene-ethanol, ethanol-pyridine, petroleum ether-benzene were tried as solvent combinations in addition to methanol-ethyl ether.

When magnesium oxide was substituted for Baker and Adamson alumina as the adsorbent it was found that the bottom pigment zone was more easily and clearly separated from the major pigment zone. The absorption spectra of the bottom pigment separated with magnesium oxide, was found to contain a second maximum in the visible region. The absorption maxima for the three zones separated from a 0.5 g. extraction, determined in carbon disulphide, are given in Table VI.

Table VI. Absorption Maxima of Wheat Stem Rust Pigments

Description of Zone	Absorption Maxima (mu)
Top pink zone	542, 507
Middle main orange zone	529.5, 496
Bottom orange yellow zone	510, 485

In another trial with magnesium oxide, the petroleum ether solution of the rust pigments was filtered through this adsorbent. Three main zones and a faint yellow top line were observed after initial development with petroleum ether and subsequent development with carbon tetrachloride. Their absorption spectra were determined. The absorption maxima obtained are summarized in Table VII.

Table VII. Absorption Maxima of Race 15B Pigments

Description of zone	Solvent Used	Absorption Maxima Obtained (mu)
Zone 1 top yellow line	carbon disulphide	385
Zone 2 pink zone	carbon disulphide	535, 505, 475
	benzene	512.5, 484, 458
	petroleum ether	480, 450
Zone 3 orange zone	carbon disulphide	528, 493
Zone 4 orange yellow	carbon disulphide	510, 484.5
	benzene	490, 462
	petroleum ether	473, 447

A considerable amount of sand ground ether extracted spores were subjected accidently to intense heat for a few minutes. A deeply colored orange

pigment extract was obtained after acid hydrolysis by the usual procedure. The short period of intense heat did not affect the pigments greatly since the usual three zones were separated.

Table VIII. Absorption Maxima of Pigments from Spores Subjected to Intense Heat

Description of Zone	Absorption Maxima (mu)
Top pink zone	535, 505
Middle orange zone	526, 493.5
Bottom yellow zone	505, 480

The middle orange zone was repeatedly re-chromatographed on micron brand magnesium oxide but the absorption maxima failed to change appreciably. A clear separation of this zone did not occur in any of the chromatograms. The absorption maxima obtained for the five trials with carbon disulphide are listed in Table IX.

Table IX. The Effect of Successive Rechromatographing on the Absorption Maxima of the Major Rust Spore Pigment

Rechromatographing Trials	Absorption Maxima (mu)
Trial 1	526, 494
Trial 2	529, 494
Trial 3	529, 493.5
Trial 4	528, 495
Trial 5	527, 494

The greyish-brown stem rust mutant Race 121 was found to contain the same carotenoid pigments as normal wheat stem rust. However the total carotenoid content from these fresh spores was very small and could only be

extracted after a preliminary acid digestion.

Approximately 325 mg. of this mutant were ground with sand in the presence of ethyl ether. A colored solution could not be obtained even after 30 minutes of intermittent grinding. The ground spores were removed from most of the sand and subjected to acid digestion. The digested mixture was centrifuged and the supernatant straw-colored liquid was poured off. The residue was treated with ethyl ether and a small amount of yellow color was obtained. This yellow solution was evaporated to dryness and the residue was taken up in carbon disulphide. The pink carbon disulphide solution gave an absorption spectra similar to the "crude" extract of rust spores previously examined. It exhibited absorption maxima at 495 and (525) mu.

A further 430 mg. of this mutant was subjected to the acid digestion without a preliminary sand grinding treatment. The colored ether extract obtained was evaporated to dryness and taken up in petroleum ether. The petroleum ether solution was filtered through a small adsorption column packed with micron brand magnesium oxide. On developing with more of the original solvent a clear separation of the pigment into three zones was observed. Two pink lines fairly close together were seen near the top of the column. The middle zone contained an orange band which held the bulk of the pigment. This band gave absorption maxima at 494 and 527 mu. The lowest zone consisted of a pale orange band which gave absorption maxima at 510 and 482 mu. On the basis of transmission data the greyish-brown mutant was calculated to contain roughly 1% of the pigment of fresh normal rust.

The 1941 wheat stem rust spores (sample No. 15) were found to contain only a degraded or oxidized lipochrome having an absorption maximum in carbon disulphide at 382 mu. Grinding 5 g. of this sample with sand in the presence of ethyl ether failed to extract any color. On acid treatment of the ground spores

brown tea colored solutions were formed. After decanting this supernatant liquid the residue was extracted with ethyl ether. A peculiar brownish ethereal solution was obtained. A carbon disulphide solution of the pigment was also brownish yellow and gave the above mentioned absorption maxima. This pigment in methanol was also the same color and contained one absorption maximum in the ultra-violet zone at 330 mu. On attempting to chromatograph this degraded pigment only one pale brown line was observed.

Sample No. 20 of wheat stem rust urediospores was composed of mixed races. It was found that this rust grown in a different locality contained the same carotenoid pigments in the same proportions. One of the reasons these spores were available for this investigation was because they had lost most of their ability to germinate. Although they were comparatively old, they still contained most of their normal carotenoid content because they had been stored with refrigeration since their collection. Less than 5% of the pigment content could be extracted from the spores by grinding with sand in the presence of ether. The bulk of the pigment content could be extracted only after the usual acid digestion. It is possible that aging of the spores has an effect on the carotenoid content since the chromatograms showed several colored lines not seen in those from fresh spores. However the three main pigments were present in their usual proportions. The results of an experiment with these spores illustrated this.

The carotenoids from 5 g. of these spores were extracted by the usual methods. These pigments were then partitioned between 90% methanol and petroleum ether. The petroleum ether layer was dried, concentrated and filtered through a large adsorption column packed with micron brand magnesium oxide. The development with 20% benzene in petroleum ether was allowed to procede for two hours. At least nine lines or bands were seen and isolated. The major orange band, No. 8 had a lighter shade on either side and some of the middle bright orange part was taken for absorption studies. No absorption maxima

could be obtained for the No. 1 purple line. However, absorption maxima in carbon disulphide determined for the other zones are given in Table X.

Table X. Absorption Maxima of the Various Zones Obtained from Sample No. 20

Description of Zone	Absorption Maxima (mu)
No. 1 purple line	indeterminate
No. 2 pink line	(495), 475
No. 3 pink line	(540), 505, 480
No. 4 pink line	(530), 503, 475
No. 5 violet line	483
No. 6 yellow line	480
No. 7 pink line	(500), 480, (470)
No. 8 heavy orange band	531, 497
No. 9 lighter orange band	508, 485

The No. 3 pink line was re-chromatographed from petroleum ether on a smaller column packed with magnesium oxide. A red line first formed at the top of the column. On developing with 20% benzene in petroleum ether, the red line moved down slightly and acquired an orange tinge. A brownish yellow band appeared above this line. The red line was eluted and gave absorption maxima in carbon disulphide at 541, 505 and 480 mu. In chloroform the absorption maxima were at 515, 484, and 459. On partitioning between 97% methanol and petroleum ether, this pigment was found almost entirely in the epiphase.

The No. 4 pink line was re-chromatographed from petroleum ether on the specially sieved aluminum oxide. The developed chromatogram contained a yellow band at the top of the column, two thin brown orange lines, one thin

purplish brown line and the main pink band. This pink band had an intense violet color in carbon disulphide. However its absorption spectra lacked definition and contained only one maximum at 510-515 mu.

The main zone from this 5 g. sample (zone No. 8) was re-chromatographed on micron brand magnesium oxide from petroleum ether. The developed chromatogram contained a wide orange zone having five different shades in color from top to bottom. However, no white spaces appeared between these shades of color. The absorption maxima in carbon disulphide determined for each of these divisions are listed in Table XI.

Table XI. Absorption Maxima of the Divisions of the Main Rust Spore Pigment Zone

Description of the Division		Absorption Maxima (mu)
No. 8a	top pale orange	529, 496.5, 469
No. 8b	light orange	529, 496
No. 8c	medium orange	529.5, 496
No. 8d	dark orange	529, 496
No. 8e	medium orange	528, 494.5

A petroleum ether solution of the No. 8d pigment was rechromatographed on the specially sieved aluminum oxide. The developed chromatogram contained two yellow lines at the top followed by a cream colored zone. There was a wide yellow orange band below this and a narrow yellow orange line still lower down. The absorption maxima were determined in carbon disulphide for all the zones and in chloroform as well for the No. 8d 4 zone.

After it was determined that γ-carotene, β-carotene and lycopene were the pigments present in stem rust spores, an experiment was carried out in order to spectrophotometrically estimate the amount of each pigment present. The

Table XII. Absorption Maxima of the Zones Obtained from Filtering No. 8d 4 Zone Through Alumina

	Description of Zone	Absorption Maxima (mu)
No. 8d 1	two yellow lines	526, 495
No. 8d 2	cream colored zone	529.5, 496
No. 8d 3	top part of main yellow orange	528, 496
No. 8d 4	main yellow orange zone (in chloroform)	530, 496 (506, 475)
No. 8d 5	bottom yellow orange line	524.5, 494

carotenoid pigments of 2 g. of fresh wheat stem rust spores were extracted by the usual method and spatially separated on a magnesium oxide column. The usual three zones were observed but the top pink zone (lycopene) was found to be oxidized and could not be accurately estimated in the spectrophotometer. However 980 γ of γ-carotene and 420 γ of β-carotene were obtained. On the basis of parts per million stem rust spores were found to contain 490 of γ-carotene and 210 of β-carotene.

(b) Carotenoid Pigments of Oat Stem Rust (Puccinia graminis avenae)

Attention was turned to the oat stem rust samples No. 3 and No. 4 about six months after they had been in the laboratory without refrigeration. These samples would not release any pigment without a preliminary acid digestion. Even then the amount of pigment released was relatively small. On chromatographing on magnesium oxide from petroleum ether only one yellow line could be seen. Its absorption spectra in carbon disulphide contained one very sharp maximum at 382 mu.

The fresh oat stem rust sample No. 9 gave very similar results to wheat stem rust. Approximately 1.5 g. of this sample were used for the first large trial. The spores were ground with sand in the presence of ethyl ether until the

final solutions obtained appeared practically colorless. This process took over thirty minutes of intermittent grinding. The ground spores were separated from the bulk of the sand and given the usual acid hydrolysis. The spore residue was stirred with ethyl ether and a much more intensely colored solution for the same amount of solvent was obtained than with over thirty minutes of the sand grinding treatment.

On filtering the colored solutions both from the "sand" and "acid" extracts, some color was left on the respective filter papers. The colors were eluted from the filter papers and their absorption spectra were determined. Both gave the same type of curves and indications of same maxima at 525 and 495 mu in carbon disulphide (taken to the nearest 5 mu). These curves were regarded as indicating the spectra of the mixed carotenoid pigments.

The pigment solution from the sand extraction was chromatographed on micron brand magnesium oxide. Three zones separated. The middle (largest) zone was re-chromatographed on magnesium oxide heavy powder and two zones separated out. Their absorption maxima in carbon disulphide are listed in Table XIII.

Table XIII. Absorption Maxima of Oat Stem Rust Spore Pigments from "Sand" Extraction

Description of Zone		Absorption Maxima (mu)
Zone 1	orange	(495), 380
Zone 2a	red orange	527, 493
Zone 2b	yellow orange	482
Zone 3	orange	485, 380

The colored solution, obtained by eluting from the filter paper through which the extract from the acid treated spores had been poured, was

chromatographed on micron brand magnesium oxide. The middle zone was re-chromatographed on the heavy powder but there was no separation. This was probably due to the initial careful separation of the middle zone. However the bottom zone was probably contaminated with some of the former zone. Their absorption curves were determined in carbon disulphide and maxima obtained are given in Table XIV.

Table XIV. Absorption Maxima of Zones Obtained from Chromatographing Filter Paper Eluate of "Acid" Extraction

Description of Zone		Absorption Maxima (mu)
Zone 1	yellow orange	505, 485
Zone 2	red orange line	527.5, 493.5
Zone 3	pale orange band	495, 475

The deeply colored "acid" extract was then chromatographed on micron brand magnesium oxide. Three zones appeared at first but after one hour's development a fourth sharp orange line appeared below the others. The No. 1 pink and No. 4 orange zones were separated from the chromatogram in a fairly pure condition. The No. 2 orange zone was re-chromatographed on a column prepared by mixing heavy powder and micron brand magnesium oxide. A yellow line separated near the top of the column while an orange band with a sharp bottom border separated lower in the column. The No. 3 brown orange zone had been imperfectly separated from Zone No. 2 and was therefore re-chromatographed. After developing for over an hour two bands separated out. The absorption maxima of all of these zones determined in carbon disulphide are given in Table XV.

The zone 2b contained the bulk of the pigment. This orange pigment was re-chromatographed on the alumina which had been prepared by sieving

Table XV. Absorption Maxima of Oat Stem Rust Spore Pigments from "Acid" Extraction

Description of Sections		Absorption Maxima (mu)
Zone 1	pink	539, 505, 481
Zone 2	orange (a) yellow line	indeterminate
	(b) orange band	528, 495
Zone 3	brown orange (a) upper clear orange	525, 494
	(b) lower pale orange	510, 480, 360
Zone 4	orange	509, 483.5

between sieves No. 60 and No. 30. The chromatogram was developed with petroleum ether and 20% benzene. Two yellow brown lines were visible above the wide pink orange zone and an orange and a yellow line appeared below it. The column was divided into six sections and the absorption maxima of each in carbon disulphide were determined as shown in Table XVI.

Table XVI. Absorption Maxima of the Sections Obtained on Filtering Zone 2b Pigment (Table XV) Through Alumina

	Description of Sections	Absorption Maxima (mu)
Section 1	two yellow brown lines	495, 470
Section 2	pink orange band, top third	528 495
Section 3	pink orange band, middle third	528.5, 495
Section 4	pink orange band, lower third	526.5, 493.5
Section 5	orange zone	527, 494
Section 6	yellow zone	527, 494.5

The pigments from sections 2 to 6 inclusive were taken up in

petroleum ether and re-chromatographed on micron brand magnesium oxide. One homogeneous sharp red line appeared on developing with pure petroleum ether. On washing the column with benzene, the line changed color from a red to an orange tinge. Development for several hours failed to separate this line. Further re-chromatographing on magnesium oxide heavy powder from pure benzene again failed to separate this orange line. Its absorption maxima were then found to be 528 and 494 mu. The initial separation of the zone 2b was probably due to the formation of trans-cis isomers. Apparently they are adsorbed as one band on magnesium oxide.

Normal carotenoid pigments could not be obtained from the 1941 oat stem rust. Five grams of sample No. 16 was ground with sand in the presence of ethyl ether but no color was imparted to the ether. These spores were then digested with 30 ml. of dilute hydrochloric acid under the usual conditions. A dark brown or tea color was imparted to the dilute acid. After decanting this solution, the residue was extracted with ethyl ether. A peculiar brownish yellow solution was obtained. This color remained the same in a variety of solvents. The carbon disulphide solution of this pigment showed one maximum at 382 mu.

The oat stem rust sample No. 21 gave similar results to those of the barley and wheat stem rust samples No. 19 and No. 20, also obtained from Saskatoon. The usual three zones with their corresponding absorption maxima were obtained.

(c) Carotenoid Pigments of Barley Stem Rust (Puccinia graminis hordei)

In this thesis barley stem rust has been listed as a separate species. As there has been no international agreement on nomenclature and classification of rust fungi, one is not bound to adhere to any set rules. Plant pathologists agree closely on fundamental terminology and classification but differ slightly on minor points. Petit (80), chief of the fungi section of the Department of

Botany and Agronomy of Tunisia, treated barley stem rust as a separate species using the special name Puccinia graminis hordei. However, officials of the Dominion Laboratory of Plant Pathology at Winnipeg have grouped barley stem rust together with wheat stem rust under the single name of Puccinia graminis tritici. Their reason for doing this is the fact (11) that the same races of wheat stem rust such as race 15B will attack either wheat or barley (but not oats). Samples No. 18 and No. 19, were marked barley stem rust. It was decided to differentiate barley and wheat stem rusts in this thesis since these urediospores were collected from different host cereals.

In view of the above it is not surprising that the carotenoid pigments in barley stem rust proved to be the same as those in wheat stem rust. Approximately 0.5 g. of barley stem rust were ground with sand in the presence of ethyl ether. Only a pale yellow solution could be obtained.

A bright yellow solution was obtained using the "acid" extraction method and the extracted pigments were taken up in carbon tetrachloride. The concentrated carbon tetrachloride solution was filtered through micron brand magnesium oxide. Fresh carbon tetrachloride was used to develop the chromatogram. The familiar three zones were seen on the developed chromatogram. There was a distinct separation between the major orange band and the bottom orange zone with a sufficiently wide white zone between them to afford room for a careful mechanical separation. In the upper pink zone yellow brown lines appeared both above and blow the main pink band. Their absorption maxima in carbon disulphide were determined and are listed in Table XVII. Extracts from wheat stem rust sample No. 20 and oat stem rust sample No. 21 were chromatographed on similar columns and connected to the same source of vacuum. Practically identical results were obtained with the barley, wheat and oat stem rust. The chromatograms were similar and absorption maxima of the corresponding zones agreed.

Table XVII. Absorption Maxima of the Pigments Obtained from Barley Stem Rust Spores

Description of Zone	Absorption Maxima (mu)
Zone 1 top pink (a) yellow brown line	465
(b) pink band	(535-540), 500, 475
(c) yellow brown line	490, 475
Zone 2 major orange band	527, 494
Zone 3 bottom orange zone	(510), 482

The "sand" extract from the barley rust sample was chromatographed on micron brand magnesium oxide. Petroleum ether was used as the original solvent and the chromatogram was developed with fresh petroleum ether. There was a yellow orange zone at the bottom, a wide orange band in the middle and a faint pink zone near the top. A yellow band appeared at the very top of the column. The main orange zone gave absorption maxima at 527 and 494 mu. while the bottom yellow orange zone gave maxima at (510) and 483 mu.

(d) Carotenoid Pigments of Crown Rust of Oats (Puccinia coronata avenae)

All of the information on the normal carotenoid pigments in crown rust was obtained from fresh crown rust (sample No. 10). Samples No. 6 and No. 14 each contained 10 g. of spores but the normal yellow orange carotenoid pigments could not be obtained from them. The orange crown rust contained the same carotenoid pigments as the red stem rusts.

Approximately 0.7 g. of fresh crown rust spores were ground with sand in the presence of ethyl ether. In ten minutes of grinding and extracting 75 ml. of a colored solution of optical density 1.72 (at 495 mu.) were obtained. The ground, extracted spores were re-extracted with fresh ether after the usual acid treatment. There was obtained 115 ml. of a colored solution of optical density

2.1 at the same wavelength. On this basis it was calculated that 37% of the total pigment content was extracted with sand treatment while the remainder was extracted after the acid treatment.

On chromatographing extracts obtained both from the initial sand treatment and after acid digestion, chromatograms similar to those obtained with stem rusts were obtained. Three familiar zones were seen on the magnesium oxide columns, viz. a top pink line, the middle main orange band and the bottom orange band. The separation of the two orange bands was accomplished easily with less development than in the case of the stem rust spores. The white space between the orange bands was quite large in relation to the width of the bands. This ease in separation was probably due to differences in the colorless components of crown rust and stem rust extracts. In the crown rust chromatograms a yellow line was sometimes observed, either above or below the pink line. Such lines have also been observed in the stem rust chromatograms.

The absorption curves of the pink line appeared to be similar to the pink pigment observed in previous studies but accurate maxima could not be obtained. On re-chromatographing the pink component from the "acid" extract, four pink lines appeared but sufficient color could not be eluted to get distinguishable spectral curves. However the absorption maxima of the two main pigments were determined accurately in a number of solvents. They are listed in Table XVIII.

It was impossible to extract any color from the 1950 samples of crown rust, which had become buff colored. A pale yellow extract was obtained after acid digestion. However on chromatographing only a single pigment zone was obtained, which gave one absorption maximum in carbon disulphide at 380 mu. Five g. of the 1941 crown rust gave similar results. The carbon disulphide solution of the "acid" extract gave one well defined maximum at 385.5 mu.

Table XVIII. Absorption Maxima of Crown Rust Pigments in Various Solvents

Solvent	Main Orange Band	Bottom Orange Band
Carbon disulphide	527.5, 494.0, 434.0 mu	(510), 483.5 mu
	527.0, 494.0 mu	510, 483.0 mu
Chloroform	502.0, 472.0 mu	(488), 462.0 mu
Petroleum Ether	487.0, 458.0, 434.0 mu	475.0, 448.0 mu

A quantitative experiment was performed on crown rust using 628 mg. of sample No. 10. The combined extracts from this sample was filtered through magnesium oxide. The pigment zones which were separated were eluted with 2 percent methanol in petroleum ether. The pigment solutions were made up to a volume of 100 ml. and the optical density for each of the three pigments was determined at the appropriate wave lengths. The lycopene zone appeared to be oxidized and its concentration could not be determined by the spectrophotometric procedure. However 685 γ of γ-carotene and 230 γ of β-carotene were found. In terms of parts per million crown rust urediospores were found to contain 1090 of γ-carotene and 370 of β-carotene.

(e) Carotenoid Pigments of Leaf Rust of Wheat(Puccinia triticina)

The carotenoid pigments of leaf rust of wheat were found to be identical with those of the other rust spores examined. The technique had been mastered to such an extent that distinct chromatograms could be obtained from as little as 86 mg. of fresh leaf rust. Normal carotenoid pigments could not be obtained from samples No. 7 and No. 17.

Using 185 mg. for the first trial with the fresh leaf rust, a bright yellow solution was obtained on grinding with sand in the presence of ether. The ground spores were given the usual acid treatment and bright yellow ether

solution was obtained. The absorption spectra of the crude extracts obtained before and after acid treatment were determined in carbon disulphide. Both showed a maximum at 495 mu but the non-acid extract had a better defined absorption curve. From the absorption data it was calculated that about one-third of the total pigment content was released during the sand grinding while the remainder was obtained after acid treatment. Two orange lines were seen on chromatographing the "sand" extraction but as the development proceeded most of the pigment disappeared and only one faint band was left. On filtering a petroleum ether solution of the "acid" extraction through [illegible] micron brand magnesium oxide and developing with 20 percent benzene in petroleum ether, the three familiar zones were observed. However a similar destruction of much of the pigment occurred before separation of the three zones could be effected. Absorption maxima could not be determined for the top pink zone but the main orange zone had maxima at 525 and 492 mu while the bottom pale orange zone had vague maxima at 510 and 482 mu.

In the second trial 120 mg. of leaf rust were used. The "sand" extraction gave maxima at 495 and (520-525) mu before chromatographing. After filtering a petroleum ether solution of the pigments through magnesium oxide, two orange lines were visible. The upper orange line gave maxima at 523 and 494 mu while the lower thin orange line gave one definite maximum at 482 mu. The proportion of color in the upper orange line to that in the lower orange appeared to be similar to that encountered with the other species of rust. The extraction from the acid treated spores was also chromatographed on magnesium oxide. The usual separation into three zones was observed. The top line was pink, the middle orange and the bottom yellow. Sufficient development to separate the zones adequately caused considerable destruction of the pigments. The bottom zone appeared to be affected least and more pigment was obtained from it than from the middle one..

Table XIX. Absorption Maxima of the Pigments Obtained from 120 mg. of Leaf Rust (of Wheat) Spores

	Description of Zone	Absorption Maxima (mu)
1	Top pink zone in carbon disulphide	380
2	Middle orange zone in carbon disulphide	525, 492
3a	Bottom yellow orange zone in carbon disulphide	508, 481
3b	Bottom zone in petroleum ether	475, 447

Only 86 mg. of leaf rust was used for the next trial but the results were excellent. The leaf rust was subjected to the acid treatment without first grinding with sand. As the supply of ethyl ether was exhausted temporarily the extraction was carried out with methanolic petroleum ether and acetone. A yellow solution was obtained in the petroleum ether phase. This solution was evaporated to dryness and the residue was taken up in carbon tetrachloride. The concentrated carbon tetrachloride solution was filtered through micron brand magnesium oxide and on addition of further pure solvent three distinct divisions were observed. There was a pink line near the top, a distinct red orange line in the centre and a pale pink orange band near the bottom of the column. There was a fairly good recovery of the zones. The proportion of pigment recovered from the top pink zone, according to absorption data, was just slightly less than the amount recovered from the bottom zone. As usual the middle zone contained the bulk of the total pigment content. Absorption maxima are listed in Table XX.

Exactly 1.0 g. of leaf rust spores were extracted by the usual procedure. A concentrated petroleum ether solution of the combined extracts from this sample was filtered through magnesium oxide. After developing the

Table XX. Absorption Maxima of the Pigments Obtained from 86 mg. Leaf Rust Spores

	Description of Zone	Absorption Maxima (mu)
1	Upper pink zone	542, 506, 478
2	Major middle red orange zone	527, 495
3	Bottom pink orange band	482

chromatogram and separating the zones, the pigments were eluted with methanolic petroleum ether and made up to a volume of 100 ml. The optical density for each of the three pigments was determined at the appropriate wave lengths. The lycopene content of the leaf rust seemed to be greater than in the other rust species. Moreover it was less easily oxidized and could be quantitatively estimated with the spectrophotometer. From the 1.0 g. of leaf rust spores 1130 γ of γ-carotene, 360 γ of β-carotene and 220 γ of lycopene were obtained.

(f) Carotenoid Pigments of Flax Rust (Melampsora lini)

This species of rust was the only non-cereal rust investigated. It was found that it contained the same carotenoid pigments as the cereal rusts.

Exactly 135 mg. of flax rust spores were ground with sand in the presence of ether and a yellow extract was obtained. A further orange solution was obtained from the "sand" extracted spores after they were subjected to the usual acid treatment. From absorption measurements, it was calculated that 25 percent of the total pigment extracted was obtained during sand grinding while the remainder was isolated after acid treatment. The unchromatographed extract gave absorption maxima at 494 and 523 mu. The extract from the acid digested material gave only one visible maximum at 492 mu.

A chromatogram obtained from the sand extraction by filtering a carbon tetrachloride solution through micron brand magnesium oxide gave the

usual three zones. There was a yellow red line at the top, a distinct orange band in the middle and a pale orange band at the bottom. The middle orange zone gave distinct maxima at 527 and 494 mu while the bottom zone gave maxima at (510) and 482 mu. The absorption maxima of the top zone could not be determined.

A concentrated carbon tetrachloride solution of the "acid" extract was filtered through magnesium oxide and the chromatogram was developed with fresh solvent. The usual three zones separated out. On prolonged development the pink zone divided into two pink lines and an orange line appeared just above the main orange zone. No separation occurred in the bottom pale orange zone. The middle main orange zone gave absorption maxima at 527 and 495 mu while the bottom zone gave maxima at (510) and 482 mu. Definite absorption maxima for the pink zones could not be obtained at first but absorption curves indicated the absorption to be at slightly longer wavelengths (in the redder portion of the spectrum) than those of the two orange pigments. On re-chromatographing the two pink lines, a pink zone was obtained having absorption maxima at 540, 500 and 475 measured to the nearest 5 mu.

One quantitative experiment was performed with the flax rust using 600 mg. of spores. The combined extracts gave a chromatogram similar to the ones described above. Visually the ratio of the bottom orange zone (β-carotene) to the main orange band (γ-carotene) did not appear much different than in the chromatograms obtained with the other species of rust. However the quantitative determination showed that there was considerably less β-carotene in flax rust than in the other species examined. The lycopene was oxidized in the separation and could not be spectrophotometrically determined. However 470γ of γ-carotene and 70γ of β-carotene were obtained. In terms of parts per million flax rust spores were found to contain 780 of γ-carotene and

I 20 of β -carotene.

6. CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC STUDIES ON THE CAROTENE FRACTION OF VEGETABLE LIPOCHROMES

Before the identity of the rust spore pigments had been determined it was decided to check on the agreement of the results of the methods used in this study with the results of other investigators on similar carotenoid material. Several litres of the carotene fraction of vegetable pigments was obtained from the Nutritional Research Institute. They had isolated this fraction from a number of green and yellow vegetables in order to determine the pro-vitamin A content. As expected this carotene fraction consisted largely of β-carotene with a smaller amount of α-carotene. Small amounts of other pigments were seen in the chromatogram but they were not separated completely nor were all identified. The absorption maxima obtained with α-carotene agreed very closely with the literature values which had been obtained by dissolving a small amount of the crystalline pigment in a given solvent. The absorption maxima obtained with β-carotene did not agree very closely with the literature values. However these values were very similar to those obtained with the lowest rust spore pigment zone.

Four main zones were observed on chromatographing a concentrated petroleum ether solution of the crude carotene on adsorption columns packed with micron brand magnesium oxide. The bottom least strongly adsorbed pigment zone was yellow orange. It was the second largest band. The large orange band which had separated out above it contained the bulk of the pigment. A third yellow zone was seen above the orange band and a fourth zone consisting of several lines was adsorbed near the top of the column. The fourth zone was re-chromatographed on zinc carbonate and nine lines separated. The third yellow zone was re-chromatographed and four lines were formed. The lowest (α-carotene) and the second lowest (β-carotene) zones were re-chromatographed but a

division of these bands did not take place. The crude carotene extract was thus divided up into the sections and lines listed in Table XXI.

The No. 8 red pink line of the top zone appeared to be either γ-carotene or pro-γ-carotene. However on attempting to purify it by re-chromatographing, most of the pink color disappeared and a green top line became visible.

There were sufficient amounts of α- and β-carotene to work with. The absorption maxima obtained for α-carotene in a number of solvents is compared with the literature values for the same solvents in Table XXII.

The values of the absorption maxima obtained agree closely with the literature except in the case of petroleum ether. The petroleum ether used in these experiments was a low boiling ($\sim$40°C.) fraction while the petroleum ether used in obtaining the literature values was a fraction boiling around 70°C. Presumably this difference accounts for a shift in the spectra from 4 to 6 mu. It is probably better to compare the petroleum ether results with the literature values for hexane since this is equivalent to light petroleum ether.

The absorption maxima for the lowest rust spore pigment zones, for the β-carotene zone obtained from vegetables, and literature values for β-carotene are compared in Table XXIII. Even after several purifications on magnesium oxide columns for two to three hours the absorption maxima for the vegetable β-carotene and the literature values did not agree very closely. There was however much closer agreement on the absorption maxima for the lowest rust spore pigment and the β-carotene zone from the vegetable extract. The spectral curves for the pigments isolated from these two sources were practically identical in a number of solvents.

Table XXI. Fractionation of Total Carotene Content from Vegetables

Fourth Zone at Top of Column	Absorption Maxima in Petroleum Ether (mu)
1. Green top band	Indeterminate
2. Brown line	405, 380
3. Yellow line	428, 408, 380
4. Wine red line	450, 430
5. Pale orange line	430, 405
6. Yellow orange line	450, 430, 405
7. Wine pink line	450, 430
8. Red pink line	465
9. Blue green line	440
Third Yellow Zone	**Absorption Maxima in Carbon Disulphide (mu)**
1. Dirty orange line	453, 428, 403, 380
2. Yellow line	478, 450
3. Pale yellow zone	454, 427.5
4. Pale orange line	503, 477
Second Orange Zone	
(β-carotene)	(510), 483
Lowest Yellow-Orange Zone	
(α-carotene)	508, 476.5

Table XXII. Experimental Versus Literature Values for Absorption Maxima of α-Carotene

Solvent	Absorption Maxima Determined (mu)	Literature Data (mu)	
Carbon disulphide	508, 476.5	509, 477	(46)
	507, 476	507, 476	(88)
Chloroform	485.5, 457	485, 454	(46)
	485, 457		
Ethanol	475.5, 447	476, 446	(88)
Hexane		475, 445	(46)
Petroleum Ether	472, 443	478, 447.5	(46)

Table XXIII. Comparison of Absorption Maxima Obtained from Bottom Rust Pigment and β-Carotene from Vegetables with Literature Values

Solvent	Bottom Rust Pigment Zone (mu)	β-Carotene from Vegetables (mu)	Literature Values (mu)	
Carbon disulphide	510, 483	(510), 483	520, 485, 450	(46)
		506, 482	511, 485	(88)
Chloroform	(488), 462	(484), 463	497, 466	(46)
		462		
Petroleum Ether	475, 448	473, 447	483.5, 452	(46)
Hexane			477, 450	(46)
Ethanol		474, 450.5	482, 452	(88)

7. CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC STUDIES OF COMMERCIAL CAROTENE

The 90 percent β-carotene, 10 percent α-carotene crystalline mixture obtained from General Biochemicals Inc., was found to be easily separated on adsorption columns packed with micron brand magnesium oxide. The absorption spectra of the β-carotene isolated was identical with the spectra from the bottom rust spore pigment.

Several mg. of the commercial carotene was dissolved in a small volume of petroleum ether. The petroleum ether solution was filtered through an adsorption column packed with micron brand magnesium oxide in the usual manner. The chromatogram was first washed with pure petroleum ether, and then further developed with 20 percent benzene in petroleum ether. A colorless zone was observed between the top orange zone and bottom yellow-orange zone. The top orange zone contained most of the pigment and it was evidently β-carotene. It gave an absorption curve similar to that obtained from the bottom rust spore pigment and to that obtained from the major fraction of the vegetable carotene extract. Absorption maxima in carbon disulphide were at 508.5 and 483 mu.

The bottom yellow-orange zone gave well defined maxima in carbon disulphide at 506 and 476 mu. It was obviously α-carotene.

8. THE IDENTIFICATION OF THE BOTTOM RUST SPORE PIGMENT AS β-CAROTENE

Until β-carotene had been obtained from a known source, it had been difficult to identify the bottom rust spore-pigment or even to consider it a single component. Mackinney (73) had determined the absorption maxima in carbon disulphide for the total carotene fraction isolated from carrot roots, redwood leaves and ivy leaves. In Table II of his paper the values for the first absorption band range between 509.5 and 512 mu while the values for the second absorption band range between 482 and 484 mu. The values obtained for the bottom rust

spore pigment are summarized in Table XXIV.

The close agreement between the values for the absorption maxima of the bottom rust spore pigment and for Mackinney's crystalline mixtures of α- and β-carotene at first served to support Lederer's contention that the bottom rust spore pigment zone (in crown rust at least) is a mixture of α- and β-carotene. Lederer (65) had obtained maxima for this pigment at 481 and 450 mu in petroleum ether while Karrer and Jucker (46) listed absorption maxima for β-carotene at 483.5 and 452 mu and for α-carotene at 478 and 447 mu. Without bothering to re-chromatograph the bottom rust pigment which he had obtained as the filtrate which passed unadsorbed through a lime column, and on the basis of one experiment only, Lederer had asserted that this pigment was a mixture of α- and β-carotene. The advances in carotenoid chemistry since Lederer performed his experiment has demonstrated the need to exercise caution when identifying a carotenoid pigment on the basis of absorption maxima only. If one refers to the stereoisomers of β-carotene reported in the literature review it can be seen that Lederer's values of the absorption maxima could be satisfied by a mixture of β-carotene geometric isomers or even a single neo-β-carotene. Similarly, on the basis of the cis-trans isomerization of the carotenoids, the absorption maxima obtained for the bottom rust spore pigment could indicate it to be a stereoisomer or a mixture of stereoisomers of β-carotene, rather than a mixture of structural isomers as are Mackinney's pigments. Mackinney had used the same micron brand magnesium oxide recommended by Strain (87) and had found it possible in all cases to separate α-carotene from β-carotene. This lowest rust spore pigment would not separate into two zones on columns of the same adsorbent. Moreover β-carotene was easily separated from α-carotene when the carotene fraction of a vegetable extract were filtered through the magnesia adsorbent. The β-carotene which was isolated had absorption maxima in various solvents

Table XXIV. Summary of Absorption Maxima for Bottom Rust Pigment Zone Isolated from the Various Rust Species

Source of the Bottom Rust Spore Pigment	Absorption Maxima in Carbon Disulphide (mu)
1. Wheat stem rust spores	510, 485
2. Wheat stem rust spores	510, 484.5
3. Wheat stem rust spores	505, 480
4. Wheat stem rust spores	510, 482
5. Wheat stem rust spores	508, 485
6. Oat stem rust spores	510, 480
7. Oat stem rust spores	509, 483.5
8. Barley stem rust spores	(510), 482
9. Barley stem rust spores	(510), 483
10. Crown rust of oats, urediospores	(510), 483.5
11. Crown rust of oats, urediospores	510, 483
12. Spores of leaf rust of wheat	508, 481
13. Spores of leaf rust of wheat	510, 482
14. Flax rust spores	(510), 482
15. Flax rust spores	(510), 482

corresponding to the values for the bottom rust spore pigment (see previous section). The final evidence which confirmed the observations indicating that thebottom rust spore pigment was β-carotene were the results of mixed chromatograms.

Petroleum ether solutions of the "bottom" rust pigment and of β-carotene (obtained from vegetables) were adjusted to the same intensity. Three identical tubes were filled with micron brand magnesium oxide and topped with celite. On two tubes solutions of the two pigments to be compared were adsorbed. On the third tube a mixture of equal parts of the unknown and of the β-carotene solutions was adsorbed. The three tubes were connected to the same source of vacuum and the same volume of liquid was allowed to pass through the columns. The three tubes were washed with equal volumes of petroleum ether and of 20 percent benzene in petroleum ether. The color (orange) and the position of the zone in each of the three tubes was the same. No new lines were formed nor separations observed in the mixed chromatogram. The three single orange zones were eluted with 2 percent methanolic petroleum ether and absorption curves for each, in this solution, were determined in the spectrophotometerfrom 500-400 mu. Making the determinations at every 5 mu interval, identical absorption curves were obtained for each of the three zones, with absorption maxima occurring at 475 and 450 mu in each case.

A mixed chromatogram was later developed from the β-carotene isolated from the commercial carotene mixture. A similar procedure was used and the results were the same. Only one orange zone developed on each of the three tubes. Identical absorption curves were obtained for each of the three zones. with the absorption maxima in the eleunt occurring at 475 and 450 mu.

The absorption spectra of the bottom rust spore pigment in carbon disulphide is shown in Figure 1.

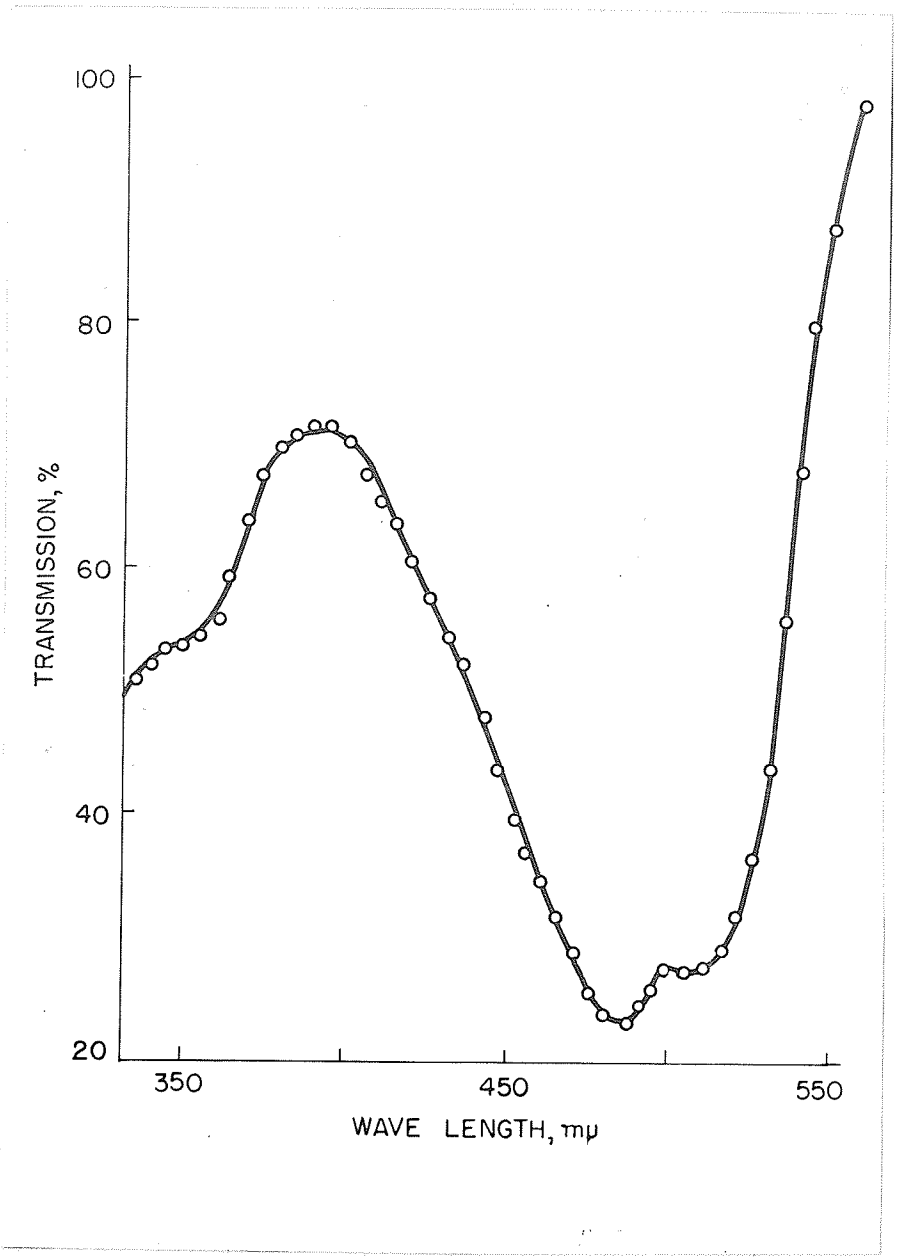


Figure I

The Absorption Spectra of the Bottom Rust Spore Pigment in Carbon Disulphide

9. ISOLATION AND INVESTIGATION OF LYCOPENE AND LYCOXANTHIN EXTRACTED FROM TOMATOES

There seemed a good possibility, from absorption spectra and the hydrocarbon nature of the top pink zone of the rust spores, that this pigment might be lycopene. It was thus decided to obtain a supply of lycopene for reference. As no commercial source of lycopene could be found, it was necessary to isolate this carotenoid pigment from a known and undisputed source. The red pigment of tomatoes has been reported by many workers to be composed predominantly of lycopene. Kuhn and Grundmann (46) reported that fully ripened tomatoes contained 7.85 mg. of lycopene, 0.73 mg. of β-carotene and 0.16 mg. of xanthophylls (including the mono-hydroxy carotenoid lycoxanthin) per 100 g. of fresh fruit. The most important step in the isolation of lycopene was the removal of the total amount of water (97 percent of the weight of fresh tomatoes) from the fresh fruit. The isolated lycopene agreed quite closely in spectral properties with the top pink zone obtained from the rust spores. The isolated lycoxanthin was partitioned between petroleum ether and various concentrations of aqueous methanol and found to agree in relative solubility properties with those given in the literature for mono-hydroxy-carotenoids.

Two pounds of fully ripened tomatoes (900 g. of fresh fruit) were used for the isolation of lycopene according to the following procedure. The seven tomatoes were cut in halves and quarters and churned to a thick soup in a Waring Blendor. This mash was centrifuged for thirty minutes and the supernatant aqueous liquid was decanted. Further dehydration and removal of xanthophylls was carried out with ethanol. The red residue from the centrifuged material was shaken with 1500 ml. of ethanol in a large brown bottle. The mixture was filtered through a fine white cloth in a Buckner suction flask. A turbid yellow solution passed through the cloth leaving a red residue. This

residue was again shaken with 1500 ml. of ethanol and the mixture was allowed to stand for one hour. It was filtered through the same cloth and firmly pressed down to remove most of the clear yellow liquid. This second washing was saved for the isolation of lycoxanthin. The red residue was spread on large sheets of paper and dried at room temperature with the aid of a current of air from a mechanical blower. In order to hasten the drying process this red material was also pressed between filter papers. The 29 g. of dehydrated material were then extracted with 225 ml. of carbon disulphide in one 75 ml. portion and three 50 ml. portions. Very intense purplish or beet red solutions were obtained.

The absorption spectra of some of the purplish pigment before purification by chromatography gave maxima at 543 and 506 mu. A concentrated petroleum ether solution of this material was filtered through an adsorption column packed with micron brand magnesium oxide. A heavy crimson red band formed at the top and an orange line formed down with a white space between these two zones. Even after 90 minutes of developing with petroleum ether, the red band failed to move down appreciably. The thin orange line gave absorption maxima in carbon disulphide at 505 and 481 mu. The absorption curves given by this line were quite similar to those given by the β-carotene zone of vegetables and to the curves obtained from the lowest rust spore pigment zone. From its position in the chromatogram this orange line can be regarded as the β-carotene portion of tomato lipochromes. Its spectral properties lend support to the contention that the lowest rust spore pigment zone is β-carotene. The top red band gave absorption maxima at 543, 506.5 and (480) mu in carbon disulphide and can only be lycopene (or a mixture of lycopene-like carotenoids). These absorption maxima differ to a slight degree from the literature values for crystalline lycopene which were reported as 548, 507.5 and 477 mu (46). However, the absorption curve as a whole, as well as

the absorption maxima, practically coincide with the absorption curves obtained from the top pink zone of the rust spore pigments.

10. THE IDENTIFICATION OF THE TOP RUST SPORE PIGMENT AS LYCOPENE

The final evidence in the identification of the top pink or red rust spore pigment as lycopene was provided by the results of a mixed chromatogram with lycopene isolated from tomatoes. The behavior of this top rust spore pigment on partition between aqueous methanol and petroleum ether, its absorption maxima obtained in carbon disulphide, and its color and position on chromatograms all pointed to its being lycopene. The partition test with 97 percent methanol had shown the top rust spore pigment to be a hydrocarbon. Karrer and Jucker (46) in Table XIII of their text listed lycopene as the only hydrocarbon carotenoid containing its first absorption band in carbon disulphide between 535 and 550 mu. Table XXV which follows below lists absorption maxima for lycopene and summarizes the values obtained for the top rust spore pigment in carbon disulphide. The most reliable values for the top rust pigment were numbers 3, 6 and 9 marked with asterisks in Table XXV. Although these maxima did not coincide with the values for crystalline lycopene given by Karrer and Jucker, they were in close agreement with the maxima obtained in this study for lycopene from tomatoes as well as with Hunter and Scott's values (41) for lycopene from palm oil.

Petroleum ether solutions of the "top" rust spore pigment and lycopene (obtained from tomatoes) were adjusted to the same intensity. Three identical tubes were filled with micron brand magnesium oxide and topped with one and one-half inches of zinc carbonate. On two tubes solutions of the two pigments to be compared were adsorbed. On the third tube a mixture of equal parts of the unknown and of the lycopene solutions was adsorbed. The three tubes were connected to the same source of vacuum and the same volume of liquid were allowed to pass through the columns. The three tubes were washed with equal

volumes of petroleum ether and of 20 percent benzene in petroleum ether. The red pigment in each case moved through the zinc carbonate and was adsorbed near the top of magnesium oxide. Only a single red line was seen in the mixed chromatogram and in the lycopene chromatogram. However two red lines close together were seen in the rust spore pigment chromatogram. The absorption spectra of each pigment zone was determined in carbon disulphide. Making the determinations at every 5 mu interval, absorption maxima were found in each case to occur at 540, 505 and 480 mu. The absorption spectra for the top rust spore pigment is shown in Figure 2.

II. THE BASIS OF THE IDENTIFICATION OF THE MAJOR RUST SPORE PIGMENT AS γ-CAROTENE

The major rust spore pigment has been identified as γ-carotene on the basis of its absorption spectra (including absorption maxima) its epiphasic nature and its position and color in adsorption columns. The absorption curves for this pigment were determined in numerous instances during the course of investigating the pigment content of the various species of rust urediospores. A representative curve is shown in Figure 3. The epiphasic nature of this pigment was determined in the partition tests. The results of chromatographic studies on the major rust spore pigment is described below.

Approximately equal amounts of lycopene (obtained from tomatoes) β- and α-carotenes (obtained from green and yellow vegetables) and the major rust spore pigment were mixed. A concentrated petroleum ether solution of these pigments was filtered through an adsorption column packed with micron brand magnesium oxide. Four lines began to separate out on initial development with petroleum ether. This separation was hastened by developing with 20 percent benzene in petroleum ether and after 90 minutes a very good separation into four distinct bands were seen. A colored illustration of this chromatogram is shown in Figure 4. The white spaces between the colored bands

Table XXV. Comparison of Absorption Maxima for "Top" Rust Pigment and for Lycopene Obtained from Various Sources

Description of Sample	Absorption Maxima in Carbon Disulphide (mu)		
1. Top pigment, wheat stem rust spores	(535),	505,	475
2. Top pigment, wheat stem rust spores	538,	505,	480
3. Top pigment, wheat stem rust spores	*542,	507	
4. Top pigment, wheat stem rust spores	535,	505,	475
5. Top pigment, wheat stem rust spores	535,	505	
6. Top pigment, wheat stem rust spores	*541,	505,	480
7. Top pigment, oat stem rust spores	539,	505,	481
8. Top pigment, barley stem rust spores	538,	500,	475
9. Top pigment, leaf rust spores	*542,	506,	478
10. Top pigment, flax rust spores	540,	500,	475
11. Unchromatographed lycopene from tomatoes	543,	506	
12. Chromatographed lycopene from tomatoes	543,	506.5,	(480)
13. Lycopene, Hunter and Scott (41)	544,	505,	(481)
14. Lycopene + neolycopene (41)	543.5,	505,	(478)
15. Neolycopene (41)	535,	496,	(469)
16. Lycopene, crystalline (46)	548,	507.5,	477

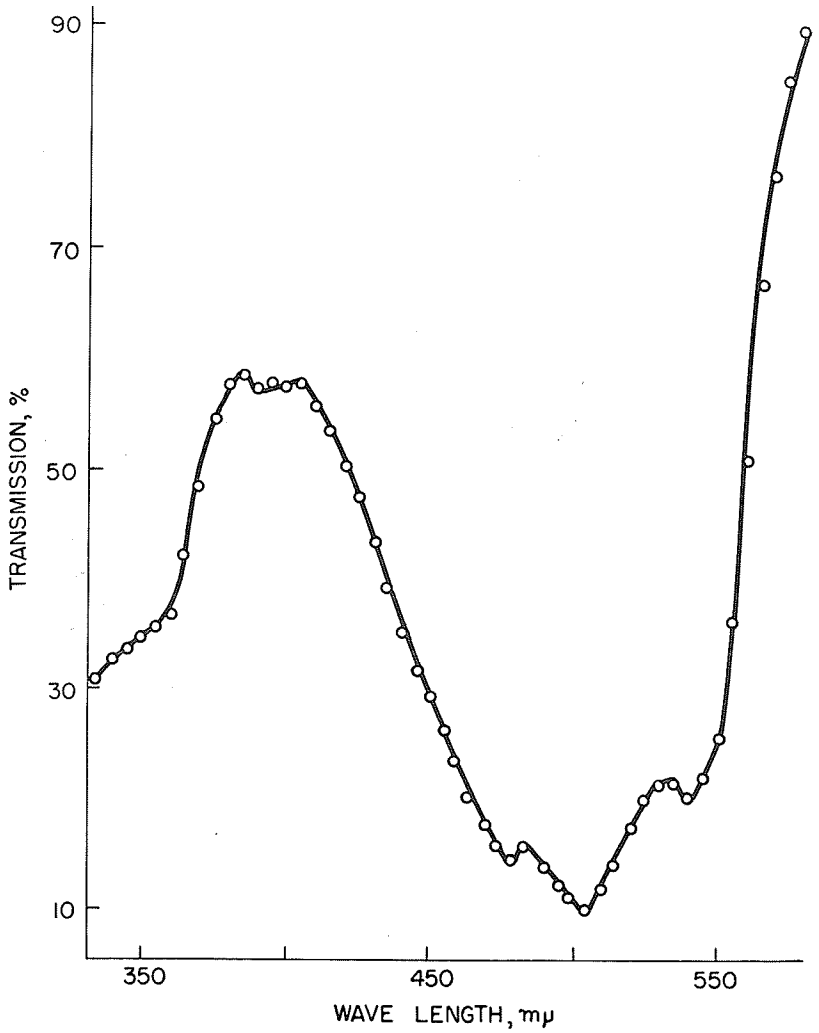


Figure 2

The Absorption Spectra of the Top Rust Spore Pigment in Carbon Disulphide

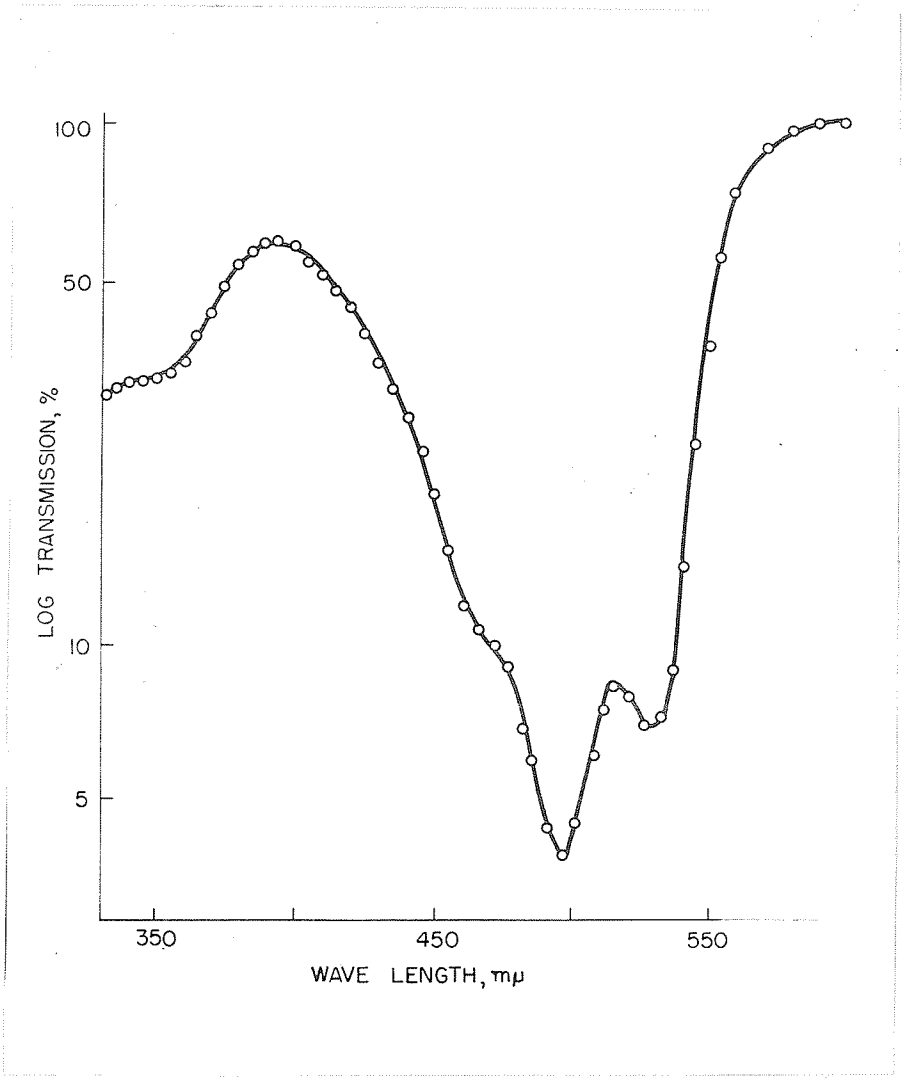


Figure 3

The Absorption Spectra of the Major Rust Spore Pigment in Carbon Disulphide

were at least twice as wide as the bands. A red band was visable almost at the very top of the column. An orange red band appeared not far below it. A yellow orange band was seen lower down in the column and below this was a yellow band. The width of each of these four bands was approximately equal. These colored bands were eluted with 2 percent methanolic petroleum ether and their absorption maxima were determined in carbon disulphide. These values are given in Table XXVI.

Table XXVI. Mixed Chromatogram, Lycopene, β-Carotene, α-Carotene and Major Rust Spore Pigment

	Zone	Color in Eluent	Color in Carbon Disulphide	Absorption Maxima (mu)
1.	Top red band	orange	red orange	542.5, 506.5 (479)
2.	Orange red band	yellow orange	orange	528, 494.5
3.	Yellow orange band	yellow	yellow orange	(510), 481
4.	Yellow band	green yellow	pale yellow orange	507.5, 476

From absorption maxima and appearance the zones 1, 3, and 4 are lycopene, β-carotene and α-carotene, respectively while zone 2 is the major rust spore pigment. Similar results were obtained when calcium hydroxide and alumina were used as the adsorbents and when α- and β-carotene isolated from a commercial mixture were substituted for the vegetable pigments.

The logical sequence for determining the identity of the major rust spore pigment began with an evaluation of the absorption maxima. Although the Beckman spectrophotometer used in these studies could determine the absorption maxima to within 0.5 mu wave length, one could not hope to obtain such close agreement to the literature due to stereoisomerism and to the fact that the pigment was not crystallized. Most of the values for the absorption maxima of the main rust spore pigment in carbon disulphide were at 529 and 496 mu.

Carotenoids with a chromophoric grouping such as that occurring in γ-carotene have absorption maxima close to these values. Besides γ-carotene epiphasic pigments which have been reported to have maxima within 5 mu are rubixanthin, gazaniaxanthin, myxoxanthol and aphanin. Both rubixanthin and gazanixanthin which are natural occurring mono-hydroxy-γ-carotenes are reported to be hypophasic on partition between 95 percent methanol and petroleum ether while the major rust spore pigment was epiphasic under these same conditions. No information was available for myxoxanthol as to its behavior in these same solvents. However this synthetic monohydroxy-γ-carotene is reported (36) not to change color on alumina columns when different solvents were added whereas the major rust spore pigment changed from a red to an orange tinge when benzene replaced petroleum ether on alumina and magnesia columns. Although aphanin was reported to have absorption maxima in carbon disulphide at 533.5 and 494 mu (46) its discoverer did not assign a γ-carotene chromophoric grouping to it. Recently Goodwin (24) pointed out that aphanin is very likely identical with myxoxanthin a mono-carbonyl-γ-carotene. The admission by Tischer that the maxima for aphanin were difficult to determine together with Goodwin's reasoning on the nature of aphanin would tend to exclude it from being the major rust spore pigment.

The position and color of the major rust spore pigment on the adsorption columns was the final evidence in favor of it being γ-carotene. According to Table VI of Karrer and Jucker (46) only γ-carotene is adsorbed between lycopene and β-carotene (apart from pro-γ-carotene and pro-lycopene. Moreover both aphanin and myxoxanthin as well as the mono-hydroxy-carotenoids are reported in the same Table to be adsorbed above lycopene. The only exception reported to the general trend in this Table is cryptoxanthin. This mono-hydroxy-β-carotene was reported (68) to be adsorbed below lycopene on calcium hydroxide but above it on alumina and calcium carbonate. However,

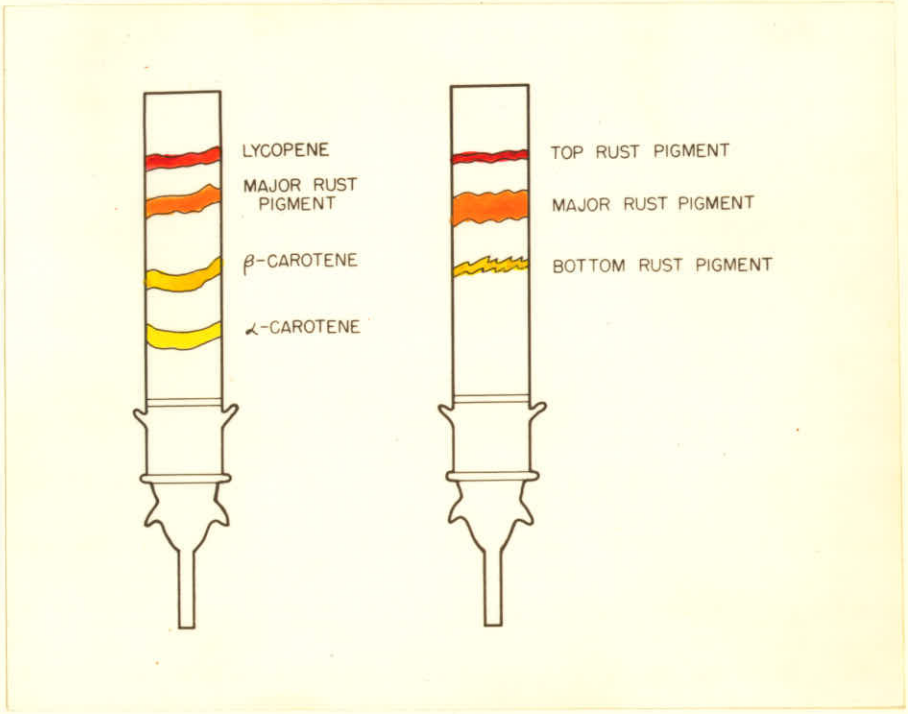


Figure 4

A Comparison of the Color and Position of the Rust Spore Pigments with Known Carotenoid Pigments on M_gO Columns

all the information in the literature states the mono-hydroxy-γ-carotenes are adsorbed above lycopene. In Table I of Strain (88) γ-carotene was reported to be adsorbed between β-carotene and lycopene on magnesium oxide columns when the original solvent was carbon tetrachloride or petroleum ether. Similar results were obtained with the major rust spore pigment using the same brand of magnesium oxide and the same solvents. According to Karrer and Jucker (46) the color of the carotenoids become redder as the number of conjugated double bonds increase in number. γ-carotene should be between β-carotene and lycopene in its shade of color. In the experiments the α-carotene band appeared yellow, the β-carotene appeared light orange, the major rust spore pigment appeared red orange while the lycopene band appeared red. The shade of color exhibited by the major rust spore pigment is the shade of color which γ-carotene would give under these conditions.

SUMMARY AND CONCLUSIONS

1. The carotenoid pigments of the urediospores of the following six species of rust fungi were investigated and identified: stem rust of wheat (Puccinia graminis tritici), stem rust of oats (Puccinia graminis avenae), stem rust of barley (Puccinia graminis hordei), crown rust of oats (Puccinia coronata avenae), leaf rust of wheat (Puccinia triticina) and flax rust (Melampsora lini). Each of the above species was found to contain γ-carotene, β-carotene and lycopene.

2. Visual observation of the three zones in a number of chromatograms for each of the species indicated that the pigments were present in each species in roughly the same proportion. Quantitative determinations of the pigments for the various species showed differences although γ-carotene in each case comprised the bulk of the total carotenoid content. Lycopene was the most easily oxidized of the rust pigments and could not be spectrophotometrically determined for most of the rust species except leaf rust.

3. Stem rust was found to contain 490 p.p.m. of γ-carotene and 210 p.p.m. of β-carotene. Crown rust was found to contain 1090 p.p.m. of γ-carotene and 370 p.p.m. of β-carotene. Leaf rust of wheat was found to contain 1130 p.p.m. of γ-carotene, 360 p.p.m. of β-carotene and 220 p.p.m. of lycopene. Flax rust was found to contain 780 p.p.m. of γ-carotene and 120 p.p.m. of β-carotene.

4. These quantitative determinations indicate that flax rust urediospores contain a smaller proportion of β-carotene while leaf rust of wheat contains the largest proportion of lycopene.

5. This study has been the first general investigation carried out on the carotenoid pigments in the plant order Uredinales using modern

carotenoid research techniques. It is hoped that further work may be carried out on other species and other life phases of the rust fungi to see if the distribution of γ-carotene, β-carotene and lycopene is general for the whole plant order.

BIBLIOGRAPHY

1. Association of Vitamin Chemists, Inc. Methods of vitamin assay. Interscience Publishers Inc., New York (1947).

2. BACHMANN, E. Botanisch-chemische Untersuchungen über Pilzfarbstoffe. Ber deut. botan. Ges. 4: 68 (1886).

3. BERTRAND, G. and POIRAULT, G. Sur la matiere colorante du pollen. Compt. Rend. (Acad. des Sci. Paris) 115: 828 (1892).

4. BONNER, J. Plant Biochemistry. Academic Press Inc., New York (1950).

5. BONNER, J., SANDOVAL, A., TANG, Y.W., and ZECHMEISTER, L. Changes in polyene synthesis induced by mutation in a red yeast. (Rhodotorula rubra). Arch. Biochem. 10: 113 (1946).

6. BULLER, A.H.R. Researches on Fungi. Vol. VII. The sexual process in the Uredinales. Royal Society of Canada. University of Toronto Press (1950).

7. CHANDA, R. and OWEN, E.C. β-carotene in goat liver and colostrum. Biochem. J. 51: iv (1952).

8. CRAIGIE, J.H. Experiments on sex in rust fungi. Nature 120: 116 (1927).

9. CRAIGIE, J.H. Increase in production and value of the wheat crop in Manitoba and Eastern Saskatchewan as a result of the introduction of rust resistant varieties. Sci. Agr. 25: 51 (1944).

10. CRAIGIE, J.H. Epidemiology of stem rust in Western Canada. Sci. Agr. 25: 285 (1945).

11. CRAIGIE, J.H. Economic Diseases of Field Crops in Manitoba. Economic Survey Board, Province of Manitoba (1939).

12. DUTTON, H.J. and MANNING, W.M. Evidence for carotenoid-sensitized photosynthesis in the diatom Nitizschia closterium. Am. J. Botany 28: 516 (1941).

13. EMERSON, R. and LEWIS, C.M. The dependence of the quantum yield of Chlorella photosynthesis on the wave length of light. Am. J. Botany 30: 165 (1943).

14. FISCHER, F.G. and LOWENBERG, K. Synthesis of phytol. Ann. 475: 183 (1929) as read in Chem. Abst. 24: 595 (1930)

15. FOX, D.L. and EMERSON, R. γ-carotene in the sexual phase of the aquatic fungus Allomyces. Proc. Roy. Soc. (London) Series B 128: 275 (1939).

16. FREY-WYSSLING, A. Die stoffausscheidungen der höheren planzen. Springer, Berlin (1935). as read in Bonner, Plant Biochemistry. Academic Press Inc., New York (1950).

17. GARTON, G.A., GOODWIN, T.W., and LIJINSKY, W. The biogenesis of β-carotene in the fungus, Phycomyces blakesleeanus. Biochem. J. 46: XXXV. (1950).

18. GARTON, G.A., GOODWIN, T.W., and LIJINSKY, W. Studies on carotenogenesis I. General conditions governing β-carotene synthesis by the fungus, Phycomyces blakesleeanus Bergeff. Biochem. J. 48: 154 (1951).

19. GILLAM, A.E. and EL-RIDI, M.S. Adsorption of grass and butter carotenes on alumina. Nature 136: 914 (1935).

20. GILLAM, A.E. and EL-RIDI, M.S. The isomerization of carotenes by chromatographic adsorption. Biochem. J. 30: 1735 (1936).

21. GILLAM, A.E. and EL-RIDI, M.S. The carotenes of milk fat (butter). Biochem. J. 31: 251 (1937).

22. GISVOLD, O. and ROGERS, C.H. The Chemistry of Plant Constituents. Burgess Publishing Co., Minneapolis, Minn. (1938).

23. GLOVER, J., GOODWIN, T.W., and LIJINSKY, W. Further observations on carotenogenesis by Phycomyces blakesleeanus. Biochem. J. 50: X (1951).

24. GOODWIN, T.W., and TAHA, M.M. The carotenoids of the gonads of the limpets Patella vulgata and Patella depressa. Biochem. J. 47: 224 (1950).

25. GOODWIN, T.W., LIJINSKY, W., and WILLMER, J.S. Nitrogen metabolism and carotenogenesis by Phycomyces blakesleeanus. Biochem. J. 49: liii (1951).

26. GOODWIN, T.W. and LIJINSKY, W. Studies in carotenogenesis. 2. Carotene Production by Phycomyces blakesleeanus: The effect of different animo acids when used in media containing low concentrations of glucose. Biochem. J. 50: 268 (1951).

27. GOODWIN, T.W. Studies in carotenogenesis. 3. Identification of the minor polyene components of the fungus Phycomyces blakesleeanus and a study of their synthesis under various cultural conditions. Biochem. J. 50: 550 (1952).

28. GOODWIN, T.W. and WILLMER, J.S. Studies in carotenogenesis. 4. Nitrogen metabolism and carotene synthesis in Phycomyces blakesleeanus. Biochem. J. 51: 213 (1952).

29. GOODWIN, T.W. Fungal carotenoids. Botanical Review 18: 291 (1952).

30. GOODWIN, T.W. The carotenoids of the berries of Lonicera japonica. Biochem. J. 51: 458 (1952).

31. GROVE, W.B. The British rust fungi (Their biology and classification). Cambridge University Press (1913).

32. HALL, J.A. A system of structural relationships in phytochemistry. Chem. Revs. 20: 306 (1937).

33. HANNA, W.F. Studies on the nature of rust resistance in wheat. V. Physiology of the host. Can. J. Research 4: 134 (1931).

34. HEILBRON, I.M. Some aspects of Algal Chemistry. Eight Hugo Müller lecture. J. Chem. Soc. (London) 1942: 79.

35. HEILBRON, I.M., and COOK, A.H. Carotenoids and vitamin A - the end of a chapter. Endeavour 10: 175 (1951).

36. HEILBRON, I.M. and LYTHGOE, B. Carotenoid pigments of Oscillatorio rubrescens. J. Chem. Soc. (London) 1936: 1376.

37. HEILBRON, I.M. LYTHGOE, B., and PHIPHERS, R.F. A new type of plant lipochrome (in Rivularia nitidia). Nature 136: 989 (1935).

38. HEILBRON, I.M., MORTON, R.A., and WEBSTER, E.T. The structure of vitamin A. Biochem. J. 26: 1194 (1932).

39. HEILBRON, I.M., PARRY, E.G., and PHIPHERS, R.F. The algae. II. The relationship among certain algal constituents. Biochem. J. 29: 1376 (1935).

40. HERZIG, J. and FALTIS, F. Zur Kenntnis des Bixins. Liebigs Ann. 431: 40 (1923).

41. HUNTER, R.F. and SCOTT, A.D. Palm oil carotenoids. Biochem. J. 35: 31 (1941).

42. JOHNSON, T. Private communication.

43. KARRER, P. Carotenoid pigments. Endeavour 7: 3 (1948).

44. KARRER, P. Organic Chemistry. Fourth English edition, 48 (1950). Elsevier Publishing Co. Inc., New York.

45. KARRER, P. and HELFENSTEIN, A. The coloring materials of plants. XVI Carotene. Helv. Chim. Acta. 12: 1142 (1929). (as read in Chem. Abst. 24: 1387 (1930)

46. KARRER, P. and JUCKER, E. Carotenoids. English Edition (1950). Elsevier Publishing Co. Inc., New York.

47. KARRER, P. and MORF, R. Plant pigments XXXI. The constitution of the second form of carotene (α-carotene). Helv. Chim. Acta. 14: 833 (1931). as read in Chem. Abst. 25: 4531 (1931)

48. KARRER, P. and MORF, R. Plant pigments XXXV. The constitution of

β-carotene and β-dihydrocarotene. Helv. Chim. Acta. 14: 1033 (1931). as read in Chem. Abst. 26: 733 (1932)

49. KARRER, P., MORF, R., and SCHOPP, K. Vitamin A from fish oils. Helv. Chim. Acta. 14: 1036, 1431 (1931). as read in Chem. Abst. 26: 4359 (1932)

50. KARRER, P., MORF, R., and WALKER, O. Plant pigments. LII. The constitution of α-carotene. Helv. Chem. Acta. 16: 975 (1933). as read in Chem. Abst. 28: 161 (1934)

51. KARRER, P. and WALKER, O. Plant pigments. LI. Pure α-carotene. Helv. Chim. Acta. 16: 641 (1933). as read in Chem. Abst. 27: 4800 (1933).

52. KARRER, P. et al. Plant pigments. XXV. The constitution of lycopin and carotene. Helv. Chim. Acta. 13: 1084 (1930). as read in Chem. Abst. 25: 519 (1931).

53. KARRER, P. et al. Plant pigments. XXIX. The symmetrical lycopin formula. Perhydrolycopin. Helv. Chim. Acta. 14: 435 (1931). as read in Chem. Abst. 25: 2733 (1931)

54. KARRER, P. et al. Consideration of the structure of carotenoid epoxides. Helv. Chim. Acta. 28: 300 (1945). as read in Chem. Abst. 40: 1505 (1946)

55. KARRER, P. et al. Function of γ-carotene in Alloymces. Helv. Chim. Acta. 26: 2121 (1943) as read in Karrer and Jucker, Carotenoids. 11 (1950) Elsevier Publishing Co. Inc., New York.

56. KARRER, P. et al. Plant pigments. XIII. Bixin. Helv. Chim. Acta 12: 741 (1929). as read in Chem. Abst. 23: 4480 (1929)

57. KUHN, R. and BROCKMANN, H. γ-carotene. (Uber das Vitamin des Wachstums, IV Mitteil). Ber. 66: 407 (1933).

58. KUHN, R. and BROCKMANN, H. Über Rhodo-xanthin, den Arillus-Farbstoff der Eibe (Taxus baccata). Ber. 66: 828 (1933).

59. KUHN, R. and GRUNDMANN, C. Uber Rubixanthin, ein neues Xanthophyll der Formel C_{40} $H_{56}O$. Ber. 67: 339 (1934).

60. KUHN, R. and GRUNDMANN, C. Uber Kryptoxanthin, ein Xanthophyll der Formel C_{40} $H_{56}O$. (Uber das Vitamin des Wachstums, V Mitteil.) Ber. 66: 1746 (1933).

61. KUHN, R. and LEDERER, E. Zerlegung des carotins in seine komponenten. (Uber das Vitamin des wachstums, I. Mitteil.) Ber. 64: 1349 (1931).

62. KUHN, R., MOEWUS, F., and JERCHEL, D. Uber die chemische natur der stoffe, welche die Kopulation der mannlichen und weiblichen Gameten von Chlamydomonas eugamentos im Lichte bewirken. Ber 71: 1541 (1938).

63. KUHN, R. and WINTERSTEIN, A. Thermischer Abbau der Carotinfarbstoffe. Ber. 65: 1873 (1932).

64. LEDERER, E. Sur les carotenoids de quelques champignons. Compt. rend. soc. biol. 117: 1083 (1934).

65. LEDERER, E. Sur les carotenoids des cryptogames. Bull. soc. chim. biol. 20: 611 (1938).

66. LEDERER, E. Sur les carotenoids des bas invertebres. Bull. Soc. Chim. Biol. 20: 554 (1938).

67. LEDERER, E. Echinenone et pentaxanthine; deus nouveaux carotenoides trouves dans l'oursin. Compt. Rend. (Acad. des Sci. Paris). 201: 300 (1935).

68. LE ROSEN, A.L. A method for standardization of chromatographic analysis. J. Am. Chem. Soc. 64: 1905 (1942).

69. LE ROSEN, A.L. and ZECHMEISTER, L. Prolycopene. J. Am. Chem. Soc. 64: 1075 (1942).

70. LE ROSEN, A.L. and ZECHMEISTER, L. The carotenoid pigments of the fruit of Celastrus Scandens L. Arch. Biochem. I: 17 (1942).

71. LUTERAAN, P.J., CHAMPEAU, M.F., and CHOAY, J. Role of certain pigments in cellular respiration. Compt. rend. soc. biol. 141: 616 (1947). as read in Chem. Abst. 42: 1986 (1948)

72. MACKINNEY, G. On the crystal structure of carotenoids. J. Am. Chem. Soc. 56: 488 (1934).

73. MACKINNEY, G. Properties of carotenes from certain roots and leaves at various stages of development. J. Biol. Chem. 108: 45 (1935).

74. MUNSEY, V.E. Carotenoid pigments in flour. J. Assoc. Official Agr. Chem. 21: 337 (1938).

75. NEWTON, M. and JOHNSON, T. Color mutations in Puccinia Graminis Tritici. Phytopathology 17: 711 (1927)

76. NEWTON, M., JOHANNSON, H., and JOHNSON, T. A study of the carotenoid pigments of the urediospores of wheat stem rust and four of its color variants. Phytopathology 25: 30 (1935).

77. NOACK, K. Photochemische wirkungen des chlorophylls und ihre bedeutung fur die kohlensäure assimilation. Z. Botan. 17: 481 (1925).

78. PALMER, L.S. Carotenoids and related pigments. Chemical Catologue Co., New York (1922).

79. PAULING, L. Recent work on the configuration and electronic structure of molecules with some applications to natural products. Fortschr. Chem. Organ. Naturstoffe 3: 203 (1939). as read in Chem. Revs. 34: 267 (1944)

80. PETIT, A. Nouvelles Observations Sur Les Rouilles Des Cereales. Moyens De Preservation. Ann. du Service Bot. Agron. de Tunisie 19: 113 (1946).

81. POLGAR, A. and ZECHMEISTER, L. Isomerization of β-carotene. Isolation of a stereoisomer with increased adsorption affinity. J. Am. Chem. Soc. 64: 1856 (1942).

82. POLGAR, A. and ZECHMEISTER, L. Action of cold conc. HI on carotenes: Structure and some cis-trans-isomerization of some reaction products. J. Am. Chem. Soc. 65: 1528 (1943).

83. QUACKENBUSH, F.W., STEENBOCK, H., and PETERSON, W.H. The effects of acids on carotenoids. J. Am. Chem. Soc. 60: 2937 (1938).

84. SCHON, K. Studies on carotenoids. V. Gazaniaxanthin. Biochem. J. 32: 1566 (1938).

85. SCHROEDER, W.A. Formation of Pro-Carotenoids in "Monkey Flowers" under some conditions. J. Am. Chem. Soc. 64: 2510 (1942).

86. SPOER, H.A. The culture of the albino maize. Plant Physiol. 17: 397 (1942).

87. STRAIN, H.H. Carotene VIII. Separation of carotenes by adsorption. J. Biol. Chem. 105: 523 (1934).

88. STRAIN, H.H. Leaf xanthophylls. Carnegie Institute of Washington. Pub. 490 (1938).

89. TISCHER, J. Carotenoids of fresh water algae. IV. Polyene pigments of the blue alga Aphanizomenon flos-aquae. I. Z. physiol. chem. 251: 109 (1938). as read in Chem. Abst. 32: 3454 (1938)

90. TSWETT, M. Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. Ber. deut. botan. Ges. 24: 384 (1906).

91. WENT, F.A.F.C. Rec. trav. botan. neerland 1: 106 (1904). as read in Karrer and Jucker, Carotenoids 10 (1950). Elsevier Publishing Co. Inc., New York.

92. WHITE, J.W., ZSCHEILE, F.P., and BRUNSON, A.M. The carotenoids of yellow corn grain. J. Am. Chem. Soc. 64: 2603 (1942).

93. WILLIAMS, T.I. An introduction to chromatography. Chemical Publishing Co., Brooklyn, New York (1947).

94. WILLSTATTER, R. and MIEG, W. Uber die gelben begleiter des chlorophylls. Liebigs Ann. 355: 1 (1907).

95. WILLSTATTER, R. and STOLL, A. Untersuchurgen uber aas chlorophyll. Berlin (1913). as read in Karrer and Jucker, Carotenoids 21 (1950) Elsevier Publishing Co. Inc., New York.

96. WILLSTATTER, R. and STOLL, A. Untersuchungen uber die assimilation der kohlensaure. Berlin (1918). as read in Karrer and Jucker Carotenoids 10 (1950) Elsevier Publishing Co. Inc., New York.

97. ZECHMEISTER, L. and CHOLNOKY, v.L. Lycoxanthin und Lycophyll, zwei naturliche Derivate des Lycopins. Ber. 69: 422 (1936).

98. ZECHMEISTER, L. and CHOLNOKY, v.L. Die chromatographische adsorptionsmethode. J. Springer, Berlin (1937).

99. ZECHEMISTER, L. and POLGAR, A. Cis-trans isomerization and spectral characteristics of carotenoids and some related compounds. J. Am. Chem. Soc. 65: 1522 (1943).

100. ZECHMEISTER, L. and POLGAR, A. Cis-trans isomerization and cis-peak effect in the α-carotene set and in other stereoisomeric sets. J. Am. Chem. Soc. 66: 137 (1944).

101. ZECHMEISTER, L. and POLGAR, A. Contributions to the stereochemistry of γ-carotene. J. Am. Chem. Soc. 67: 108 (1945).

102. ZECHMEISTER, L. and SANDOVAL, A. Phytofluene. J. Am. Chem. Soc. 68: 197 (1946).

103. ZECHMEISTER, L. and SCHROEDER, W.A. Pro-γ-carotene. J. Am. Soc. 64: 1173 (1942).

104. ZECHMEISTER, L. and SCHROEDER, W.A. Cis-trans isomerization and spectral characteristics of gazaniaxanthin. Further evidence of its structure. J. Am. Chem. Soc. 65: 1535 (1943).

105. ZECHMEISTER, L. and TUZSON, P. Spontaneous isomerization of lycopene. Nature 141: 249 (1938).

106. ZECHMEISTER, L. and TUZSON, P. Isomerization of carotenoids. Biochem. J. 32: 1305 (1938).

107. ZECHMEISTER, L. Cis-trans isomerization and stereochemistry of carotenoids and diphenylpolyenes. Chem. Revs. 34: 267 (1944).

108. ZECHMEISTER, L. et al. Spectral characteristics and configuration of some stereoisomeric carotenoids including prolycopene and pro-γ-carotene. J. Am. Chem. Soc. 65: 1940 (1943).