STUDIES ON THE METABOLISM OF LIPIDS IN PLANTS

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ABSTRACT

This work is concerned with some physiological and biochemical studies of the lipids of higher plants, a subject in which only an extremely limited number of studies have previously been made.

From a study of plants grown under controlled conditions, it was found that both character and amount of fat and wax produced by a plant may be affected by a factor of two or three by day and night temperatures and soil moisture. The effect of increased day or night temperature on the yield of fat was different for different species. Plants generally responded to increased temperature by producing less wax. The fats and waxes from plants at high temperatures were of a higher melting point than those from plants at low temperatures. Water stress plants also produced large amounts of fat, or in Larrea, resin. Although the wax content was only slightly affected by low soil moisture, in Nicotiana glauca an abundant formation of cuticle occurred under this condition. These and other effects of climate on lipids were discussed.

Whereas changes in climate effect up to three-fold changes in lipid yield, a series of recessive genes in corn was found to control ten-fold changes in wax yield. The genetic factors also affect the character and yield of the fats.

A system was obtained for studying the synthesis of fats in a higher plant. Preliminary results show that short chain

compounds (ethyl alcohol, acetate, acetone, acetoacetate) may be rapidly utilized in the synthesis of fat. These substrates are readily used only when an energy source such as sugar and the vitamin biotin are supplied. The effects of other substrates and vitamins on fat synthesis were also studied and found to be small or altogether absent.

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I. INTRODUCTION

The lipids constitute a large and heterogeneous group of substances of great biological importance, for lipids of one or more types occur in all living cells and in many metabolic capacities. The lipids, in general, may be defined as those naturally occurring substances which contain higher fatty acids or their immediate derivatives as major components. They are characterized by their general insolubility in water and solubility in organic solvents such as ether, petroleum ether, benzene, and chloroform. Chemically the group may be divided as follows:

Fats - esters of fatty acids with glycerol, usually triglycerides.

Waxes - esters of fatty acids with alcohols other than glycerol, usually long chain aliphatic monohydric alcohols; also related hydrocarbons, alcohols, and ketones.

Phospholipids - substituted fats containing phosphoric acid and a nitrogenous base; some phospholipids contain inositol in place of glycerol.

Phosphatidates - phospholipids without the nitrogen base, usually a calcium or magnesium salt.

Free components - fatty acids, glycerol, long chain alcohols, hydrocarbons, ketones, etc.

In addition to these compounds, certain other non-fatty substances often are associated with the true lipids in plant extracts. These are the sterols, which may occur free or as esters of fatty acids; terpenes, resins, resin acids, and

carotenoids, all of which are isoprenoid compounds with solubilities very similar to the lipids; and the chlorophylls. Although these substances are not related to lipids, with the exception of those sterols which are esterified with fatty acids (wax-like in nature), it is for all practical purposes impossible to avoid contamination of a plant lipid extract with these cellular constituents. This fact has led to considerable confusion and error in the literature. Thus the "lipid" fraction has often been extracted by solvents not specific for the true lipids so that other substances, particularly the resins, have been included in the "lipid" fraction.

Plant fats have been studied in great detail, notably by Hilditch, Jamieson, Markley, and Ralston, yet much remains to be learned. The component fatty acids of many fats are well known chemically, but the arrangement of these acids in the glyceride has been very difficult to determine since all fats are mixtures of various mixed triglycerides. The method generally used to determine the glyceride composition of a fat first involves hydrolysis of the fat and then the quantitative identification of the constituent fatty acids by fractional crystallization or fractional distillation of their methyl or ethyl esters. On the basis of such analytical data several theories of triglyceride structure have been proposed, notably the even distribution theory of Hilditch (1948) and the theory of partial random pattern of Doerschuk and Daubert (1948).

The even distribution theory of Hilditch (1948) states that if one-third of the total fatty acids consists of FA_1, then one molecule of this fatty acid will occur in most of the triglyceride molecules; if FA_1 accounts for 33 to 67 percent of the total fatty acids then two molecules of FA_1 will occur in most of the triglyceride molecules; if FA_1 makes up more than 67 percent of the total fatty acids then some simple triglycerides containing three molecules of FA_1 will occur in the fat. Although the even distribution theory is in accord with some of the analytical data, it is not in agreement with all. Doerschuk and Daubert (1948) have therefore modified this theory and formulated the concept of the partial random pattern of glyceride composition. The principle of this theory is that the more predominant a certain fatty acid is in a fat, the more likely that this fatty acid will occur one or more times in each triglyceride. Thus the concept of the partial random pattern removes the restriction of Hilditch's theory that if a fatty acid amounts to 33 percent of the total fatty acids it must occur once in each triglyceride. Furthermore, if a fatty acid (FA_1) is present to the extent of 33 to 67 percent of the total fatty acids, three molecules of FA_1 may constitute a simple triglyceride in a few cases, although some triglycerides will also occur which contain no molecules of FA_1. In support of their concept of partial random distribution, Doerschuk and Daubert present data on corn oil, and it is quite remarkable how closely the observed values agree with those calculated on the basis of their theory. Moreover, their data do not agree with the

even distribution theory of Hilditch. It may well be that this new theory of partial random distribution offers a more nearly correct solution to the problem of triglyceride arrangement and composition than any of the preceding theories.

The fatty acids which occur esterified with glycerol in higher plant fats are chemically well known. They are all long straight chain aliphatic monocarboxylic acids containing an even number of carbon atoms. The most common fatty acids are palmitic (CH_3-$(CH_2)_{14}$-COOH), stearic (CH_3-$(CH_2)_{16}$-COOH), oleic (CH_3-$(CH_2)_7$-CH:CH-$(CH_2)_7$-COOH), linoleic (CH_3-$(CH_2)_4$-CH:CH-CH_2-CH:CH-$(CH_2)_7$-COOH), and linolenic (CH_3-CH_2-CH:CH-CH_2-CH:CH-CH_2-CH:CH-$(CH_2)_7$-COOH) acids. Other fatty acids may occur in rather high quantities in certain plant families, for example, myristic acid in the Myristicaceae, lauric and myristic acids in the Lauraceae, etc., but the amounts of these acids produced in the world each year do not approach the world production (both natural and commercial) of the C_{16} and C_{18} acids. Hydroxylated fatty acids also are of rather common occurrence, but they rarely occur in any significant quantity. Many of the most common acids differ only in degree of unsaturation, and this is undoubtedly the most important chemical difference between the fatty acids which occur in fats.

Fats and the constituent fatty acids are solids or liquids depending upon chain length and degree of unsaturation. The solid fats are commonly referred to as fats, whereas the liquid fats are called oils. Fats and fatty acids, with the exception of the

lower fatty acids of eight carbons or less, are quite insoluble in water but are readily soluble in ether, acetone, and other organic solvents. Although there have been many quantitative chemical studies of fats and fatty acids, as mentioned earlier, these studies require extremely difficult and laborious procedures of fractional distillation or crystallization, and as yet only macro methods requiring 10 to 100 grams of lipid are available (Hilditch, 1947). Chromatographic techniques may in the future be very useful in fat analysis, but as yet no very acceptable methods are available, particularly for the study of small quantities of lipid.

Because of the absence of any generally useful micro methods for characterization of the fatty acid of a fat, several qualitative tests have been used to characterize fats and fatty acids. Each of these tests serves to measure the over-all average of a particular chemical property. Thus the iodine number indicates the degree of unsaturation, the acetyl number indicates the degree of hydroxylation, the acid number indicates the amount of free acid in the sample, the saponification number indicates the amount of saponifiable matter (esters and free acids), and the melting point in conjunction with the iodine number indicates the chain length. There are other determinations of lesser importance. Although none of these tests tells us the exact chemical constitution of the lipid, they do serve to indicate by means of rapid, and in some cases micro, techniques

the over-all nature of the complex lipid. For these reasons they have received wide acceptance and use among fat chemists and in the fat industry.

The vegetable waxes differ from fats mainly in chain length of the constituents and because the waxes do not contain any glycerol. A wax has been defined as an ester of a long chain fatty acid with a long chain monohydric alcohol, but most waxes are more correctly complex mixtures of esters, free acids and alcohols, hydrocarbons, and other constituents such as resins, lactones, etc. Thus the economically important palm tree wax, carnauba wax, consists of 80 to 81 percent alkyl esters of wax acids, 1 to 1.5 percent free wax acids, 9 to 10 percent free monohydric alcohols, 3 to 5 percent lactones, 1 to 2 percent free polyhydric alcohols, up to 1 percent hydrocarbons, and 3 to 4 percent resin (Warth, 1947).

Because of the complexity of plant waxes, detailed studies on wax constituents have not been numerous. Analysis is quite difficult because the constituents are of great chain length, sometimes up to 34 or 36 carbons, thus making the homologues of the fatty acid or alcohol series very similar chemically. Other complications also arise such as the occurrence of branched chain acids in the waxes of tubercle bacilli (Velick, 1944; Ginger and Anderson, 1944ab).

Although the straight chain acids and alcohols which occur in waxes all contain an even number of carbon atoms, the wax

hydrocarbons are distinguished by the fact that they all have an odd number of carbon atoms. Chibnall and Piper (1934) have postulated that the wax hydrocarbons arise by decarboxylation and reduction of the next higher beta-keto acid, but it is also possible that they are derived by direct decarboxylation of the corresponding wax acid.

Waxes are usually rather hard or brittle, colored, and are more difficultly soluble in organic solvents than are fats. Waxes are, in fact, insoluble in cold acetone, in which fats are readily soluble, and this has been the basis of separation of the two groups by the method of Chibnall et al. (1931).

Again, as in the case of fats, because detailed analysis is extremely difficult, most workers have made use of general characterization tests such as melting point, iodine number, etc. These, as before, give a rapid convenient guide to the nature of the waxes.

Phospholipids occur widely in the plant kingdom, but their chemistry is as yet very incomplete. In general their fatty acid composition is similar to that of the fats, while the nitrogenous bases are usually choline, as in the lecithins, or ethanolamine as in the cephalins. Other nitrogenous bases undoubtedly occur but no broad search has been made for them. Phospholipids show many properties similar to those of waxes, particularly in regard to their insolubility in many organic solvents, as for example, acetone. It is therefore quite difficult to separate phospholipids and waxes in a total lipid extract, but in general the phospholipids constitute only a minor fraction of the total

lipids so that this problem is not a serious one.

As mentioned earlier, lipids of one type or another occur in every living cell. For the most part the lipids constitute a small part of the total dry weight of the cell, but certain tissues and cytoplasmic structures may attain a high lipid content. Thus many fatty seeds, such as flax, castor bean, and sunflower, contain up to 50 percent dry weight of fat, and even in the corn fruit, which is best known for its starch content, the embryo is about half oil.

Fats occur in every organ of the plant, but the vegetative organs contain low concentrations as compared to the seeds and fruits. In vegetative tissues fats are dispersed as very small droplets in the cytoplasm, or they may be intimately associated with proteins and other substances, as in mitochondria (Newcomer, 1951) and chloroplasts (Rabinowitch, 1945). In storage tissues, in which the fat content may reach high proportions, fat droplets of 20 to 30 microns in diameter often occur (Pack, 1925). The plasma membrane has been demonstrated to contain phospholipids, but the role of these substances in regard to permeability of the membrane remains obscure. Undoubtedly fats, phospholipids, and their constituent compounds also are dissolved in the cytoplasm, at least to a limited extent.

Phospholipids occur in the same organs and plant parts as the fats, but they do not occur as droplets as do the latter. Although the phospholipids never occur in such high concentrations as fats, in some fatty seeds, such as soybean, phospholipids

containing either inositol or glycerol constitute up to 4 percent of the dry weight (Jamieson, 1943). In seeds a large part of the phospholipid present may be in the form of the K, Ca, or Mg salt of the phosphatides. This fraction disappears as the seed germinates and does not reappear to any great extent in the seedling, thus indicating that it is a storage substance in the seed. Chloroplasts and mitochondria also contain a high proportion of phospholipids. Here again, as in the case of fats, they do not occur in the free state, but rather are bound intimately with the proteins of these structures (Rabinowitch, 1945; Nyman and Chargaff, 1949). In view of these facts it appears that, although phospholipids may function as reserve material in seeds, in the vegetative shoots they are closely associated with the structural morphology of the cell and the functions of the cytoplasm.

Most textbooks state that wax occurs as a rather thick layer on the epidermal surface of leaves, stems, flowers, and fruits. Although this outer layer or cuticle is certainly the site of most abundant wax accumulation, the status of waxes in plants is definitely more complex.

Cuticular structure has received considerable study in recent years from several experimental approaches including x-ray patterns, electron microscopy, chemical analysis, and anatomical studies. Submicroscopic studies have shown that the cuticle is composed of several substances, including waxes,

cellulosic material, cutin*, and pectins, each of which is laid down by the plant in a rather orderly fashion (Anderson, 1934; Meyer, 1938; and others). Thus a typical cuticle consists of alternate layers of cellulosic material (including pectins) and cutin, with wax interspersed within the cutin layers. Close to the epidermal wall the cuticle largely consists of cellulosic material and pectins with very little wax, and as one proceeds toward the outer surface of the cuticle the polysaccharide content decreases while the component waxes and cutin increase.

A more thorough study by Kreger (1948) of the submicroscopic structure of the cuticle of sugar cane indicates an even more elaborate arrangement of the wax molecules. Kreger has concluded from x-ray diffraction work that wax occurs in rods which are arranged perpendicularly to the surface of the cuticle. This is in accord with the studies just cited. Furthermore, each rod consists of aggregates or bundles of aggregates of ribbon-like crystallites lying parallel to the longitudinal direction of the rods. The ribbons are about 2000 to 5000 Å broad and 200 to 500 Å thick, and the individual wax molecules are packed adjacent to each other with the longitudinal axis of each wax molecule lying perpendicular to the longitudinal direction of the ribbon. It is very likely that many variations of this cuticular structure

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* The chemical nature of cutin is very indefinite. Most likely cutin is closely related chemically to suberin, which is known to be composed of long chain dicarboxylic acids and hydroxy acids.

exist, particularly in the chemically very complex cuticles such as of the carnauba palm, but Kreger's work is definitely an important step toward elucidating the structure of this physiologically important layer.

The mode of cuticle formation has not been demonstrated, but there are some important considerations to be noted.

Many workers have described various anatomical aspects of the cuticle, but the work of Skoss (1951) perhaps best shows the gross cuticular characteristics. The technique of Skoss utilizes the bacterium Clostridium acetobutylicum, a polysaccharide digesting organism. When this bacterium is incubated with leaves or other plant material it digests the cell walls but the cuticle remains more or less intact. Obviously the cellulosic constituents of the cuticle will also be attacked, but the remaining cutin and wax components are sufficient to retain the original shape and form of the cuticle. After careful washing the pure cuticle can be studied. The following features have been observed by Skoss: 1. The cuticle is more or less continuous over the epidermal surface including the guard cells and the substomatal chamber. It may even surround some of the chlorenchyma adjacent to this cavity. 2. Cuticular pegs penetrate deeply between the epidermal cells. 3. Plasmodesmata or similar pores frequently extend from the epidermal cell through the cuticle to the external surface. 4. The cuticle may be stratified. These observations, which are in excellent accord

with the earlier literature (see Kreger, 1948), show that both the epidermal and chlorenchyma tissues may secrete wax and other cuticular constituents, and that these materials are exuded through minute pores to the external cuticle surface. Indeed, Pohl (1928) has observed a liquid secretion of wax through minute cuticular pores in two species. Such secretion may further account for the rod and ribbon type structure of the cuticle.

It is now of interest to determine what role the epidermal and mesophyll cells play in wax formation and secretion. Anderson (1934) suggested that in Clivia the cuticular materials are all the result of the activities of the epidermal cells. The ability to form a cuticle cannot be restricted to the epidermal cells, however, in view of the observation of Skoss that the mesophyll cells which line the substomatal chamber may be heavily cuticularized. Apparently the formation of a cuticle is much more complex than previously supposed.

If it is a general fact that the wax is secreted in a liquid state, then it must be assumed that either the solvent evaporates or condenses to leave the solid wax rods, or that the wax polymerizes or is otherwise chemically changed at the external surface so as to become a solid wax. The evidence favors the first hypothesis. It was shown by Channon and Chibnall (1929), Pollard et al.(1931, 1933), and Chibnall et al.(1933) that the leaves of cabbage, wheat, and others contain cytoplasmic waxes to a limited extent. The liquid seed storage wax of Simmondsia

californica is also well known (Warth, 1947). Thus the cytoplasm is capable of constructing the long carbon chains of waxes, and as will be shown in Part III, the genetic constitution of the plant exerts control over this formation and ultimate exudation. It is also apparent that any climatic factors which affect the growth and metabolism of the plant will have an effect on the formation of the cytoplasmic wax, the rate of wax exudation, and possibly the oxidation or polymerization of the waxes at the cuticle surface. Such effects of environment on waxes and related lipids will be discussed in Part II.

As we have seen there is a large body of knowledge on the chemical constitution of plant fats, waxes, and other lipids. We have, however, but little understanding of the biological aspects of the plant lipids. In view of the great economic importance of plant lipids to the fields of food technology, detergents, polishes, human nutrition, paints, cosmetics, and many others, research on the biochemical, physiological, and genetic aspects of the lipids is certainly warranted. The present study has been an attempt to partially elucidate three fundamental problems of plant lipid metabolism. This introduction, Part I, is followed by Part II which discusses some effects of environment on lipids; Part III covers the genetic control of wax formation by a series of genes in corn; and Part IV takes up some problems of the synthesis of fats in seeds.

II. ENVIRONMENTAL EFFECTS ON LIPIDS

A. Introduction

Climate exerts a profound influence on the growth rate and ultimate shape and form of plants, but little is known about the effect of climate on the substances of which the plant is composed. In spite of the great economic importance of a knowledge of the climatic control of plant growth and resultant composition, most of the data available are derived from observations in the field, and few experiments have been designed with the principal objective of correlating a specific climatic factor with the yield and type of compound formed by the plant. That a single climatic variable may control plant composition was shown in the case of the rubber plant, young guayule. It had been known that the accumulation of rubber from guayule is more or less cyclical, with production taking place during fall and winter. The climatic factor associated with this plant response was shown by Bonner (1943) to be the night temperature. Thus it was found that if the temperature is kept constant at 80° F. day and night there is only slight rubber accumulation, although the plants grow rapidly, but as the night temperature is lowered rubber accumulation becomes more rapid, the optimum being 40 to 45° F. Further studies have also shown that the period of low night temperature must be greater than ten hours, even at the optimum temperature, in order to initiate rubber accumulation (in Bonner and Galston, 1947). The effect on rubber accumulation of light

intensity, nutrition, water, and other climatic factors are also discussed in this reference. The ecology of rubber accumulation is perhaps the best understood of plant constituents.

In regard to the ecology of plant lipids the facts are more obscure, a few field observations and the literature surveys of McNair being the principal contributions. McNair attempted to correlate climate with the distribution and composition of fats and waxes (McNair, 1929, 1930, 1931, 1945). He grouped the yield and composition of the known plant lipids according to whether the species concerned were tropical, subtropical, temperate, or widely distributed. There are two obvious objections to this approach: 1. As noted earlier, some data in the literature are not reliable because plant extracts termed "lipid" were more correctly mixtures of true lipids and other non-lipid substances. Therefore the methods of extraction and separation of the lipid components must be carefully studied in reviewing the literature, and those data which involve non-lipid extracts must be discarded. This was not done by McNair. 2. The classification of a species into large climatic groups such as tropical, subtropical, etc. may be accepted only in the broadest sense.

In spite of these limitations McNair came to some interesting conclusions which may serve as a basis for experimental work:

a, oils in seed coats are of higher melting point than oils which occur in seed kernels; b, tropical and subtropical plants generally have non-drying oils, that is, their oils have higher melting points and are less unsaturated than temperate fats; c, shade decreases oil content; d, increased soil moisture increases fat content and iodine number; e, more wax producing plants occur in the tropics than in the temperate zone; f, wax hydrocarbons, acids, and alcohols from tropical species have lower melting points and greater molecular weights than those of temperate origin; and, g, the melting points of wax esters of tropical plants are higher than those of temperate plants. In connection with the last two points, McNair is concerned with the fact that tropical fats have higher melting points and are more saturated than temperate ones, whereas the reverse is true of wax hydrocarbons, acids, and alcohols. Although this may be true, it should also be noted that because most waxes contain wax esters as their greatest constituent, the whole wax will have a melting point very close to that of the ester fraction. For example, carnauba palm wax and others which contain 50 to 80 percent of esters have a melting point very close to that of the ester fraction (Warth, 1947). Therefore McNair's observation that tropical wax esters have higher melting points than temperate wax esters may be revised to include the melting point of the whole wax in relation to climate. Thus it may be concluded that whole waxes, like fats, have higher melting points when derived from tropical plants than when derived from temperate zone species.

Experimental confirmation of McNair's conclusions is not abundant, although agronomic experiments and observations tend to support his ideas in regard to fats and oils. Cartter and Hopper (1942) in the course of a USDA cooperative soybean program observed that the iodine number of oil from several varieties showed a rather marked decrease when the soybeans were grown in warmer environments. The Manchurian variety showed the greatest variation with an iodine number of 141.9 and 116.5 from plants grown, respectively, in Maine (temp. 56 to 64° F.) and Kentucky (temp. 70 to 76° F.). These authors did not come to any definite conclusions as to the effect of soil on oil formation except that such effects, if they do exist, are probably very minor in comparison to climatic effects. Similar observations were made by Painter and Nesbitt (1943) on the oil of flax seed. Apparently this plant is more greatly influenced by climatic factors since the iodine number dropped from 202.8 to 127.8 when the flax was grown under adverse conditions of high temperature and insufficient soil moisture.

The effect of soil types on lipid production in cotton, soybean, and peanut was studied by Garner, Allard, and Foubert (1914), but they found only slight effects and their conclusion was that climate is more important than soil type in controlling oil content. The effect of soil nutrients perhaps needs further investigation in view of the recent results of Milner (1951). He found that by varying the nitrogen supply of Chlorella

cultures he could vary fat yields between 7 and 69 percent dry weight. Whether such effects are possible with higher plants is not known.

In a more direct approach to the study of the effect of temperature on lipids, Pearson and Raper (1927) found that by increasing the culture temperature from 18 to 35° C. the iodine number of the fatty acids of an Aspergillus dropped from 149 to 95, whereas in Rhizopus a temperature change of 12 to 25° C. decreased the iodine number from 88 to 78. McNair (1945) discusses several other similar but less marked changes in fats of lower plants, but no experiments on higher plants have been reported.

Experimental data on the effects of environment upon plant fats are thus neither extensive nor satisfactory.

Data regarding the effect of climate on the plant waxes are essentially non-existent. There have been no experiments designed to test the conclusions of McNair, and the existing works deal only with wax yield and cuticle thickness. For example, Dahlgren (1933) compiled some data on yields of carnauba wax during years of differing rainfall. The waxes were removed by the ordinary "thrashing" technique, which, although done uniformly each year, does not give the total wax from a leaf. From Dahlgren's data it can be calculated that in a year of average rainfall the yield of wax from carnauba palm leaves collected over a three-month period was about seven grams per leaf. In a wet year the yield decreased to five grams of wax

per leaf, and in a drier than average year the yield increased to eight grams per leaf.

Howes (1936), in disagreement with Dahlgren, found that if the rainfall in the wet season just preceding leaf cutting is lower than usual a lower yield of wax is obtained. He also found that in very dry years the yield may only be one-tenth of that obtained under favorable conditions. Although these facts are undoubtedly correct, their interpretation may, however, be in error. If, for example, the period before leaf cutting is of very low rainfall, the heavy cuticular wax exudate becomes dry, cracks, and is sloughed off the leaf, thus decreasing the yield by a large factor, as was observed by Howes. The sloughing of wax may become quite a problem even in average wet years, since if the leaves are not removed at the proper time but rather they are allowed to remain on the tree until completely mature and partly dry, the yield of wax by the usual methods of extraction becomes negligible.

Howes (1936) agrees with Dahlgren that the yield of candelilla, another commercially important wax plant, when grown in well-watered areas is less than when grown in the more arid districts.

From an anatomical standpoint these changes in wax content with variation of soil moisture may be associated with changes of cuticle thickness, which has been demonstrated by Cooper (in Lee and Priestley, 1924). Measurements of both upper and lower

cuticles of broad sclerophyll vegetation of California indicate that as one passes from regions of low soil moisture and high light intensity to regions of high moisture and low light intensity, the cuticle thickness decreases by a factor of five.

It is clear that we have little information of a quantitative nature concerning the effects of individual climatic factors on lipid production by higher plants. Temperature and soil moisture are apparently quite important, but the effects of these factors have not been well defined. The present investigation, a detailed study of the effect of environment on plant lipids, is a beginning in the direction of filling this gap in our knowledge. The whole work has been made possible by the facilities of the Phytotron, the Earhart Laboratory (Went, 1950).

B. Methods

Four species were used in this study, Nicotiana tabacum L. and Nicotiana glauca Graham, both of the Solanaceae, Larrea tridentata (DC) Coville (Zygophyllaceae), and Zea mays L. (Gramineae). Mature plants from a wide range of experimental conditions of the first species were generously furnished by Dr. Guy Camus. N. glauca, or tree tobacco, is commonly observed in certain native habitats to possess a white powdery bloom on its leaves, whereas in other environments the leaves are a dull green. Since some climatic factor appears to cause changes in the cuticle, the plant appeared to be good material for the present study. Larrea tridentata, or creosote bush, is a

typical xerophyte of the southwestern United States and Mexican deserts and is therefore at home in a hot, dry environment. Because both factors of high temperature and low soil moisture have been implicated in the ecology of fat and wax formation, Larrea was another reasonable species for study of the effects of these factors. Since it had been shown (Kurtz, 1949) that both N. glauca and L. tridentata contain rather large quantities of fats and waxes, the usual Phytotron techniques of culture of a small number of plants could be employed. Seed of both species was kindly provided by Dr. F. Went.

The fourth experimental plant, corn, was used because it was of interest to determine whether the factors affecting lipids of the dicots (Nicotiana and Larrea) would similarly affect a monocot species. Although the plant is admirably suited to study in the Phytotron, and although the growth data are, in general, of statistical significance, it was found that there was considerable variation in the lipid content of the plants, probably because of genetic variability in the seed stock used. Nevertheless, some important results were obtained and these will be included in the present discussion. I wish to thank Dr. H. Teas for furnishing the seed material (Bikini out-crossed).

A broad range of day and night temperatures and day length was used and a detailed account of these climatic conditions will be presented with the discussion of each species.

In the introduction to Part II it was noted that high or low soil moisture may be important as factors in lipid formation,

and it therefore appeared necessary to include soil moisture among the variables studied. This was achieved by culturing two equivalent sets of plants under all of the temperature and light conditions. One set was maintained as close to field capacity as possible by frequent supplies of water and nutrient. The second set of plants, which was allowed to remove moisture to the wilting point, was flooded with Hoagland's nutrient solution only when the plants were severely wilted. The latter set of plants will be referred to as the set under water stress (WS). Although in some temperature conditions the water stress plants received nutrient and water only at intervals of two or four weeks, no mineral deficiency symptoms were observed.

Uniform seedlings were selected at the beginning of the experiments, and four (6 to 8 in L. tridentata) such plants were then placed in each experimental condition. Analytical results represent a pooling of each set of four plants, with the exception of N. tabacum in which case only two plants were available from each condition.

Only the leaves of the four species were examined for their lipids because this is the only plant organ that provides a sufficient yield of both fats and waxes from a small quantity of material. As has been shown previously (Kurtz, 1949), the yield of wax is best expressed as the weight of wax per unit leaf area since the greatest proportion of a leaf wax occurs superficially in the cuticle. Therefore leaf areas were deter-

mined for all species studied except corn.

After harvest the leaves were dried in an air-draft oven at 70-80° C. They were then shattered to a coarse mulch and the fats and waxes extracted according to the modified method of Chibnall et al. (1931) used previously (Kurtz, 1950). In this method the petroleum ether (B.P. 60-70° C.) total lipid extract is separated into two fractions by acetone. The acetone soluble fraction contains the fats, whereas the acetone insoluble fraction contains the waxes. The two fractions are admittedly crude, but the separation is quantitative and the fractions exhibit typical physical and chemical properties of fats and waxes. As shown in Table I, the yields of both fats and waxes show a variation of $\pm$ 10 percent or less between replicates which is reasonable in view of the relatively small quantities of plant material used.

Another extraction procedure that was used by Conrad and Neely (1943) for the study of the wax of cotton fibers was tested on some tobacco leaves but it failed to give any separation of fats and waxes. The fraction which corresponded to Conrad and Neely's "wax" fraction was exactly equal, on a percent dry weight basis, to the total crude petroleum ether extract obtained by the method of Chibnall et al.

The two fat and wax fractions were not further fractionated for reasons discussed earlier and because only minute quantities of each fraction were available. The fractions were, however,

TABLE I. Percent yields of fats and waxes from leaves of various sources to show the variation due to extraction method.

Plant	sample no.	wt.(gm.) leaves extr.	wt. wax (gm.)	% wax	wt. fat (gm.)	% fat
Nicotiana tabacum	1	31.1	0.225	0.72	1.787	5.74
"	1	11.6	0.083	0.72	0.569	4.90
"	2	32.0	0.250	0.78	1.303	4.07
"	2	3.0	0.026	0.88	0.105	3.50
"	3	32.5	0.231	0.72	1.255	3.86
"	3	24.1	0.173	0.72	0.878	3.64
"	4	34.5	0.322	0.93	1.676	4.86
"	4	18.9	0.147	0.78	0.719	3.80
Corn	1	50.3	0.938	1.86	0.882	1.75
"	1	30.7	0.468	1.52	0.613	2.00
"	2	43.3	0.705	1.63	0.889	2.05
"	2	37.2	0.674	1.81	0.679	1.83

characterized by melting point and iodine number determinations. Determinations of the melting point and iodine number have been frequent in earlier work on lipids so that determination of these values in the present study makes comparisons possible with the literature. The melting point was determined by the micro-drop melting point method (Kurtz, 1949) which gives values very close to those obtained by the macro method generally used (anonymous, 1947). The iodine number was determined in all cases by the micro method of Yasuda (1931). Although this method gave consistently close values for each sample, the values were always much higher than would be expected. Yasuda did not report this difficulty. In order to make the iodine numbers determined in the present study comparable to published values, all determinations were corrected by a factor of 0.37. This factor represents the theoretical iodine number of oleic acid (90) divided by the value determined by the method of Yasuda (242).

C. Results

1. Nicotiana tabacum variety Cuba White

Mature plants (102 days in the thermal conditions) of this variety all received 8 hours of natural light per day and were grown under the complete range of night temperatures from 2° to 30°C.*, with a constant day temperature of 26°. A detailed discussion of the growth data may be found elsewhere (Camus and

* All temperatures are in degrees Centigrade.

Went, 1951), but it should be mentioned here that this variety is particularly affected by a 17° night temperature, as shown in Fig. 1. This phenomenon is repeatible and the cause is unknown.

Table II summarizes the analytical data on the lipids. The yields of both fat and wax were greatly affected by the 17° dip, particularly in the case of the fats (Figure 2). Apparently the physiological effect of the dip on the fats is different from the effect on the waxes. The yield of fat remained constant above 17° night whereas the yield of wax, on a leaf area basis, increased again at 20° night and then fell off to a minimum at 30°. The over-all trend of both fat and wax yield on a leaf area basis was a decrease as the night temperature increased. It has been shown (Kurtz, 1950) that plant age has a strong influence on lipid fractions. Age is probably not a factor in this study, however, because all the plants had visible inflorescence shoots with optimum development at 14° and 26°. Since the plants were therefore of similar maturity, it may be concluded that there was a definite effect of night temperature on the yield of lipids in this species and variety.

As judged by the melting point, night temperature also affected the type of wax formed. There was, however, no effect on the properties of the fat fraction, and in particular no significant effect of the 17° dip (Table II). The fat fractions from all the night temperatures were rather viscous liquids, and

Figure 1. Mature plants of N. tabacum var. Cuba White grown under controlled conditions. Day temperature 26°, 8 hours daylight, night temperature (left to right) 2, 6, 10, 14, 17, 20, 23, 26, and 30° C. (from Camus and Went, 1951).

TABLE II. Yields and characteristics of the fats and waxes of N. tabacum variety Cuba White.

Temp. °C. Day	Night	% wax	gm. wax/m^2	% fat	M.P. wax °C.	Iodine number wax	fat
26	2	0.65	0.127	4.78	69.0	31.1	214
26	6	0.69	0.123	5.44	79.0	56.1	204
26	10	0.88	0.113	5.61	78.2	54.8	217
26	14	0.72	0.089	5.32	76.0	49.7	207
26	17	0.88	0.095	3.79	91.0	66.7	223
26	20	0.93	0.146	4.34	110.0	80.1	224
26	23	0.72	0.092	3.75	-----	57.1	220
26	26	0.86	0.108	4.33	-----	69.3	207
26	30	0.50	0.063	3.91	-----	49.5	206

this was reflected in the constant iodine number. The waxes on the other hand were greatly affected. The melting point rather steadily increased as the night temperature increased, but the degree of unsaturation showed a significant optimum at 17 to 20°. Thus the continuous rise of the melting point with increased night temperature is not a reflection of a lower degree of unsaturation. Other marked changes must therefore have occurred in the constituents, the most likely one being greatly increased chain length at the higher temperatures.

2. Nicotiana glauca

This species, which proved to be very admirable for study in the Phytotron, was grown under different conditions of day

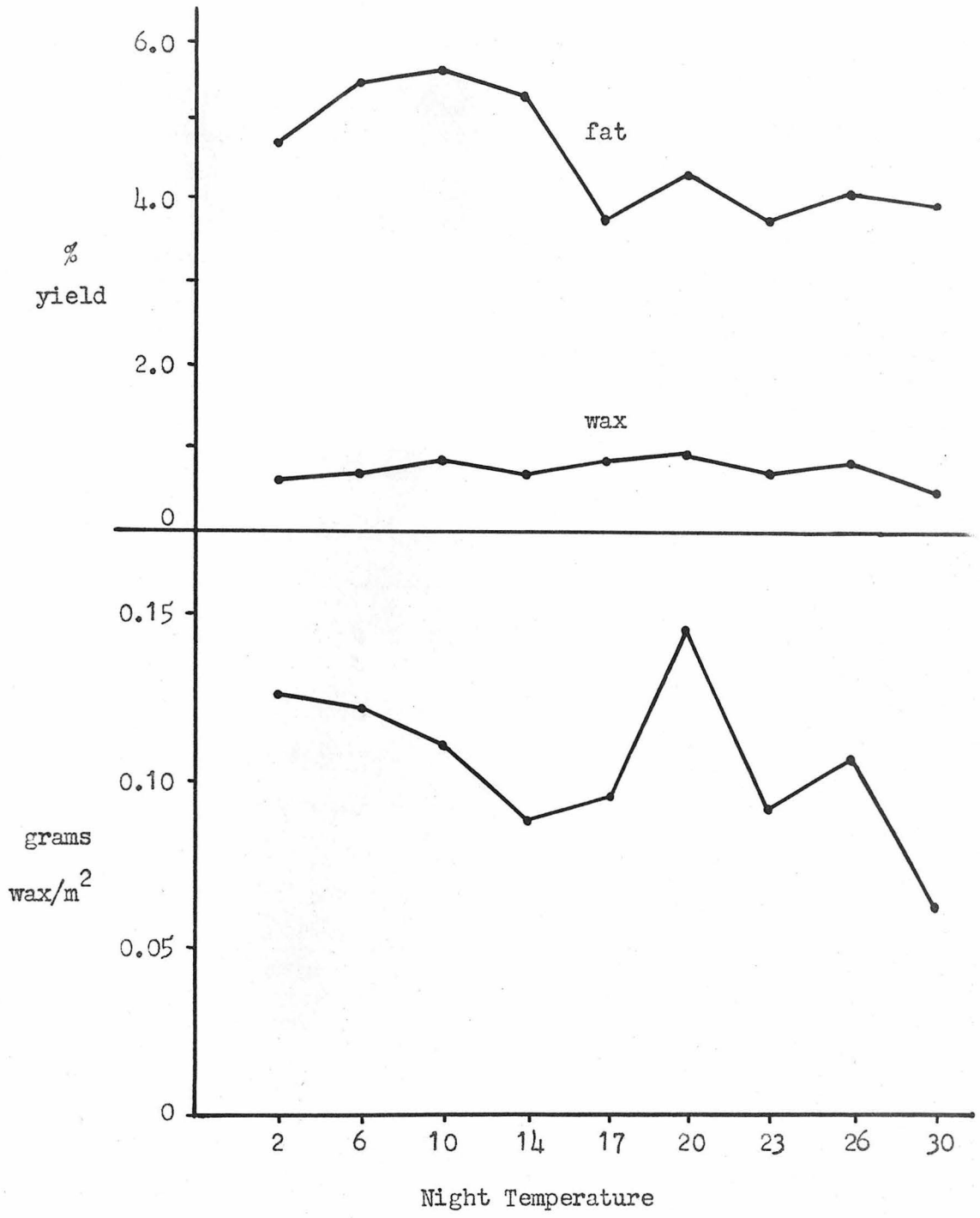


Figure 2. Yields of lipid fractions from N. tabacum under controlled conditions. Above, percent yields; below, grams of wax per square meter of leaf surface. Day temperature 26°, 8 hour daylength.

(8 hours) and night (16 hours) temperature and soil moisture content. As Fig. 3 shows, the growth effects of these factors were very pronounced. Other growth data may be found elsewhere (Liverman and Kurtz, 1951), but in general terms stem length, number and size of leaves, and degree of axillary branch formation were most greatly affected. No mature flowers were produced under any condition, and floral primordia formed only in the 23° day and 6° night condition (no water stress).

The yields of fat and wax (Table III) show some interesting variations in response to night temperature and water stress. Whether the results are calculated on a dry weight or on a leaf area basis, the yields of both fat and wax were consistently greater from water stress plants. In water stress plants the percent fat remained at a constant level, whereas in the non-water stress plants there was a slight increase with rising night temperature. The yields of wax on the other hand illustrate quite well the effect of method of presentation. On a percent dry weight basis there appears to be an optimum 20° night temperature for wax formation, whereas on a leaf area basis there was no significant change in relation to day or night temperature. The latter relationship is probably the more correct.

It is also of interest that although plants in the various conditions had extremely different growth characteristics, etc., on a per plant basis the yield of fat or wax was remarkably constant from one condition to another (Table III). Thus the

Figure 3. Plants of N. glauca just before harvest. Left, plants that received normal water and nutrient supply. Right, plants that received water stress conditions. Eight hours daylength.

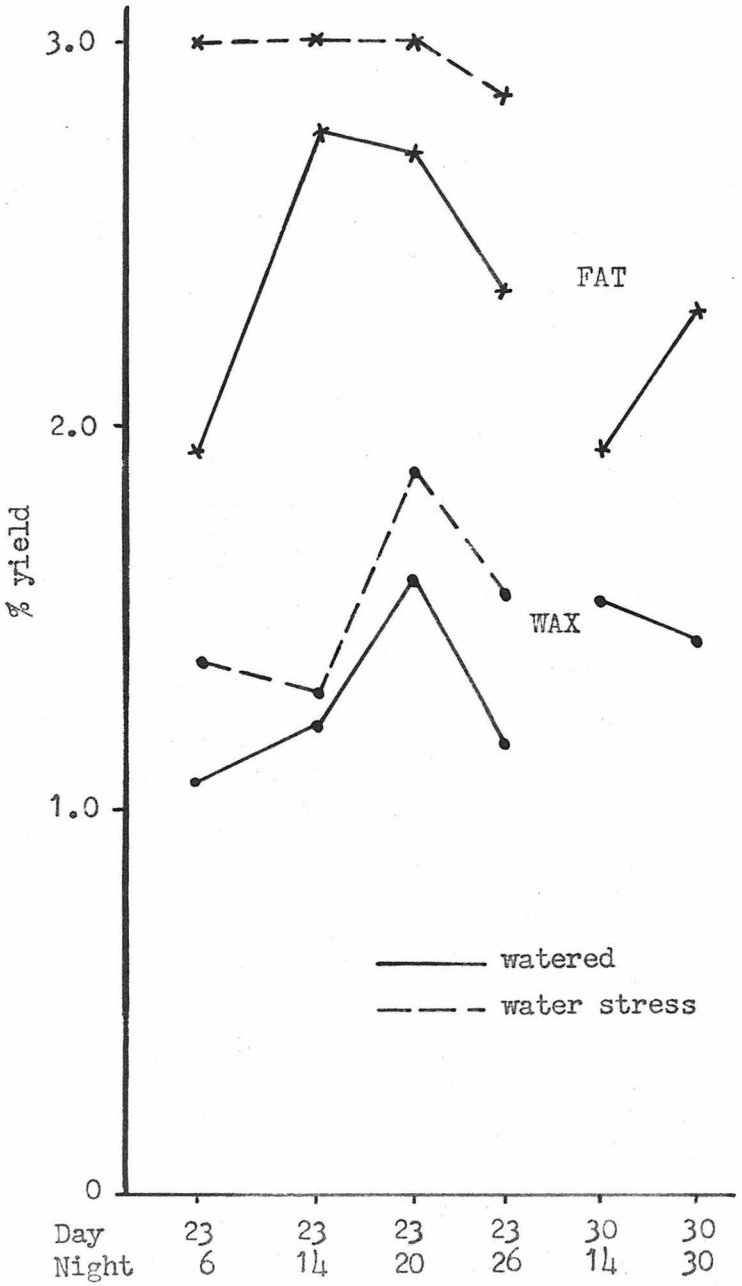
TABLE III. Yields of wax and fat from N. glauca.

temp. °C. day	night	gm. wax per plant		% wax		gm. wax/ m^2 leaf		gm. wax/m^2 WS / gm. wax/m^2 W	gm. fat per plant		% fat	
		W	WS	W	WS	W	WS		W	WS	W	WS
23	6	0.059	0.030	1.08	1.38	0.250	0.284	1.14	0.48	0.75	1.94	3.00
23	14	0.056	0.021	1.22	1.30	0.198	0.228	1.15	0.69	0.75	2.77	3.01
23	20	0.072	0.052	1.61	1.89	0.237	0.331	1.40	0.68	0.75	2.71	3.00
23	26	0.056	0.020	1.17	1.56	0.183	0.244	1.33	0.59	0.71	2.37	2.85
30	14	0.069	-----	1.53	----	0.230	-----	----	0.48	----	1.94	----
30	30	0.035	-----	1.43	----	0.205	-----	----	0.58	----	2.31	----

yield in grams of fat per plant under water stress was constant from 6° to 26° night temperature. The non-water stress plants responded similarly but contained less fat than those under water stress. The significance of these relationships cannot be ascertained at this time.

With a day temperature of 30°, as the night temperature was raised from 14° to 30° the plants became somewhat similar to high night temperature water stress plants in appearance and height. This was reflected in the yield of wax. For example, at 30° day and 14° night the yield of wax from watered plants was 0.069 grams per plant, whereas at 30° both day and night the value was only 0.035 grams per plant, which is now in the yield range of water stress plants. There is again some indication that a 20° night temperature may be optimum for wax formation in this species. The data on yields are presented graphically in Fig. 4.

During the course of the experiment it was observed that all the water stress plants, and also the 30° both day and night non-water stress plants, had an abundant white bloom on the stems and leaves, and, indeed, determination of the amount of cuticle per square meter of leaf surface shows that there was considerably more cuticle on those plants which had the white bloom (Table IV). If now it is assumed that all of a plant wax is derived from the cuticular layer, the percent of wax in the cuticle may be calculated (Table IV). Although the data are rather variable, the



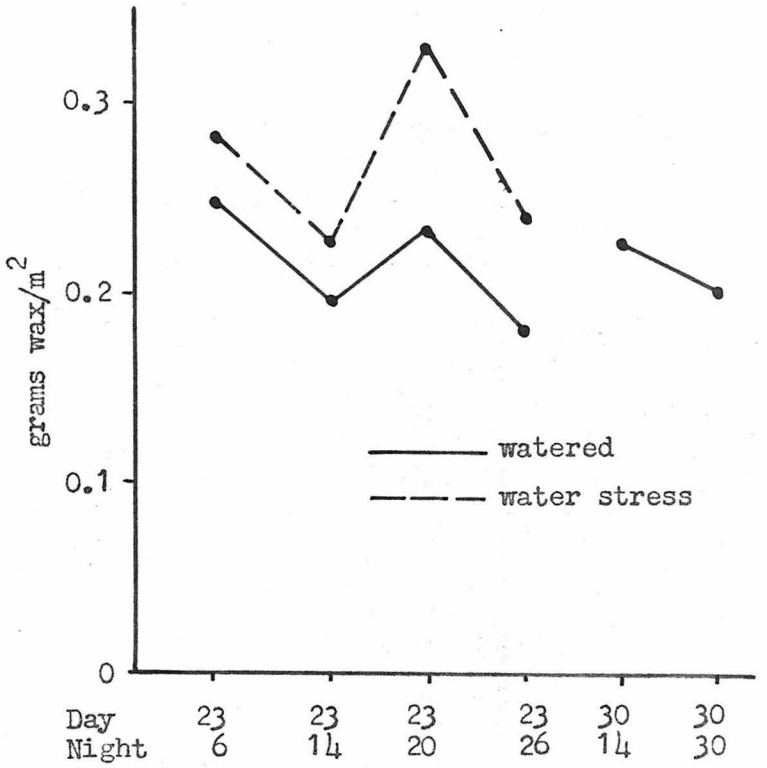


Figure 4. Yields of fat and wax of N. glauca under different temperature and water stress conditions. Left, percent yields. Above, grams wax per square meter leaf surface.

TABLE IV. Yields of cuticle and percent of wax in the cuticle from N. glauca.*

temp. °C. day	night	gm. cuticle /m²		gm. wax/m² / gm. cuticle/m² x 100	
		W	WS	W	WS
23	6	0.724	0.653	34	43
23	14	0.547	1.317	36	17
23	20	0.695	1.004	34	33
23	26	0.779	1.555	24	16
30	14	0.740	-----	31	—
30	30	0.923	-----	22	—

* I wish to thank Mr. J. Skoss for the cuticle determinations.

cuticle contained less wax as the day or night temperature increased. Furthermore, the cuticle of water stress plants did not contain a larger proportion of wax.

Characterization of the fats and waxes showed again, as in N. tabacum, that lipids are significantly modified by climate (Table V). The fat fractions of N. glauca were all solids at room temperature. The melting points of the fats from water stress plants were rather variable from one condition to another, but they were consistently higher than those of the non-water stress plants (Figure 5). As the night temperature was increased, plants receiving a normal supply of water formed fats of a higher melting point. Thus, as before, high temperatures bring about a physiological response similar to that of water stress. The change in melting point values is not

TABLE V. Effect of temperature and water stress on the melting point and iodine number of the fats and waxes of *N. glauca*.

temp. °C.		wax				fat			
		M.P.°C.		Iodine no.		M.P.°C.		Iodine no.	
day	night	W	WS	W	WS	W	WS	W	WS
23	6	67.4	68.4	32.8	17.0	37.6	46.5	244	254
23	14	68.0	68.1	27.4	12.6	34.2	54.2	226	227
23	20	69.7	69.8	35.3	16.6	36.0	45.5	230	266
23	26	70.1	70.2	15.6	19.2	51.4	52.4	209	215
30	14	69.3	----	26.2	----	40.3	----	238	---
30	30	69.9	----	22.8	----	45.9	----	231	---

associated with the degree of unsaturation for there was no significant relationship between iodine number and temperature or water stress.

The wax fractions showed a significant and steady increase in melting point with increased day and night temperatures, and there was no difference in values between watered and water stress plants (Figure 6).

The degree of unsaturation was markedly different between the waxes of watered and water stress plants, but again as the night temperature was increased the waxes of watered plants became similar to the waxes of water stressed plants (Figure 6). The chemical factors involved in the change of wax melting point in relation to temperature and water stress apparently do

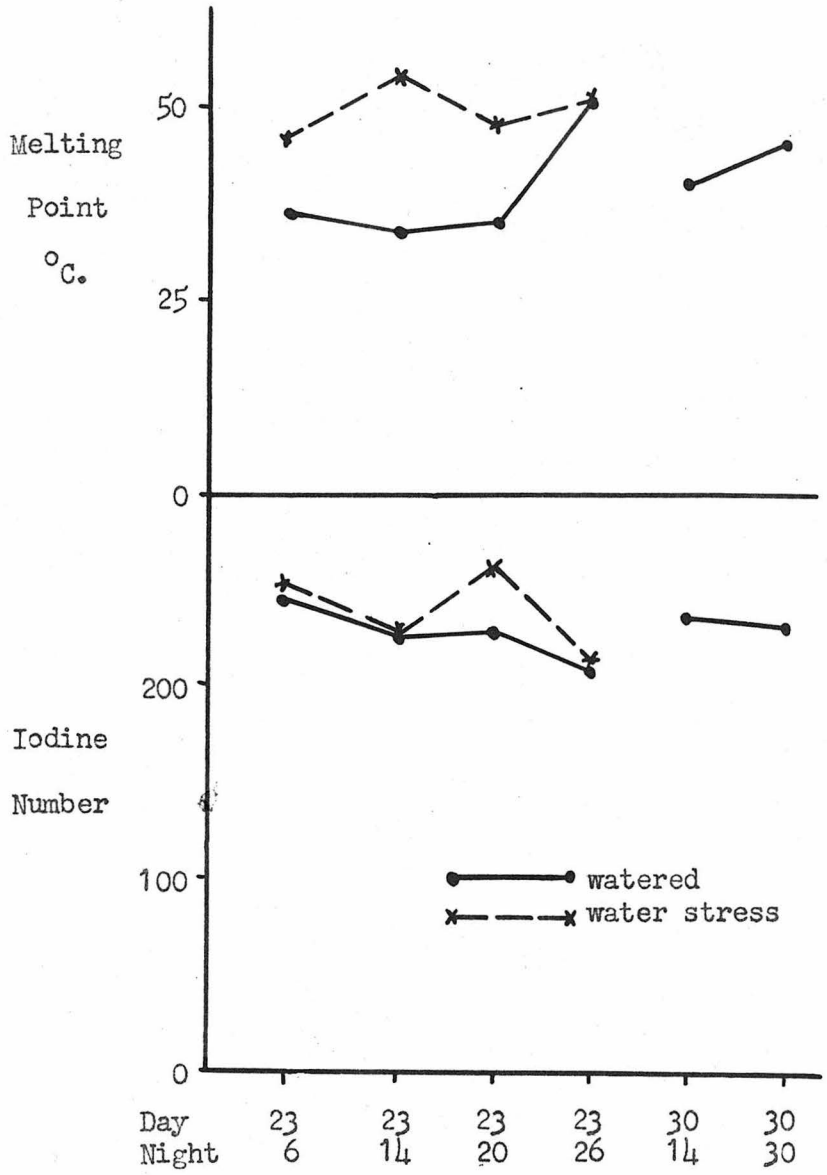


Figure 5. Relationship of the melting point and iodine number of the fat of N. glauca to temperature and soil moisture. Eight hour daylength.

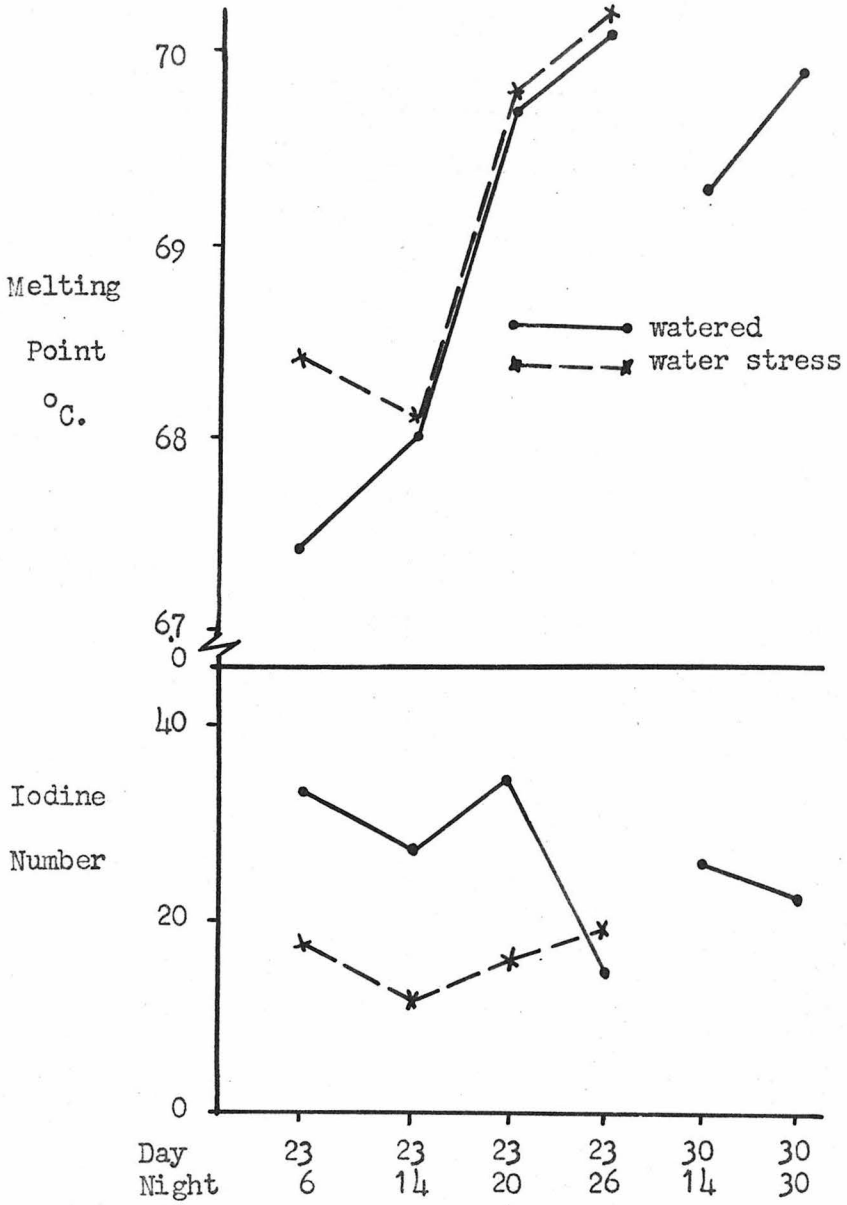


Figure 6. Relationship of the melting point and iodine number of the wax of N. glauca to temperature and soil moisture. Eight hour daylength.

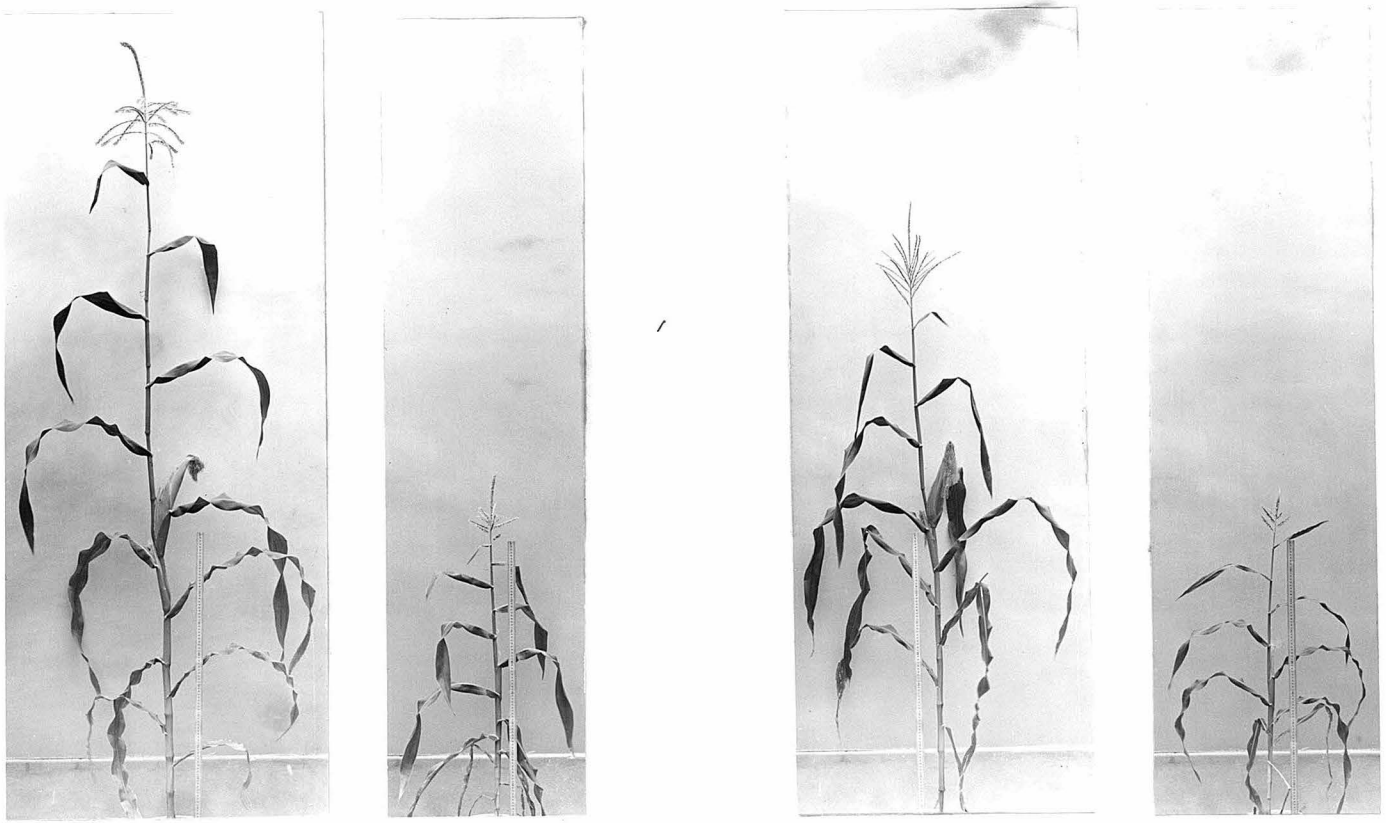
not involve the degree of unsaturation, to any great extent, as a comparison of Figures 6a and 6b indicates.

3. Zea mays

Although the effects of climate on the growth of corn were highly significant (Figure 7, Liverman and Kurtz, 1951), the effects on the lipids were not as definite due to the considerable genetic variation in the plants used. This is not surprising in view of the fact that corn lipids are greatly affected by the genetic background (see Part III). Nevertheless, the results indicate some general trends in regard to the effect of climate on lipids.

As shown in Table VI, water stress drastically reduced the percent of leaf wax, this response being just opposite to the effect of water stress on N. glauca. There is some indication that as day or night temperatures increase the yield of wax decreases, but because of the erratic values no definite conclusion can be drawn on this point.

The yield of fat, on the other hand, was greatly increased in response to water stress. In most cases there was a two-fold increase in fat yield which is a more striking response to water stress than that found with N. glauca. Corn apparently responds to water stress by an abundant formation of fats rather than by the accumulation of both fats and waxes. No relationship between temperature and fat content is shown by



W WS W WS

Day 20°, night 14° Day 30°, night 14°

Figure 7. Mature corn plants at harvest showing some growth effects of temperature and soil moisture. All plants under natural daylight and daylength. W = watered plants. WS = water stress plants.

TABLE VI. Yields and melting points of the fats and waxes of corn grown under different climatic conditions.

temp. °C. day	temp. °C. night	light	% wax		M.P. wax, °C.		% fat		M.P. fat, °C.	
			W	WS	W	WS	W	WS	W	WS
20	14	N.D.*	0.13	0.13	70.5	—	0.73	1.53	43.5	39.7
23	17	"	0.28	0.01	66.3	—	0.63	1.40	32.0	55.5
26	20	"	0.19	0.13	77.0	—	0.66	1.82	27.0	50.7
30	14	"	0.21	0.07	——	—	1.04	1.86	33.0	38.0
30	30	"	0.10	0.02	76.0	—	1.11	1.44	42.0	59.0
23	14	8 hr.	0.23	0.09	67.7	—	1.08	1.59	53.0	41.0
23	20	"	0.22	0.04	72.0	—	0.89	2.30	27.0	47.0
23	26	"	0.03	0.15	——	—	0.35	0.81	53.0	56.0

* N. D. = natural daylight and daylength.

these data. The wax and fat melting points increased with temperature, which is in accordance with previous results. Also the fats from water stress plants had higher melting points than those from watered plants. No effects of photoperiod are discernible from the data.

In general it appears that, in regard to lipids, corn responds to water stress and temperature in a manner very similar to the two species of tobacco studied.

4. Larrea tridentata

Creosote bush, Larrea tridentata, a rather large xerophytic shrub in its natural habitat, was found to lend itself well to the cultural techniques used in the Phytotron. Growth was slow, but after seven months under the various climatic conditions plants sufficiently large to yield enough lipid for analysis were obtained. A wide range of temperature conditions was employed, and as before a parallel series of water stress plants was grown in addition to the series receiving water regularly. Because the leaves of Larrea are small and thick, it was very difficult to ascertain at what time the plants had become severely wilted. Generally, when the water stress plants had survived from several days up to two or three weeks without water, the lower leaves turned yellow and dropped from the plant. At this stage the roots were flooded with water and fresh nutrient. Some examples of plants grown under these conditions are shown in Figure 8.

Details of the growth data are given elsewhere (Liverman and Kurtz, 1951), but a few pertinent facts may be mentioned here. Creosote bush in the desert has a very characteristic aromatic odor and the leaves are sticky, both being caused by a resinous material in the leaves. These characteristics are particularly true of plants growing in washes or near an area of abnormally high soil moisture. Such was also the case in the Phytotron. The plants which received water daily had a more aromatic odor and stickier leaves than did the water stress plants. Another factor, temperature, is also important in this respect since plants receiving a temperature of 20° or less during the day or night were extremely sticky and aromatic. In fact, the leaves of these plants became so resinous that they appeared glossy and greenhouses containing them were well perfumed in spite of the rapid exchange and circulation of air.

An accident occurred during the course of the experiment and the pots containing the water stress plants under 20° day and 20° night were knocked to the floor. The plants survived but they were very slow in regaining their earlier vigor, and growth and analytical data on them may therefore be in error.

Examination of Table VII shows that the fat and wax fractions were not greatly affected by the climatic conditions used. In general, however, the plants responded to water stress by producing less wax and fat. The yield of wax appears to be lowered by an increase in temperature, particularly by increased

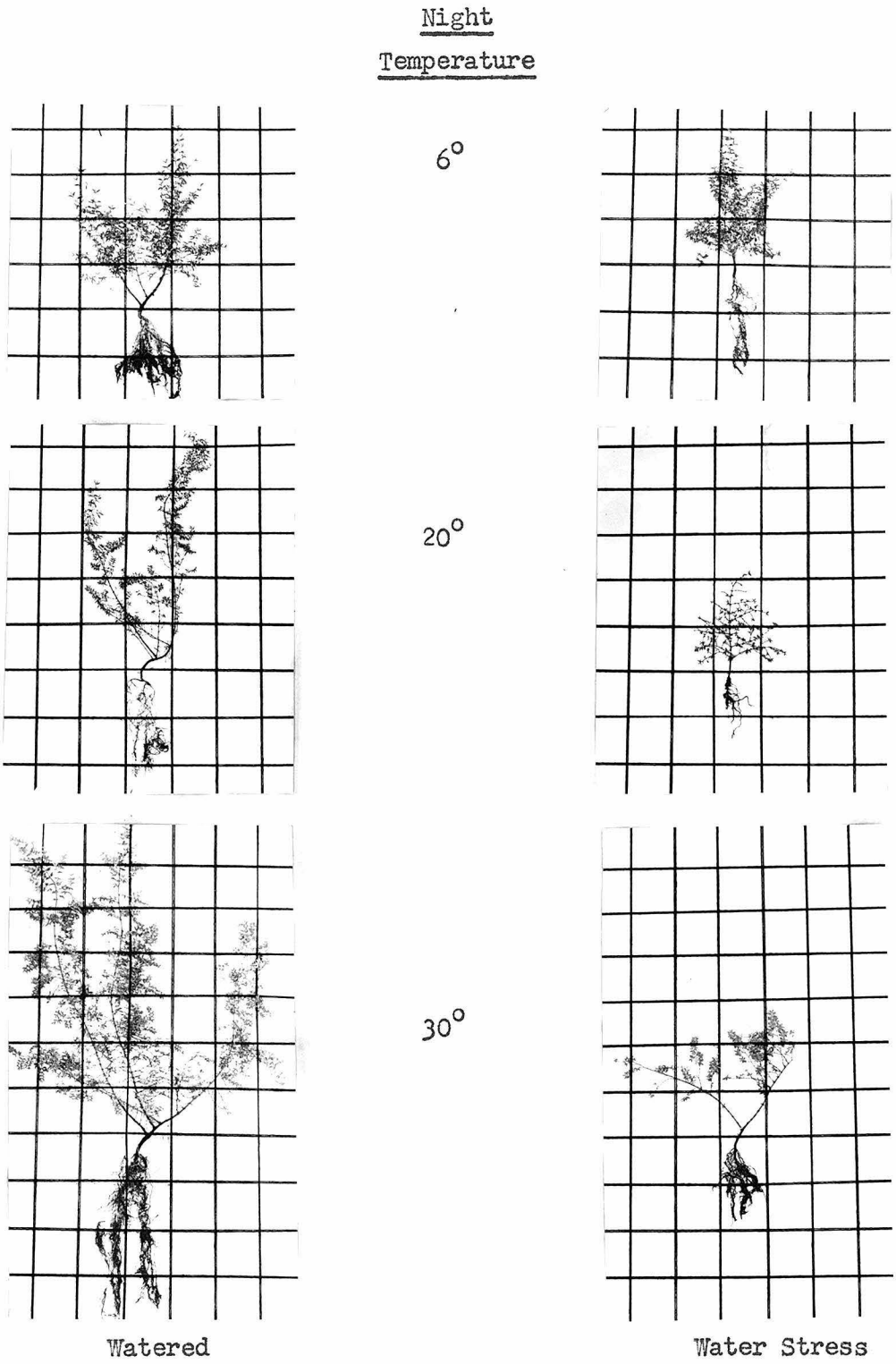


Figure 8. Plants of Larrea tridentata at harvest showing some effects of temperature and soil moisture. Day temperature 20° C., night temperature as indicated. 8 hour daylength. Each square represents 10x10 cm.

night temperature, although the relationship is not clear-cut. No conclusions can be drawn from the data on the effect of temperature on fat content.

Inasmuch as there was such a striking difference between low and high temperature plants in regard to odor and stickiness of the leaves, a study of the resinous substance was undertaken. The resin, which is completely soluble in acetone (Gardner and Sward, 1942), was extracted from the lipid-free dry leaves with this solvent. The yields of acetone soluble extract (resin) are expressed in Table VIII as percent of dry weight and on a leaf area basis. The latter method of expression is warranted since Runyon (1934) has shown that the resin accumulates as a layer on, or as a deposit in, the epidermal cells.

These results, presented graphically in Figure 9, show that temperature and water stress greatly affected the resin content of the leaves. Water stress plants, particularly at low (6°) night temperatures, contained considerably more resin than the watered plants. An increase in night temperature caused a lower resin content, but this effect was not as marked at the higher day temperatures (23° to 30°). Further, increased night temperature may have no effect on resin content if the plants are watered daily and are grown at high day temperatures. Day temperature modifies the resin content, the effect again being most marked at low night temperatures. Increased day length has no apparent effect on resin content according to these results.

TABLE VII. Yields of fat and wax from L. tridentata under different climatic conditions.

Temp. °C. Day	Temp. °C. Night	Light	% wax		gm. wax/m^2		% fat	
			W	WS	W	WS	W	WS
20	14	N.D.*	0.39	0.21	0.151	0.095	1.54	0.91
23	17	N.D.	0.37	0.26	0.118	0.098	1.92	1.07
30	22	N.D.	0.44	0.27	0.165	0.100	1.63	0.73
20	6	8 hrs.	0.28	0.37	0.120	0.200	1.41	1.42
20	20	"	0.52	0.70	0.177	0.272	1.58	1.24
20	30	"	0.28	0.29	0.078	0.082	1.08	2.17
23	23	"	0.73	0.42	0.217	0.136	2.52	1.68
23	30	"	0.45	0.31	0.120	0.083	1.75	1.47
30	6	"	0.28	0.51	0.080	0.211	1.24	0.94
30	23	"	0.39	0.37	0.123	0.134	1.28	2.42
30	30	"	0.48	0.31	0.115	0.084	1.43	2.48

* N.D. = natural daylength.

These results contradict the statement made previously that plants receiving much water were stickier and had a stronger odor than the water stressed plants. This apparent contradiction may be related to the water solubility of the resinous fraction. That is, the pure resin is slightly soluble in water and the aqueous solution has the characteristic odor of creosote bush. Also, when the resin is moistened it becomes more tacky than in the dry state. The resin in watered plants may be hydrated and the leaves therefore become sticky and odoriferous. Resin in water stressed leaves on the contrary may be dry so that very little odor or stickiness can be associated with these leaves.

Climate also affected the type of wax, fat, and resin formed, as shown by determination of the iodine number (Table IX). The amount of change was small, but increased night temperature caused an increase in degree of unsaturation of the fats. Day temperature and water stress did not have any obvious effect. Similarly, there was no significant effect of water stress or temperature on the unsaturation of the waxes. The iodine number of the resin fraction, on the other hand, was markedly increased as the day temperature increased. The effect of night temperature is rather indefinite from the data, but in some cases the plants responded as they did to day temperatures. Water stress had no significant effect.

Thus in creosote bush the effect of climate is primarily upon the resin fraction rather than upon the lipids.

TABLE VIII. Yield of resin from L. tridentata.

temp. °C. day	temp. °C. night	light	% resin W	% resin WS	gm. resin/m^2 W	gm. resin/m^2 WS
20	14	N.D.	19.05	23.37	7.33	9.74
23	17	N.D.	13.55	16.38	4.38	6.23
30	22	N.D.	9.96	15.51	3.71	5.76
20	6	8 hr.	15.07	20.65	6.51	11.17
20	20	"	16.12	14.49	5.47	5.61
20	30	"	15.98	14.52	4.43	4.04
23	23	"	10.80	14.08	3.21	4.55
23	30	"	12.75	12.11	3.40	3.26
30	6	"	6.78	16.94	1.93	7.06
30	23	"	12.30	13.00	3.83	4.68
30	30	"	12.02	11.70	2.88	3.21

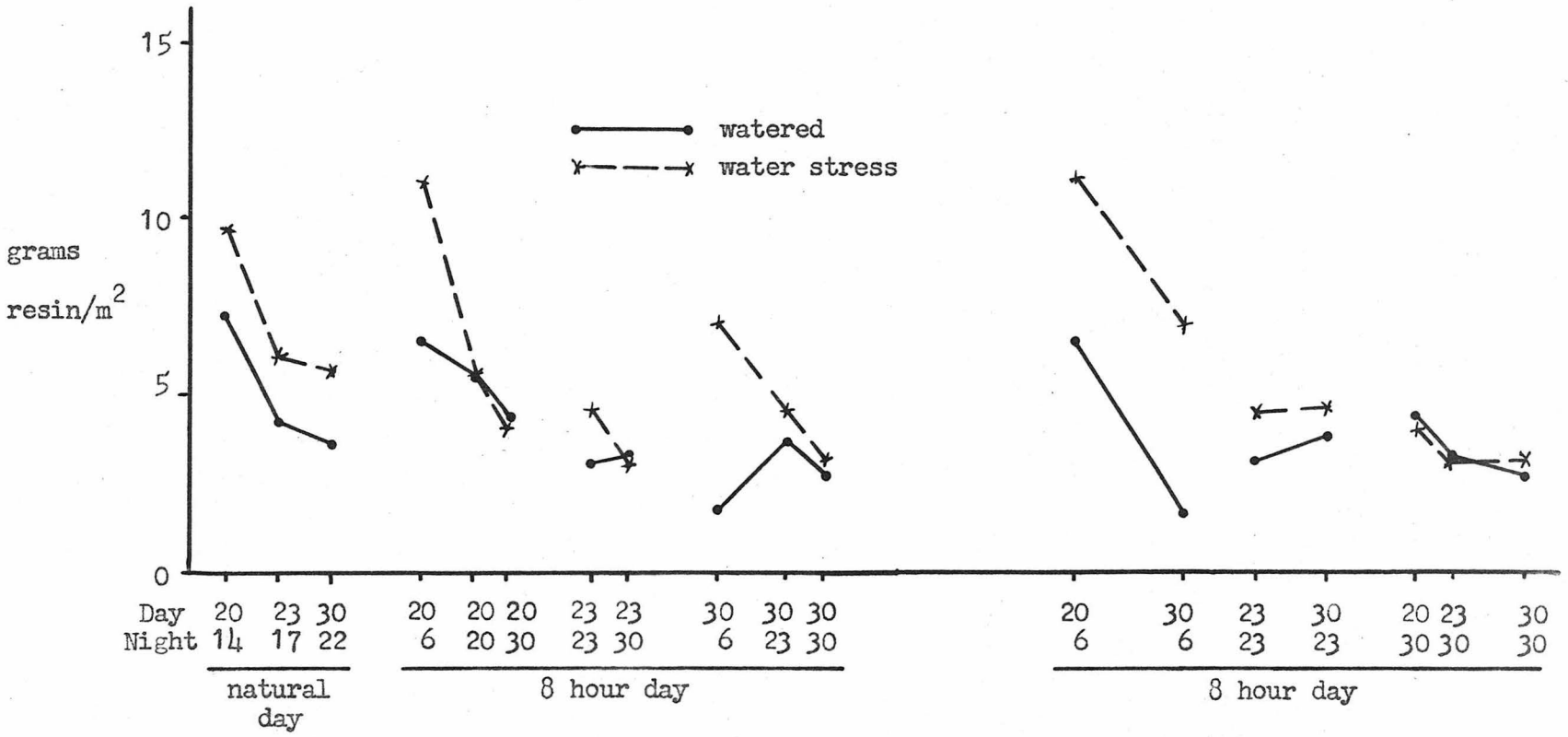


Figure 9. Yield of resin from L. tridentata from various conditions of temperature and water stress. Left above, yield at variable night temperatures. Right above, yield at variable day temperatures.

D. Discussion and Conclusions

We have seen that certain climatic factors may influence the type and quantity of two groups of lipids formed by four species. We may now make a few comparisons between these species.

The yield of leaf fat was greatly affected by temperature, but the effect depended upon the species. Thus N. tabacum produced less fat at the higher temperatures, whereas under the same conditions N. glauca formed more fat. No influence was observed in the case of corn and Larrea. Soil moisture also affects the fat content of leaves, and, contrary to the conclusions of McNair (1945), low soil moisture increased the fat content in N. glauca and corn. No difference in fat yield between watered and water stress plants was observed in Larrea. The reason for the lack of agreement with McNair is possibly due to the fact that in the present study leaf fats were studied whereas McNair referred only to seed fats. Under water stress the plant responds by producing more fat (or in Larrea, resin), and this higher fat content in the leaves may increase the capacity of the plant to endure desiccation. Thus the appearance of large amounts of fat in the water stress plants may merely be an adaptive response to the low soil moisture. Transeau (in Shields, 1950) observed such a formation of resin or oil in leaves of Rumex acetosella during conditions of water shortage. The formation of excess leaf fat under water stress may therefore be a survival factor. Seed fats, on the contrary, function as storage material, and it is quite

TABLE IX. The wax, fat, and resin iodine numbers of L. tridentata.

temp. °C. day	temp. °C. night	light	Iodine number wax W	wax WS	fat W	fat WS	resin W	resin WS
20	14	N.D.	37.6	19.0	258	237	200	217
23	17	N.D.	28.2	30.3	274	255	248	293
30	22	N.D.	30.2	29.3	262	256	321	290
20	6	8 hr.	19.7	40.7	253	253	267	235
20	20	"	27.8	30.0	259	228	327	305
20	30	"	23.6	41.9	268	275	253	232
23	23	"	24.8	19.5	242	221	221	236
23	30	"	56.2	31.7	260	264	246	283
30	6	"	13.9	36.2	223	237	356	286
30	23	"	32.6	34.7	251	286	302	303
30	30	"	28.5	28.3	263	292	284	325

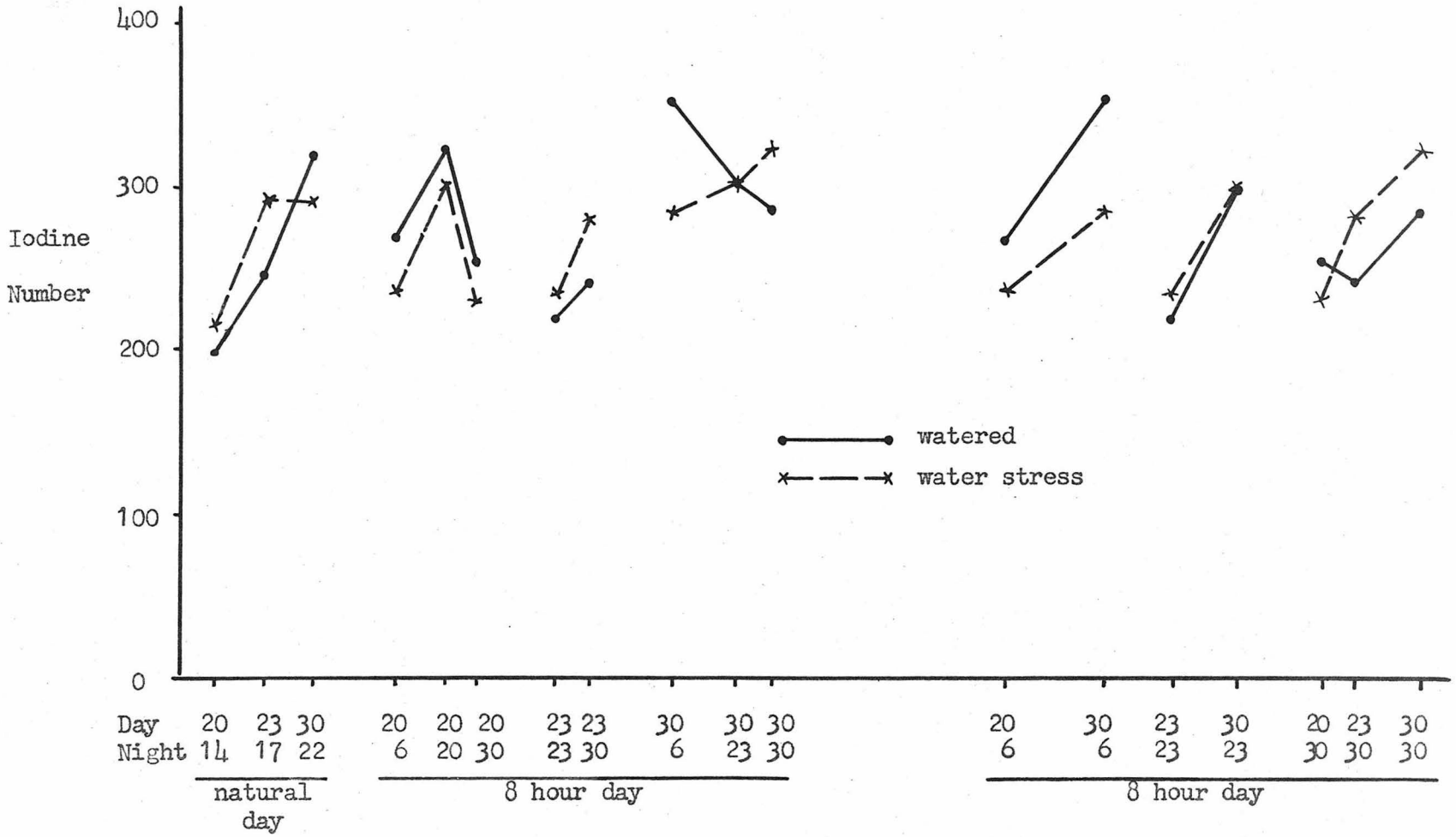


Figure 10. Resin iodine numbers of L. tridentata from various conditions of temperature and water stress. Left above, at variable night temperatures. Right above, at variable day temperatures.

probable that seeds of water stress plants would be low in fat content as a result of the poor growth status of these plants.

The type of fat formed is markedly affected by climate, particularly in regard to the melting point. In the two species in which it was determined, N. glauca and corn, the melting point increased with a rise of temperature just as McNair (1945) had found from his literature survey. Water stress had a similar and even more striking effect. Of further interest is the fact that there was no correlation between this rise of melting point and the iodine number. Under the conditions used no effect of temperature or water stress on the iodine number of leaf fats was apparent. This indicates that under conditions of high temperature or water stress the plant responds not by making more saturated fatty acids, but rather that some other chemical change occurs such as the formation of fatty acids of much longer chain length. This problem certainly warrants further investigation when good microanalytical procedures for fat analysis become available.

The results of iodine number determinations do not substantiate the conclusions of Cartter and Hopper (1942) and Painter and Nesbitt (1943) discussed earlier. This is undoubtedly because these workers studied seed fats which apparently are differently affected physiologically than are leaf fats.

According to the present study, the effects of climate on the production and type of wax largely depend upon the particu-

lar species. Temperature, however, affected each species rather similarly in that the general trend was toward a lower wax content at higher temperatures. In N. tabacum the yield of wax was affected in much the same manner as was growth, for the characteristic 17° dip was very pronounced. The physiological basis of this phenomenon is unknown.

Water stress did not have a general influence, for it caused increased wax formation in N. glauca, decreased formation in corn, and had no effect in Larrea. Thus the conclusions of McNair (1931), Dahlgren (1933), and Howes (1936) may all be correct. McNair states that tropical plants produce more wax than temperate plants, and this may indeed be correct if the species are considered as a whole. Dahlgren and Howes, on the other hand, state that during dry years the yield of carnauba, palm and candelilla may be greater. This may also be true for these species, but the present study indicates that such a response is not general.

In agreement with the work of Cooper (in Lee and Priestley, 1924), water stress, particularly when combined with high temperatures, caused abundant cuticle formation in N. glauca, but lipid analysis showed that on a leaf area basis such cuticles contained no greater concentration of wax than did cuticles of plants from conditions of low temperature or normal water supply. Certainly more data are necessary to verify these results, but they are suggestive and in part substantiate the familiar statement in

plant physiology and ecology textbooks that plants from very dry and hot areas have thick waxy cuticles. The present data are in agreement in regard to the thick cuticle, but the cuticle from such a climate does not necessarily contain a high proportion of wax. Indeed, a survey of many species indigenous to the very dry and hot areas of Arizona, of which many possess abundant bloom on the leaves and stems, showed that such species generally yield only slightly greater quantities of wax as compared to species from more moist areas (Kurtz, 1949).

Only those climatic effects have just been discussed which were marked or which exhibited a more or less general trend among the four species studied. Other conclusions may be reached from the data, but these do not appear to be warranted in view of possible genetic variability of the plant material and of the precision of the extraction and analytical methods.

From the foregoing discussion one may conclude that temperature, particularly night temperature, and soil moisture may rather greatly affect the yield and type of fat, wax, or resin formed by a plant. It will be shown in the next section, however, that genetic control of lipid formation may be even more potent.

III. GENETIC CONTROL OF LIPID FORMATION

A. Introduction

It has been recognized for many years that the genetic constitution of an organism determines, to a large extent, its

growth and ultimate phenotypic character. More recently it has been shown, principally by means of the red bread mold, Neurospora, that many biochemical reactions in the organism are controlled by single genes, and further that series of synthetic or degradative reactions are controlled by linked series of enzymes which are genetically controlled.

In higher plants biochemical genetics has not proceeded as far as in the case of Neurospora, but good progress has been made. In this connection it should be noted that for the most part biochemical genetic studies of higher plants have been done with corn or one of its close relatives. To cite but a few examples, the following problems have been attacked in corn: anthocyanin synthesis (Zarudnaya, 1951); respiration in green and albino plants (Groner, 1936); amylose-amylopectin relationship in waxy endosperm (Kramer and Whistler, 1949; Sprague, Brimhall, and Hixon, 1943; Mangelsdorf, 1947; Brimhall, Sprague, and Sass, 1945); the relationship between nicotinic acid and carbohydrates in the endosperm (Cameron and Teas, 1948); chlorophyll formation (Smith and Koski, 1951); photoperiodic response (Rogers, 1950); blue fluorescence and anthranilic acid content (Teas and Anderson, 1950); and riboflavin and phototropic response (Bandurski and Galston, 1951).

In addition to the above problems, the relationship between oil content and genetic constitution has received considerable study, both in corn and other species.

McNair (1945) observed that, in general, species of the same genus or family have similar oils, and he further concluded that there is a phylogenetic relationship. Cartter and Hopper (1942) have also shown that varieties possess rather similar oils, but that in some cases, as in flax, the yield and degree of unsaturation may be sufficiently low so as to make oil of a particular variety of little economic value as a drying oil.

A more direct genetic study, on corn, was begun by Hopkins in 1899. Hopkins began with an unselected strain of corn having a kernel oil content of 4.7 percent, and from this population the ears having the highest or lowest oil content were selected for further study. After twenty-nine years of continuous selection for high and low oil content, Winter (1929) reported that the oil content was now up to 9.86 percent and down to 1.51 percent. He stated that the high oil strain showed no tendency of having reached a limit, but that the low oil strain was approaching a physiological limit because many of the kernels were germless. The work was continued and after forty-seven years of selection Woodworth (1948) stated that the high-low oil strains were now 14 and 1.3 percent, respectively, as compared to the original content of 4.7 percent.

Using the data of Winter, Student (in Sprague, 1946) calculated that at least 20 to 40 genes and possibly 200 to

400 genes are involved in fat synthesis. These figures are based on certain assumptions, however, and are open to question. Brimhall and Sprague (1951), from a study of high and low oil content inbreds and various backcrosses, etc., concluded that the inheritance of iodine number is conditioned by four or more genes. Miller (1951) has also studied the inheritance of oil in the corn kernel, and from a series of chromosomal interchanges he concluded that high oil content is conditioned by a rather large number of genes, each having small and more or less equal effects. His results also indicate that the genes are distributed somewhat at random over the ten pairs of chromosomes. All these results would be anticipated in view of the known complexity of the fat synthesis mechanism in animals and microorganisms.

The inheritance of waxes is very poorly understood. As we have already seen, the wax yield and characteristics of a species are rather constant except for small fluctuations induced by the environment. Sex may alter this species control of the waxes (also the fats) as was shown from a study of three dioecious desert species (Kurtz, 1949, 1950). There appeared to be no relationship between the lipids and male or femaleness.

The only important genetic study of wax inheritance is that of Conrad and Neely (1943) on the heritable relation of wax content and green pigmentation of lint in cotton. They found that white lint cotton has a wax content of 0.48 to 0.63 per-cent, whereas the green lint parent has 12.64 to 15.04 per cent

wax. The F_1 generation is intermediate green and has a wax content also intermediate but closer to the white parent. It should be recalled, however, that their method of wax extraction was found unsatisfactory for the separation of leaf fats and waxes (see Part II), and therefore the results of Conrad and Neely may be open to question.

One other study by Wellensiek (1928) on the "wax" of pea shoots should also be mentioned here. He found that the glaucous ("wax") covering of the leaves and stems is conditioned by two sets of allelomorphs, and that five different gradations of waxiness can be distinguished. He did not, however, make any chemical determinations of the wax content, the five types being distinguished only by visual means. Therefore the work of Wellensiek probably does not add much to our knowledge of wax inheritance.

Because of this dearth of information on the inheritance of lipids, particularly the waxes, it was of interest to obtain from Dr. E. Anderson, California Institute of Technology, a series of recessive genes of corn which causes the seedling leaves to show various degrees of glossiness. The glossy (gl) seedlings are characterized by their shiny leaves, and by the fact that droplets of water will adhere to their leaf surface whereas no droplets accumulate on normal leaves (Figure 11). Anderson had suggested that although glossy leaves appear waxy, because water adheres to these same leaves their cuticle may

contain less wax than normal leaves; this was indeed found to be the case. The present discussion deals with the lipid constitution of this series of glossy and normal seedlings.

A study of two corn strains reported to possess abnormally high wax contents (according to appearance of the leaves) in the seedling leaves will also be discussed briefly.

B. Methods

Glossy seedlings (gl_1) were first described by Kvakan in 1924 (in Emerson, Beadle, and Fraser, 1935), and since that time many more have been found, some of which may be allelic, however. Intercross tests for allelism are being carried out by Dr. Anderson and his staff at the present time.

Seed of well-defined genetic background of glossy types were kindly furnished for study by Dr. Anderson. Table X summarizes the glossies studied, in decreasing order of glossiness, and the data on chromosome position.

TABLE X. Types of glossy corn studied, arranged in order of decreasing glossiness from top to bottom.

glossy type	remarks
1	chromosome 7
6, allelic to 8	chromosome 5
2	chromosome 2
3	chromosome 4
5321, allelic to 5534	chromosome unknown
5249	chromosome unknown
5, allelic to 10	probably chromosome 1; a late developing glossy; leaves no. 1 and 2 are normal, no. 3 glossy, no. 4 glossy, etc.

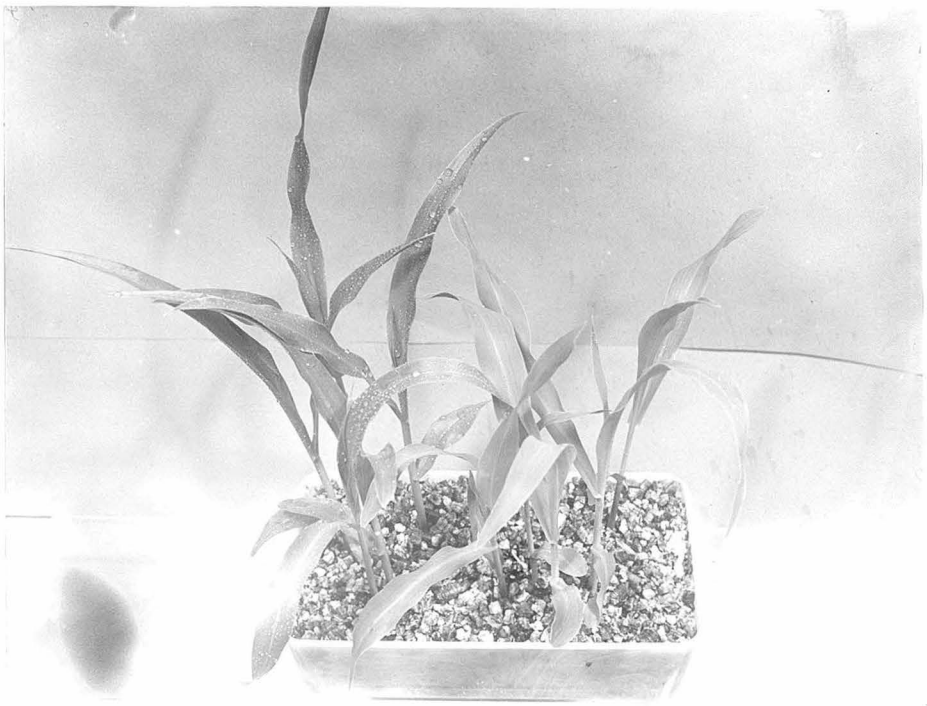


Figure 11. Three week corn seedlings segregating gl_1 (left) and normal (right). The plants have been sprayed with water which adhered only to the glossy seedlings.

All seedlings were grown in vermiculite with Hoagland's nutrient solution in the Phytotron under controlled conditions of 23° day and 17° night with natural day length. Thus environmental variations were held at a minimum. The seedlings, with one exception, segregated for glossy and normal so that the genetic background of each normal and its glossy counterpart are exactly comparable. Unless otherwise noted, all plant material was 21 days old at the time of harvest. For the survey of glossy vs. normal the whole aerial shoot was studied, but in the age study to be discussed later only leaf blades were used. The analytical procedures of preparation of material, extraction, and characterization of the fat and wax fractions are the same as those used in the study of environmental effects on lipids, Part II of this thesis. Inasmuch as the seedlings were all of the same age, except in the age study, it was believed that expression of yields on a dry plant weight basis would be satisfactory.

The two corn strains (H.I. 19 and B4-4E6) reported to possess an abnormally high wax content were obtained by Dr. Anderson from the Pioneer Hybrid Seed Company. The genetic background of these strains is not known. As an interesting comparison with the study of the effect of glossy genes, the two strains were also grown and extracted similarly to the glossy material.

C. Results and Discussion

1. Glossy and Normal corn

As Table XI shows, the yield of wax of glossy seedlings was always less than normals. Furthermore, as the ratio of percent wax in glossy to percent wax in normal indicates, the amount of wax in the glossies follows very closely the phenotypic glossiness ascribed to the glossy type by appearance and wettability. For instance, gl-1 is phenotypically the most glossy of the group, and analysis shows that leaves of gl-1 seedlings may contain as low as one-tenth as much wax as gl-1-normal leaves. There is one notable exception, gl-10, which is believed to be an allele of gl-5 (Anderson, et al., 1951). Glossy-10-gl leaves contained only 0.6 percent wax as compared to 1.65 percent for gl-5-gl. Both of these are late-developing glossies, and possibly there is a difference of time of appearance of very glossy leaves which cannot be detected visually. As will be shown later, the first leaf of gl-5 to be definitely glossy on the basis of wax analysis is the fourth leaf, although on the basis of external appearance the third leaf is quite glossy. Unfortunately no age study of gl-10 was possible, but it may be predicted that the first leaf to be chemically glossy is the third leaf rather than the fourth, as in gl-5. At the age of twenty-one days, which is the age of seedlings referred to in Table XI, the fourth leaf has not yet appeared.

TABLE XI. Yields of glossy vs. normal.
All plants 21 days old.

Glossy Type	% wax		% wax glossy / % wax normal	% fat		% fat glossy / % fat normal
	Glossy	Normal		Glossy	Normal	
1	0.28	1.86	0.149	2.00	1.75	1.14
1	0.28	1.52	0.182	2.00	2.00	1.00
1	0.17	1.82	0.092	2.08	2.65	0.78
6	0.42	1.82	0.230	2.00	1.48	1.35
8	0.66	1.62	0.407	1.79	1.67	1.07
2	0.53	1.66	0.318	2.34	2.76	0.85
2	0.46	2.07	0.221	2.28	1.88	1.22
2	0.90	2.07	0.433	2.17	2.23	0.98
3	0.79	1.96	0.405	2.01	2.00	1.00
5321	0.70	1.67	0.420	1.34	1.09	1.22
5534	0.84	1.63	0.515	1.73	2.05	0.85
5534	0.84	1.81	0.463	1.73	1.83	0.95
5249	1.08	1.56	0.700	2.23	1.85	1.20
5	1.65	2.27	0.725	1.69	1.03	1.64
10	0.63	2.04	0.313	2.05	1.91	1.07

Table XI also includes the yields of fat, and in this connection it is interesting that in both glossy and normal plants the fat content is quite similar, the glossies generally yielding a slightly higher quantity.

Not only the yields, but also the types of wax and fat formed are controlled by the glossy genes (Table XII). Wax of seedlings segregating glossy was generally of lower melting point than the wax of normals, and as the glossiness was increased the difference between the melting points of the normal and glossy types became greater. This is, in many cases, correlated with the iodine number, the glossy waxes being of greater unsaturation, but the relationship is not strict. One may conclude that the glossy genes are affecting both degree of unsaturation, chain length, and possibly other as yet unknown molecular characters.

The fat fraction is also affected by the genes for glossy. For the most part the fats of normal seedlings are liquid at room temperature, whereas the fats of the glossies are mostly solids. This again is not strictly associated with the iodine number.

These results suggest that some long chain or more saturated fatty acids accumulate in the fat fraction of glossy seedlings instead of being exuded to the leaf surface and cuticle as wax. This hypothesis is in agreement with the greater fat content and lower wax content of glossy leaves, the lower melt-

TABLE XII. The wax and fat melting points and iodine numbers of glossy vs. normal.

Glossy Type	Wax				Fat			
	M.P. °C.		Iodine No.		Liquid (l) or solid(s) at 27° C.		Iodine No.	
	Glossy	Normal	Glossy	Normal	Glossy	Normal	Glossy	Normal
1	76.5	85.0	-	22.5	s	l	156	221
1	76.5	84.0	-	14.0	s	s	156	225
1	-	84.0	53.1	24.1	l	l	207	225
6	73.9	80.6	16.4	25.1	s	l	181	230
8	79.6	84.6	18.8	17.3	l	l	210	169
2	75.2	85.5	23.6	14.4	s	l	215	228
2	-	78.6	-	27.8	s	l	237	243
2	77.4	79.6	22.2	32.0	l	l	186	224
3	77.2	81.2	51.4	45.9	s	l	169	227
5321	81.9	81.7	23.1	25.4	sl	l	185	208
5534	82.5	87.8	27.8	9.2	sl	l	176	222
5534	82.5	86.2	27.8	11.3	sl	l	176	195
5249	81.7	80.3	17.2	38.2	l	l	228	244
5	82.6	79.2	11.6	46.8	s	l	199	187
10	79.6	80.5	40.3	54.6	s	sl	198	185

ing point and greater iodine number of glossy waxes, and the more solid fats of lower iodine number of glossy leaves. The gene action of the recessive glossy character may therefore be principally to alter the fate of the fatty acids (or long chain alcohols). The genetic background, other than the glossy genes, appears to have an effect on lipid formation also because there was considerable variability of yield, melting point, and iodine number of the fats and waxes of the normal seedlings.

Because some of the glossy types (gl_5, gl_{10}) are late-developing, it was of interest to examine these glossies in order to find out if the yield of wax inversely parallels the appearance of the glossy features. Other glossies (gl_1 and gl_2) were also studied at different ages on the hypothesis that possibly these too may become glossier at later stages but that such changes are undetectable by visual means.

Leaf blades of gl-1-gl and gl-1-normal were examined at seedling ages of 12,22, and 30 days (Table XIII). From a comparison of wax content in glossy to that of normal leaves it can be seen that when the leaves were young the glossies contained more wax in relation to the normals, but as the leaves became older the ratio of glossy wax to normal wax decreased. Thus gl_1 shows some tendency toward the late-developing characteristic. The glossy leaves usually contained more fat than did the normals.

A similar study of gl-2-gl and gl-2-normal at ages of 12 and 30 days showed that in this case there is only a slight late-

TABLE XIII. The relationship of wax and fat content of glossy-1 to age. The leaves are numbered with the first leaf to appear as No. 1.

Age in days	1st	2nd	3rd	4th	1st	2nd	3rd	4th
	% wax gl-1-gl				% wax gl-1-normal			
12	1.20	1.18	-	-	1.71	1.74	-	-
22	1.22	0.61	0.50	1.45	2.40	2.07	1.97	3.55
30	1.59	0.88	0.48	0.46	3.45	2.34	1.70	1.68
	% fat gl-1-gl				% fat gl-1-normal			
12	2.45	3.36	-	-	1.94	2.38	-	-
22	2.00	2.27	2.06	2.98	2.04	2.14	1.72	2.12
30	1.85	2.05	2.12	1.77	1.55	2.33	1.81	1.96
	% gl. wax/% normal wax				% gl. fat/%normal fat			
12	0.70	0.68	-	-	1.37	1.41	-	-
22	0.51	0.29	0.25	0.41	0.98	1.07	1.20	1.41
30	0.46	0.38	0.28	0.28	1.19	0.94	1.17	0.90

developing tendency. The data are on the boundary of significance of the extraction methods. Glossy-2 seedlings usually yielded more fat than their normal counterparts (Table XIV).

Glossy-5, which is visually of the late-developing type, was studied at ages of 12 and 30 days. The seed was homozygous recessive so that only glossy seedlings were available for examination. As will be noted from Table XV, the first and second leaves had a high wax content, but in the third leaf the wax content dropped. The third leaf is glossy in appearance whereas the other two appear normal. The wax content of the fourth, fifth, and sixth leaves was very low, and it is these leaves that show to a maximum the glossiness and wettability. Thus the appearance of glossiness in gl_5 is correlated closely with the disappearance of wax. These results also indicate that much of the wax derived from a corn leaf by means of whole leaf extraction procedures is derived from the cuticle.

2. "High" wax content corn.

Twenty-one day old seedlings of strain H.I. 19 did, indeed, appear waxy. The leaves of this strain had a dense white bloom, but when sprayed with water the water accumulated in very fine droplets. Strain B4-4E6 was without any bloom, it wet easily with a spray, and showed no "waxy" characteristics.

Analysis of these strains indicates that they contain less

TABLE XIV. The relationship of wax and fat content of glossy-2 to age. The first leaf to appear is No. 1.

Age in days	1	2	3	4	5	1	2	3	4	5
	% wax gl-2-gl					% wax gl-2-normal				
12	1.35	1.12	1.65	-	-	1.90	2.04	2.04	-	-
30	4.35	1.52	1.21	1.07	0.91	5.32	2.65	1.92	1.69	-
	% fat gl-2-gl					% fat gl-2-normal				
12	3.19	3.08	2.71	-	-	2.55	3.06	2.74	-	-
30	3.30	2.44	2.29	2.14	2.06	2.74	2.59	2.15	2.06	-
	% gl.wax/% normal wax					% gl.fat/% normal fat				
12	0.71	0.55	0.81	-	-	1.25	1.01	0.99	-	-
30	0.82	0.57	0.63	0.63	-	1.21	0.95	1.06	1.04	-

TABLE XV. The relationship of wax and fat content to age in late-developing glossy-5. The first leaf to appear is No. 1.

Age in days	1	2	3	4	5	6
			% wax gl-5			
12	2.22	1.91	1.50	-	-	-
30	6.51	2.14	1.19	0.66	0.65	1.05
			% fat gl-5			
12	2.96	3.16	3.22	-	-	-
30	1.11	2.00	2.40	1.73	1.93	2.22

than the normal quantity of wax, even in H.I. 19 which has the white bloom (Table XVI). The fat content is as in normal corn. It therefore appears, at least in strain H.I. 19, that the genetic makeup may affect the formation of non-waxy cuticle constituents.

TABLE XVI. Yields of fat and wax from "high" wax corn strains.

	% wax	% fat
H.I. 19	1.65	1.96
B4-4E6	1.33	2.13
Ave. normal	1.8	2.0

D. Conclusions

The series of recessive genes related to glossiness in corn seedlings has been shown to control the wax content of the cuticle. The genes also influence the type of wax formed and the yield and type of fat of the leaves. From the data it appears that the gene action is to detour the long-chain fatty acids (or alcohols) from exudation into the cuticle to accumulation in the fat fraction of the leaves.

The effect of genetic constitution on lipid content is much greater than are the effects of the environment. Plants responded to various climatic factors by a two or three-fold change of wax or fat content, but in the glossy series up to a

ten-fold change of wax yield is possible. This effect of hereditary factors is in agreement with previous work in which kernel oil content was varied over a range of 1.3 to 14 percent through long-term selection. It is therefore apparent that the genetic constitution of plants holds great potentialities in regard to increased yields of lipids, and other constituents may respond similarly. This in combination with the optimum climatic factors would permit great control over the type and quantity of lipid formed.

Two strains of corn reported to have a high wax content were found to contain less than the normal quantity of wax. One strain, however, had an abundant white bloom, thus indicating that some genetic factors were affecting cuticular constituents other than wax. A heavy cuticular bloom may therefore be brought about by both genetic factors and environment (high temperature and water stress).

IV. STUDIES ON THE SYNTHESIS OF FAT

A. Introduction

The mechanism of fatty acid synthesis in organisms has been a challenging problem to biochemists for nearly one hundred years. Only in the past decade, however, has a substantial contribution been made to the understanding of the pathways involved, and this work has been confined to animals and microorganisms. Almost no progress has been made in the study of fat synthesis in higher plants, and, indeed, there have been in general no studies on intermediary metabolism of fats in higher plants. In the present work a start has been made in this direction, and a system has been obtained in which certain biochemical aspects of fatty acid synthesis in a higher plant may be studied.

Any satisfactory theory of fatty acid synthesis must be in accord with several well known facts: 1, all naturally occurring fatty acids of higher plants contain an even number of carbon atoms; 2, the C_{18} acids predominate although others do occur; and 3, double and triple bonds generally occur in positions 9, 12, and 15. The existing theories all attempt to account for the even-numbered carbon chains, but no suggestions as to the mechanism which controls chain length or position of unsaturated bonds have been advanced.

The older theories of fatty acid synthesis, which are

extensively reviewed by Hilditch (1947), are briefly as follows. Emil Fischer in 1890 proposed that hexoses condense directly to form C_{12}, C_{18}, and C_{24} acids. This readily accounts for the predominance of these acids in nature, but it does not account for the general occurrence of other acids, such as the common palmitic acid (C_{16}). Aldol condensation and reduction of acetaldehyde was suggested on the basis that certain two-carbon substances had been shown to be effective in supporting fat formation in microorganisms. This theory came close to present views on this problem, but it was never subjected to experimental proof. The other early theories were similar to the aldol condensation theory except that the polymerizing substances were variously considered to be pyruvic acid or aldehydes other than acetaldehyde.

Our present views of fatty acid synthesis are based on experiments which demonstrate that some C_2 fragment, probably a very close derivative of acetic acid, is utilized in a multiple condensation reaction to form even-numbered fatty acid chains. Smedley-Maclean and Hoffert (1924) first suggested that such a common C_2 precursor might be involved in fat synthesis in yeast, but it was not until isotopic techniques were applied to biological problems that their hypothesis could definitely be proved. In 1944 Rittenberg and Bloch showed that when carboxyl labeled acetate is fed to mice the resultant fatty acids are not only labeled, but that the carboxyl group of the fatty

acids contained twice as much activity as the average of the carbons of the whole molecule. This suggested that the fatty acid is derived by multiple condensation in a head-to-tail manner of acetate or some active C_2 fragment derived from acetate. Other work by Rittenberg and Bloch (1945), Wood, Brown and Werkman (1945), and White and Werkman (1947) have verified these results, but only in the past two or three years has an insight been gained as to the mechanism of the reaction.

Although much remains to be demonstrated, particularly in regard to the higher fatty acids, the work of Stadtman, Barker, and co-workers has furthered our knowledge of this subject considerably. By means of an enzyme preparation from Clostridium kluyveri it was shown (Stadtman, Stadtman, and Barker, 1949) that ethyl alcohol and butyric acid can be condensed and reduced to form caproic acid (C_6). The reaction involved the carboxyl group of butyric acid and the methyl group of ethyl alcohol. Stadtman and Barker (1949abcd, 1950) then began an excellent series of studies in which they demonstrated the anaerobic condensation of acetate, probably as acetyl acetate, and simultaneous reduction of the intermediates to form butyric and caproic acids. Adenosine triphosphate was required for the reaction. The occurrence of acetoacetate as an intermediate is shown by their work, and, indeed, poly-keto acids may well be intermediates in the synthesis of the higher fatty acids

(Stotz, 1949). The reducing power required for the reduction of the keto acid to the corresponding fatty acid is derived in their system from the oxidation of ethyl alcohol to acetyl phosphate. Whether the findings of Stadtman and Barker are directly applicable to higher plants is not known, however.

Studies of fatty acid synthesis in higher plants have been confined to measurements of respiratory quotients and fat content of developing or germinating seeds and have not concerned intermediary metabolism. These studies all indicate that as fat is synthesized in fruits and seeds carbohydrate disappears and the respiratory quotient is therefore 1.5 or greater, as would be expected. When fat is degraded, as in germinating seeds, the fat content decreases, the carbohydrate content increases, and the respiratory quotient is very low (Eyre, 1931; Burr and Miller, 1938; Miller, 1910).

B. Methods

The problem of rubber synthesis in guayule has been studied by Bonner and Arreguin (1949) by means of feeding possible substrates to seedlings. If the rubber content increased in relation to suitable controls then the substrate was considered to be a possible intermediate in rubber synthesis and was then subjected to more detailed study. It appeared that a similar technique could be applied to the problem of fat synthesis, provided, of course, that a suitable plant tissue could be found.

Developing seed and fruits of flax, Linum usitatissimum satisfied the experimental requirements. Mature flax seed contains 40 percent oil, the greater part of which accumulates over a period of about fifteen days (Figure 12). It was hoped that isolated fruits, collected at the beginning of the period of rapid fat synthesis (about 10 day old fruits), could be fed substances which would specifically support fat formation. In this way the fruits could continue to make fat, the amount of fat formed being a measure of the activity of the substrate as a possible precursor of fats.

Fruits for this study were obtained from mature plants grown in the Phytotron and at Orlando Greenhouse. Fruits were obtained from both sources from May to August, 1951. Material from the two sources both responded well to the treatments, although the Phytotron fruits were generally more satisfactory. Reference has been made in each table of data in regard to the source of the experimental material. It should also be mentioned that the best plant material is obtained by the use of a 23° day, 10° night (8 hr. light and 8 hr. dark), and 16 hour photoperiod. The low night temperature is particularly important since at any higher temperature the fruits do not mature. Under these cultural conditions flax flowers profusely, and if the fruits are removed periodically the plants will not go into senescence for many months. In this way 6000 to 8000 fruits have been collected

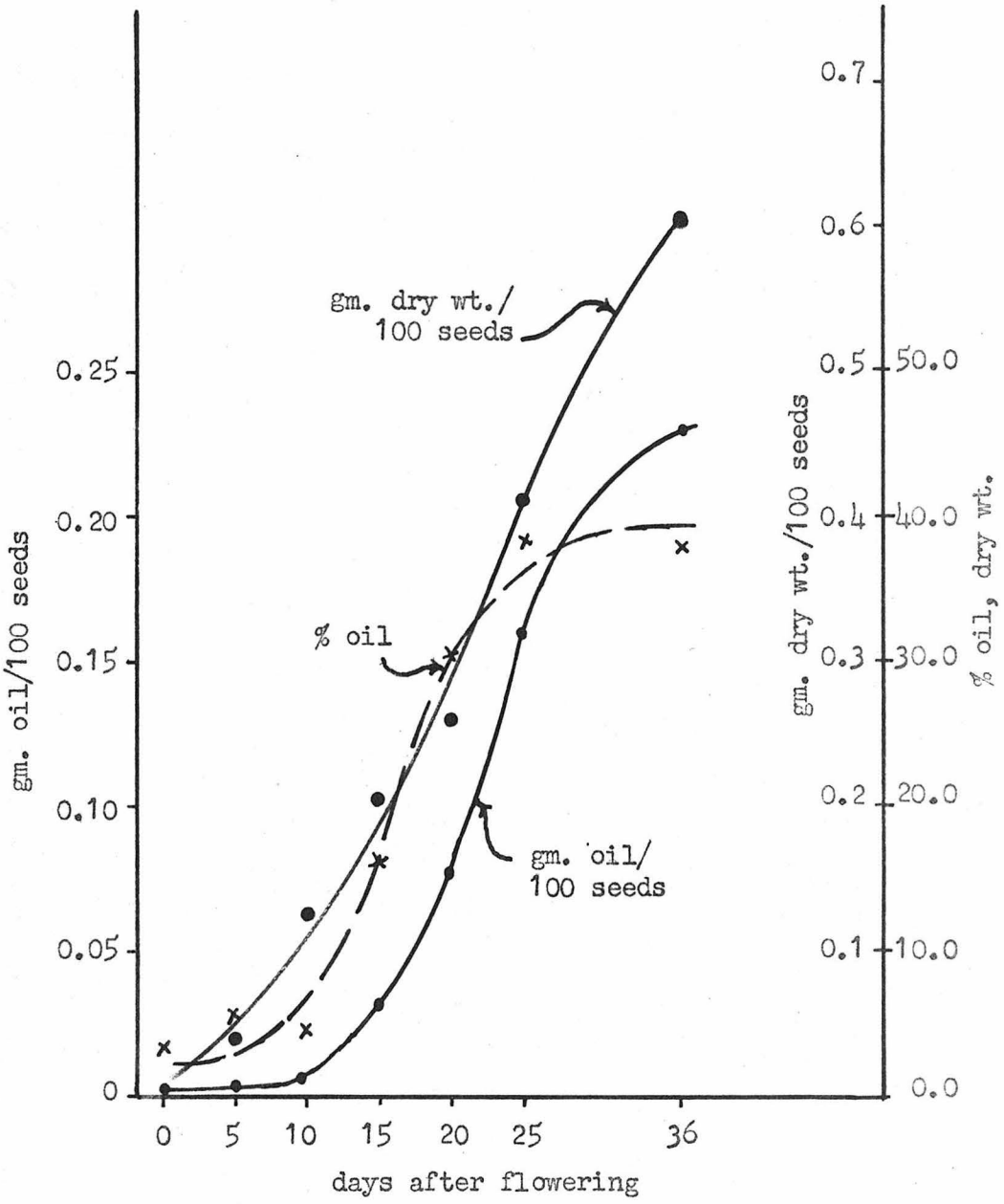


Figure 12. Growth and accumulation of fat in developing flax seeds. Fruits from Orlando Greenhouse.

continuously over a period of four months from approximately 200 plants. There were no signs of decreased fruit production at that time.

Fruits of uniform age were obtained in the following manner. All the flowers and fruits were removed from the plants and discarded the first day. About ten days later all the fruits of uniform size (about 6 mm.), shape, and color were then detached from the plants with a razor so as to leave 1 cm. of pedicel attached to the fruit. These fruits were used for the experiment. The remaining flowers and underdeveloped fruits at this time were then removed so that 10 days later another uniform crop of fruits could be obtained. The fruits were then floated on 100 cc. of culture solution, the culture flasks plugged with cotton, and placed in a controlled temperature room (the tissue culture room) at 28.5° C. A representative lot of initial fruits was collected and immediately dried for estimation of the initial oil content. After 30 to 48 hours of culture the fruits were rinsed and dried in an air-draft oven at 70° C. As will be shown, the fruits and seeds generally lose dry weight over the experimental period so that expression of the fat content on a dry weight basis becomes meaningless. All results are therefore expressed as milligrams of fat per 100 seeds. To obtain yields on a per seed basis the dried fruits were gently rolled and crushed, the intact whole seeds freed of fruit debris and then counted. Each fruit generally contained 7 to 9 seeds. The fat

was extracted with petroleum ether (B.P. 60-70° C.) for two hours in a micro-Soxhlet extractor. The solvent was then evaporated and the residue of fat dried and weighed.

Unless otherwise stated, all fruit cultures received a mineral nutrient supply (pH 6.5) to which were added the substances to be tested (Table XVII).

TABLE XVII. Composition of mineral nutrient solution used to culture flax fruits.

salt	conc. mg./l.	salt	conc. mg./l.
$Ca(NO_3)_2 \cdot 4H_2O$	400	$Fe_2(SO_4)_3$	50.0
KNO_3	100	$MnSO_4$	2.0
$MgSO_4 \cdot 7H_2O$	100	KI	0.5
KH_2PO_4	100	$NiCl_2$	0.05
		$CoCl_2$	0.05
		$TiSO_4$	0.2
		$ZnSO_4$	0.1
		$CuSO_4$	0.05
		$BeSO_4$	0.1
		H_3BO_3	0.05
		H_2SO_4	1.0

The variability of plant material which may be expected in the present experiments may be judged from the replication experiment for which data are presented in Table XVIII. In this experiment the standard error in oil content per 100 seeds was 11% of the mean. Each lot of fruits in the experiment of Table XVIII contained 30 fruits (about 250 seeds), but in the experi-

TABLE XVIII. Reproducibility of replicate lots of flax fruits (30 per lot) with respect to dry weight and fat content of the seeds. Expt. 5-27. Fruits grown 48 hours in light, in water and mineral nutrient, pH 6.5. Fruits from Phytotron.

lot	gm. dry wt./100 seeds	% oil	mg. oil/100 seeds
initial	0.4140	4.89	7.0
final 1	0.4005	11.24	16.0
2	0.3705	8.50	11.0
3	0.3780	10.05	13.5
4	0.3785	10.17	14.2
5	0.3720	8.33	11.0
6	0.3740	10.16	13.2
7	0.3970	10.45	14.6
Ave.	0.3815 $\pm$ 0.0098	9.84 $\pm$ 0.97	13.3 $\pm$ 1.5

ments to be reported later a greater number of fruits (40-70) were used. The experimental error should therefore be lower in the later experiments. Statistical analysis of each experiment was not possible, however, because all the fruits of each lot were required for fat analysis.

C. Experimental Results

Several preliminary experiments were necessary in order to find the optimum experimental conditions for fat formation by excised flax fruits. The first factor investigated was light (Table XIX). In general fruits grown in light (700-800 f.c., from 3 white fluorescent tubes, distance from culture flasks 12 to 15 inches) increased in fat content whereas those in dark lost fat. The addition of sucrose to the medium failed to support

fat formation in light or dark. It appears that light is required for some specific role (other than photosynthesis) essential to the growth and formation of fat in the seed. This is not unreasonable since the young developing seeds and fruits are green. All later experiments were carried out in light.

The effect of nutrient pH on fat yield is not marked, as

shown in Table XX. All the fruits except those at pH of 6.5 or 7.0 became soft, however, and this was accompanied by a browning of the sepals and fruit base. In view of this fact all later experiments (except those of Tables XXI and XXII) were conducted at pH 6.5.

It is generally believed that the immediate precursors of fat are the fat constituents, fatty acids and glycerol. The enzyme which catalyzes the esterification of these two components, lipase, has been found in fatty seeds and its

TABLE XIX. Effect of light on fat content. Expt. 5-15. All solutions with mineral nutrient, pH 6.5, 48 hours. Fruits from Phytotron.

lot	condition	addenda	mg. oil/100 seeds
initial			5.5
1	light	none	7.3
2	"	20 gm./l. sucrose	7.6
3	dark	none	5.3
4	"	20 gm./l. sucrose	4.2

TABLE XX. Effect of pH on fat content. Ext. 6-7. All solutions with mineral nutrient. in light, 48 hours. Fruits from Orlando Greenhouse.

lot	pH	addenda	mg. oil/100 seeds	gm. wt./ 100 seeds
initial			12.0	0.149
1	4.5	none	12.8	0.130
2	5.5	"	13.4	0.131
3	6.5	"	12.4	0.128
4	7.0	"	13.3	0.131
5	7.5	"	10.9	0.124

properties have been studied. It was therefore of interest to determine if developing flax fruits could utilize fatty acids and glycerol to form fat. When glycerol, stearate, oleate, and octanoate were supplied individually in the nutrient solution the seeds failed to increase in fat content over the initial amount and the seed weight decreased markedly (Table XXI). If, however, sucrose was fed in addition to glycerol and oleate, a significant increase in fat formation occurred (Table XXII). Other fatty acids may function similarly to oleate, although they were not tested. The effect of the immediate fat precursors on fat formation is small as compared to the effects of sucrose, acetate, and biotin which will be discussed later. This may be due in part to the slow penetration of the fatty acids or to toxic effects which they may exert.

The fact that sucrose is required for utilization of glycerol and oleate in fat formation suggests the use of sucrose by the seeds as an energy source. In any case, sucrose when fed alone does not support fat formation (Table XXIII).

It has been mentioned above that pyruvic acid, ethyl alcohol, acetoacetate, and acetone have been suggested as precursors in fatty acid synthesis. As shown in Table XXIV, these substances, with the exception of pyruvate, did indeed increase fat formation, although the extent of utilization was not great. Ethyl alcohol and acetone were active even without added sucrose.

TABLE XXI. Effect of fat constituents on fat formation. All solutions with mineral nutrients, pH 7.5, 48 hours in light. Expt. 6-2. Fruits from Orlando Greenhouse.

lot	addenda	mg. oil/ 100 seeds	gm. wt./ 100 seeds
initial		5.6	0.127
1	none	7.1	0.140
2	750 mg./l. glycerol	5.2	0.100
3	4500 mg./l. Na stearate	5.0	0.108
4	4500 mg./l. Na oleate	6.1	0.106
5	2000 mg./l. Na octanoate	5.6	0.106

TABLE XXII. Effect of sucrose, glycerol, and oleate on fat synthesis. Expt. 4-7. All solutions with mineral nutrient, pH 7.5, 48 hours, in light. Fruits from Phytotron.

lot	addenda	mg. oil/ 100 seeds
initial		16.2
1	none	15.8
2	20 gm./l. sucrose	15.0
3	5000 mg./l. glycerol, 5000 mg./l. Na oleate	16.6
4	20 gm./l. sucrose, 5000 mg./l. glycerol, 5000 mg./l. Na oleate	19.2

The rapid permeation of these substances may materially promote their utilization in fat synthesis. Pyruvic acid, on the other hand, may have not been used as a substrate because of its toxicity. Although it was not possible to investigate further the role of these substances in fat formation, the results indicate that C_2, C_3, and C_4 compounds such as ethyl alcohol, acetone, and acetoacetate may be used to support fat synthesis. Whether they are actual intermediates cannot as yet be stated.

TABLE XXIII. Effect of sucrose (20 gm./l.) on fat formation. All solutions with mineral nutrient, pH 6.5, in light.

Expt. No.	Time	Addenda	mg. oil/ 100 seeds	gm. wt./ 100 seeds
5-15	initial		5.5	-
(Ph.)*	48 hrs.	none	7.3	-
	"	sucrose	7.6	-
4-7	initial		16.2	-
(Ph.)*	48 hrs.	none	15.8	-
	"	sucrose	15.0	-
7-17	initial		36.4	0.172
(O.G.)*	31 hrs.	none	33.8	0.144
	"	sucrose	33.8	0.147

*Ph. = fruits from Phytotron; O.G. = fruits from Orlando Greenhouse.

Acetate has been shown above to be a precursor of fatty acids in animals and microorganisms. The effect of acetate as a fat precursor in the higher plant, flax, was therefore studied. It is shown in Table XXV that the addition of acetate (as Na or K salt) to the culture solution failed to significantly increase the amount of fat formed. Acetate, even in large doses, did not appear to be toxic. Furthermore, acetate in combination with sucrose does not greatly increase fat formation, although in some cases (Expts. 5-15, 7-3, and 7-20) the amount of fat is slightly greater than in the absence of added acetate (Table XXVI).

It is worthy of note that although the seeds generally lose weight during the culture period, fat formation continues and the final fat content of seeds fed acetate and sucrose may

TABLE XXIV. Effect of various substrates on fat formation. All solutions with mineral nutrient, pH 6.5, in light.

Expt. No.	Time	Addenda	mg. oil/ 100 seeds	gm. wt./ 100 seeds
7-26	initial		9.4	0.095
(O.G.)	30 hrs.	none	5.9	0.078
	"	20 gm./l. sucrose, 900 mg./l. pyruvic acid (0.01M)	6.6	0.078
	"	20 gm./l. sucrose, 1310 mg./l. ethyl acetoacetate (0.01M)	12.1	0.089
8-13	initial		16.8	-
(Ph.)	31 hrs.	none	18.8	-
	"	460 mg./l. ethyl alcohol (0.01M)	23.3	-
	"	580 mg./l. acetone (0.01M)	22.6	-

even exceed the content of mineral nutrient controls. This suggested that the culture medium was deficient in one or more growth factors essential to fruit growth. It was also anticipated that better fruit growth might have a direct effect on fat synthesis. The following data show this to be the case.

The B-vitamin, biotin, has been implicated in fatty acid synthesis by several workers. Thus Gavin and McHenry (1941) found that biotin when added to the diet of rats increased body weight and produced fatty livers. In lactic acid bacteria oleic acid may replace the essential growth factor biotin (Williams and Fieger, 1946; Williams, Broquist, and Snell, 1947; Potter and Elvehjem, 1948). It has also been demonstrated that biotin, even in extremely minute amounts, may function in succinic acid oxidation (Ajl, Hart, and Werkman, 1950), and in the carboxylation of pyruvic acid to form oxalacetic acid (Lardy, Potter, and Elvehjem, 1947; Shive and Rogers, 1947). In view of these facts it seemed advisable to investigate the effect of biotin on fat synthesis in flax fruits. Biotin does, indeed, have pronounced effects in this process (Table XXVII).

The addition of biotin to the medium used for the culture of excised flax fruits may cause either an increase or a decrease of fat content, depending upon the age of the fruits used. Biotin, even when fed with sucrose and acetate, may

TABLE XXV. Effect of acetate (Ac) on fat formation. All solutions with mineral nutrient, pH 6.5, in light.

Expt. No.	Time	Addenda	mg. oil/ 100 seeds	gm.wt./ 100 seeds
6-2	initial		5.6	0.127
(O.G.)	48 hrs.	none	7.1	0.140
	"	500 mg./l. NaAc	5.0	0.102
4-7	initial		16.2	-
(Ph.)	48 hrs.	none	15.8	-
	"	2000 mg./l. NaAc	17.4	-
6-10	initial		4.6	0.125
(O.G.)	24 hrs.	none	5.1	0.105
	"	5000 mg./l. NaAc	5.9	0.110
	"	5880 mg./l. KAc	6.9	0.112
	48 hrs.	none	5.6	0.099
	"	5000 mg./l. NaAc	4.9	0.096
	"	5880 mg./l. KAc	6.8	0.105
7-17	initial		36.4	0.172
(O.G.)	31 hrs.	none	33.8	0.144
	"	5880 mg./l. KAc	37.3	0.155
6-12	initial		15.6	0.140
(Ph.)	48 hrs.	none	17.5	0.126
	"	5880 mg./l. KAc	16.4	0.123

TABLE XXVI. Effect of acetate and sucrose on fat formation. All solutions with mineral nutrient, pH 6.5, in light.

Expt. No.	Time	Addenda	mg. oil/ 100 seeds	gm. wt./ 100 seeds
5-15	initial		5.5	-
(Ph.)	48 hrs.	none	7.3	-
	"	20 gm./l. sucrose, 1000 mg./l. NaAc	9.2	-
	"	" " , 4000 mg./l. NaAc	6.8	-
	"	" " , 8000 mg./l. NaAc	5.8	-
7-3	initial		3.3	0.141
(O.G.)	41 hrs.	none	3.1	0.135
	"	20 gm./l. sucrose, 5880 mg./l. KAc	4.4	0.155
7-31	initial		2.2	0.080
(Ph.)	48 hrs.	none	5.9	0.079
	"	20 gm./l. sucrose, 5880 mg./l. KAc	5.8	0.087
7-20	initial		11.1	0.104
(O.G.)	30 hrs.	none	13.4	0.100
	"	20 gm./l. sucrose, 5880 mg./l. KAc	16.8	0.108

inhibit the formation or cause the breakdown of fats in young fruits which have not yet entered the period of rapid fat synthesis (Expt. 7-31). In slightly older fruits biotin has no apparent effect on fat synthesis or breakdown (Expt. 7-3). Biotin has, however, a pronounced effect in increasing fat synthesis in fruits which are more mature and which have entered the grand period of fat accumulation (Expts. 6-18, 7-17). During this period biotin alone has no effect, but when fed in addition to sucrose and acetate rapid formation of fat occurs (Table XXVII). A comparison of the data of Table XXVII with those of Figure 12 shows that when biotin, sucrose, and acetate are fed simultaneously the formation of fat in cultured fruits parallels that of fruits growing on the plant. The cultured fruits receiving biotin and the necessary substrates still lose dry weight in the course of the experiment, but the loss is usually not as great as when no biotin is supplied. Thus, again, the fat synthesis mechanism remains intact and functional even though the seed as a whole exhibits a negative weight balance.

The exact role of biotin in fat synthesis cannot be inferred from these data. Both the present experiments and the studies referred to earlier indicate, however, that biotin is essential to one or more steps in fat synthesis, and the low concentration of biotin required suggests that this vitamin may be a cofactor in an enzyme system involved.

TABLE XXVII. Effect of sucrose, acetate, and biotin (5 gamma/l.) on fat formation. All solutions with mineral nutrient, pH 6.5, in light.

Expt. No.	Time	Addenda	mg.oil/ 100 seeds	gm.wt./ 100 seeds
6-18	initial		7.4	0.126
(Ph.)	48 hrs.	20 gm./l. sucrose, 5880 mg./l. KAc	10.0	0.107
	"	20 gm./l. sucrose, 5880 mg./l. KAc, biotin	17.1	0.124
7-17	initial		36.4	0.172
(O.G.)	31 hrs.	none	33.8	0.144
	"	biotin	33.2	0.152
	"	20 gm./l. sucrose, biotin	37.4	0.160
	"	5880 mg./l. KAc, biotin	34.6	0.150
	"	20 gm./l. sucrose, 5880 mg./l. KAc	34.8	0.158
	"	20 gm./l. sucrose, 5880 mg./l. KAc, biotin	42.3	0.167
7-31	initial		2.2	0.080
(Ph.)	48 hrs.	none	5.9	0.079
	"	20 gm./l. sucrose, 5880 mg./l. KAc	5.8	0.087
	"	20 gm./l. sucrose, 5880 mg./l. KAc, biotin	2.7	0.068
7-3	initial		3.3	0.141
(O.G.)	48 hrs.	none	3.1	0.135
	"	20 gm./l. sucrose, 5880 mg./l. KAc	4.4	0.155
	"	20 gm./l. sucrose, 5880 mg./l. KAc, 0.5 gamma/l. biotin	3.9	0.138
	"	20 gm./l. sucrose, 5880 mg./l. KAc, 5 gamma/l. biotin	4.4	0.151
	"	20 gm./l. sucrose, 5880 mg./l. KAc, 50 gamma/l. biotin	3.5	0.148

Inasmuch as biotin produced such marked effects, several other vitamins were also tested. These included pantothenic acid, which is a coenzyme for acetylation (Lipmann, et al., 1947); adenine sulfate, a growth factor in etiolated peas (Galston and Hand, 1949); and pyridoxine (as pyridoxal phosphate) which is associated in some unknown manner with fat metabolism in animals (Sherman, 1950). Unfortunately the fruits used were rather variable so that no conclusions could be drawn.

Thiamine, riboflavin, and nicotinic acid are known to be involved in the conversion of carbohydrate to fat (Boxer and Stetten, 1944; Nord, et al., 1949), as would be expected in view of the present knowledge of the glycolytic pathway. A B-vitamin mixture containing these and other vitamins was therefore tested. The mixture contained the following components:

	mg./l.
thiamine.HCl	0.1
riboflavin	0.05
pyridoxine.HCl	0.1
Ca pantothenate	0.8
p-aminobenzoic acid	0.05
niacin	0.5
inositol	0.5
choline chloride	0.1

Ascorbic acid and indole acetic acid (IAA) were also tested. Various combinations of these growth factors in addition to biotin, sucrose, and acetate were added to nutrient solutions and tested (Table XXVIII). The mixture of B-vitamins employed

TABLE XXVIII. Effect of various growth factors on fat synthesis in flax. All solutions with mineral nutrient, pH 6.5, 48 hours, in light. Expt. 6-18. Fruits from Phytotron.

lot	addenda	mg. oil/ 100 seeds	gm.wt./ 100 seeds
initial		7.4	0.126
1	20 gm./l. sucrose	11.4	0.120
2	20 gm./l. sucrose, 5880 mg./l. KAc	10.0	0.107
3	20 gm./l. sucrose, 5880 mg./l. KAc, 5 gamma/l. biotin	17.1	0.124
4	20 gm./l. sucrose, 5880 mg./l. KAc, B-vit. mixture	10.5	0.109
5	20 gm./l. sucrose, 5880 mg./l. KAc, 25 mg./l. ascorbic acid	12.1	0.117
6	20 gm./l. sucrose, 5880 mg./l. KAc, 100 mg./l. IAA	16.9	0.123
7	20 gm./l. sucrose, 5880 mg./l. KAc, B-vit. mixture, 5 gamma/l. biotin, 25 mg./l. ascorbic acid	14.1	0.122
8	5880 mg./l. KAc, B-vit. mix., 5 gamma/l. biotin, 25 mg./l. ascorbic acid	12.2	0.113
9	5880 mg./l. KAc, B-vit. mix., 5 gamma/l. biotin, 25 mg./l. ascorbic acid, 100 mg./l. IAA	10.5	0.110

failed to increase fat formation, even in the presence of sucrose, acetate, and biotin. Some member of the B-vitamines may therefore be inhibitory to fat formation or the action of biotin. Ascorbic acid had little or no effect on fat synthesis. Indole acetic acid in combination with sucrose and acetate markedly increased fat formation. This effect may possibly warrant further study. The maximum rate of fat formation under the present conditions has apparently been attained by the use of acetate, sucrose, and biotin.

D. Conclusions

Flax fruits, properly selected for age and uniformity, provide excellent material for the study of the fat synthesis system in higher plants. Over short time periods of culture, 48 hours or less, substantial production of fat may take place in excised fruits provided that certain substrates and growth factors are supplied to them. The substrates found to be most effective are compounds containing two, three, or four carbon atoms. All are materials which are, in general, readily oxidized by living tissues to acetate or related acetyl derivatives. The data presented offer no proof that the active substrates acetate, acetoacetate, ethyl alcohol, or acetone are actual intermediates in fat synthesis. Such proof might, however, be obtained from experiments with isotopically labeled materials.

The immediate fat precursors glycerol and oleic acid are

effective in supporting fat formation. It is not known whether glycerol and oleic acid are used directly for fat synthesis, or whether the fatty acid fed is first broken down and then re-synthesized to appropriate fatty acids which are then converted to glycerides. Sucrose is also required for utilization of glycerol and oleic acid, suggesting that the latter possibility is the more probable.

Energy is required for the synthesis of fats, as shown by the fact that light and an energy source such as sucrose are necessary for rapid production of fats at the expense of short chain carbon substrates. The requirement of both light and sucrose suggests that light is essential to this process through reactions other than a photosynthetic one.

For maximum fat formation in excised fruits, certain growth factors are required in addition to appropriate carbon sources. Biotin is particularly effective in this capacity. Biotin, in extremely small quantities (5 gamma/l.), when added to a medium of sucrose and acetate, accelerated fat formation to such an extent that the amount of fat formed in cultured excised fruits paralleled the amount formed in fruits left on the plant. This effect is in agreement with other studies on animal and microbiological systems in which it has been found that biotin promotes fat formation. Biotin is only effective when the fruits are 10 to 20 days old, that is, when the fruits are rapidly

accumulating fat. Biotin is apparently a limiting factor in fat synthesis only during this stage of development.

No clear role of other growth substances (Ca pantothenate, adenine sulfate, pyridoxal phosphate, and others) is apparent from the present study.

Flax fruits appear to offer an excellent system for the study of fat synthesis in a higher plant. Although the fruits generally lose dry weight during the experiment, the fat synthetic mechanism remains more or less intact. Also the system is not overly sensitive to toxic effects of the added substrates or to pH of the medium. Further study of additional substrates, particularly isotopically labeled acetate, is certainly warranted, and ultimately attempts should be made to isolate and study the individual enzyme systems involved.

Part V. SUMMARY

In spite of the vast knowledge of the chemical technology of lipids and in spite of the great importance of these substances both commercially and biologically, extremely little is known about the lipids of plants from a physiological or biochemical standpoint. Work of recent years, notably on animals and microorganisms, has begun to rectify this situation, but the biochemistry of lipids in higher plants has received practically no attention. It has been of interest, therefore, to investigate some of the many problems involved. The present study concerns three aspects of the general problem: the environmental (Part II) and genetic (Part III) effects on lipids, and the mechanisms involved in the synthesis of fats (Part IV).

From a study of four species grown under controlled conditions of temperature, light, and soil moisture, it was found that these climatic factors exert marked effects on the yield and on the composition of leaf fats and waxes.

Plants responded to increased temperature, particularly to night temperature, by producing less wax. This wax was of a higher melting point than that from plants grown at lower temperatures. The degree of unsaturation of the waxes, as indicated by the iodine number, showed no correlation with temperature. The waxes from the high temperature plants must

therefore owe their high melting point to molecular changes other than saturation of double or triple bonds. It is most likely that at the higher temperatures the chain length of the component substances is greatly increased.

The effect of soil moisture on wax production varied from one species to another. A striking response to low soil moisture (water stress) was that of the formation of an abundant white bloom in Nicotiana glauca. High temperature also favored the bloom formation. The bloom was found to consist of a large deposit of cuticle, but the amount of wax in this thick cuticle was not greater than that in thin cuticles from plants grown under conditions of high soil moisture or low temperature. Thus the bloom so typical of plants native to hot and dry climates may be accounted for, at least partly, as a response to low soil moisture and high temperatures. However, the cuticle of these plants with abundant bloom, either from controlled conditions or from desert climates, does not contain an abnormally high quantity of wax, a property which has often been ascribed to it. Perhaps the familiar concept that transpiration of desert plants is retarded by their thick waxy cuticles should also be revised.

Climate affects the fat fraction of plants quite strikingly. The yield of fat, which may vary as much as 300

percent over a temperature range of 6° to 30°, was affected differently in different species. Low soil moisture increased the fat content, however. High temperature and water stress also caused the melting point of the fat to increase as compared to the fats of plants under low temperatures and high soil moisture. This increased melting point was not related to changes of the iodine number, thus indicating again that molecular changes other than simple hydrogenation must have occurred.

For the most part the conclusions drawn from the present study in regard to the fats and waxes agree with the findings of McNair and others obtained from literature surveys and field observations.

The resinous substance of Larrea tridentata, a typical xerophyte of North American deserts, showed extreme variation under the climatic conditions used. This resin, which imparts the characteristic odor and stickiness to leaves of Larrea, was most abundant in water stress plants and in plants at low day or low night temperatures. In fact, at 20° day and 14° night in plants under water stress the resin constituted almost one-fourth the dry weight of the leaves. Such data support the widely held view that the resin is a factor in drought resistance, but the fact that little resin occurs in plants at high day or night temperatures should certainly provoke further

interest in the physiological significance of this substance.

Although plants respond to changes of certain climatic factors by producing two or three-fold changes in the character and yield of the lipid fractions, it was found that genetic makeup of the plant may exert a much greater control of the lipids formed by the plant. This was made possible by a series of recessive genes in corn which cause the seedling leaves to have a glossy nonwettable surface. It was found that leaf glossiness is directly related to the wax content, the most glossy type (gl_1) possessing only one-tenth the normal quantity of wax. The series of glossy genes also affects the type of wax formed, as shown by the lower melting points and generally greater degree of unsaturation of the glossy seedling waxes than of the waxes of normal plants. The fats are also modified in glossy as compared to normal seedlings, the glossies usually yielding more fat (of a higher melting point) than do the normal plants.

These results suggest that the glossy genes alter the site of deposition of long chain fatty acids (and alcohols). In normal seedling leaves these substances are exuded to the cuticle on the leaf surface and the yield of wax is therefore high and the wax has a high melting point. In glossies it appears that the long chain compounds are in some way modified so that they are prevented from being exuded to the surface,

with the result that they appear in the fat fraction. The fat from glossies is therefore of greater yield and of a higher melting point than that from normal seedlings.

Two strains of corn reported to possess a high wax content failed to yield even the normal quantity of wax. One strain did, however, possess an abundant white bloom on the leaves which indicates that some hereditary factor or factors also control the production of cuticular constituents other than wax.

The results of this study, together with previous work, definitely indicate that knowledge of the genetics of plant constituents offers great promise with respect to increasing crop yields, particularly in regard to the yield of lipids. There is a great need for further work on this problem since there are many plants that produce high yields of either carbohydrate or protein, but there are no species that produce good yields of fat or other lipid. It appears from the present study that through genetic control and the simultaneous use of an optimum environment it should be possible to greatly improve fat yields under agricultural conditions.

There have been no studies on the intermediary metabolism of lipids in higher plants. By means of a short term culture of developing flax fruits, it has now been shown that acetate and certain C_2, C_3, and C_4 carbon compounds may be utilized for fat synthesis. This is in agreement with the existing knowledge

of fatty acid synthesis in animals and microorganisms. Subsequent studies involving labeled acetate should elucidate the exact role of this compound as an intermediate in fatty acid synthesis. Light and an energy source such as sucrose were found to be required for rapid conversion of acetate to fat. Maximum rate of synthesis occurs only when a minute amount of biotin (5 gamma/liter) was also added to the culture medium. Biotin apparently is active as a cofactor in some enzyme system closely involved in the fat synthesis mechanism, which is in accord with studies on the effects of biotin on fat synthesis in animal and microbiological systems.

VI. REFERENCES

Ajl, S.J., W.R. Hart, and C.H. Werkman. 1950. Biotin in succinic acid oxidation. Enzymologia 14:1-7.

Anderson, D.B. 1934. The distribution of cutin in the outer epidermal wall of Clivia nobilis. Ohio J. Sc. 34:9-19.

Anderson, E., et al. 1951. Maize Genetics Cooperation News Letter 25:2.

Anonymous. 1930. Method of test for melting point of petrolatum, D 127-30. Am. Soc. Testing Materials, Part II, pp. 532-533.

Bandurski, R.S., and A.W. Galston. 1951. Maize Genetics Cooperation News Letter 25:5.

Boxer, G.F., and DeWitt Stetten, Jr. 1944. The role of thiamine in the synthesis of fatty acids from carbohydrate precursors. J. Biol. Chem. 153:607-616.

Bonner, J. 1943. Effects of temperature on rubber accumulation by the guayule plant. Bot. Gaz. 105:233-243.

_____, and B. Arreguin. 1949. The biochemistry of rubber formation in the guayule. I. Rubber formation in seedlings. Arch. Biochem. 21:109-124.

_____, and A.W. Galston. 1947. The physiology and biochemistry of rubber formation in plants. Bot. Gaz. 13:543-596.

Brimhall, B., and G.F. Sprague. 1951. Unsaturation of corn oil--inheritance and maturity studies. Cereal Chem. 28:225-231.

_____, _____, and J.E. Sass. 1945. A new waxy allel in corn and its effect on the properties of the endosperm starch. J. Am. Soc. Agron. 37:937-944.

Burr, G.O., and E.S. Miller. 1938. Synthesis of fats by green plants. Bot. Gaz. 99:773-785.

Cameron, J.W., and H.J. Teas. 1948. The relation between nicotinic acid and carbohydrate in a series of maize endosperm genotypes. Proc. Natl. Acad. Sc. 34:390-398.

Camus, G.Ch., and F.W. Went. 1951. Studies on the thermoperiodicity of three varieties of Nicotiana tabacum. in press.

Cartter, J.L., and T.H. Hopper. 1942. Influence of variety, environment, and fertility level on the chemical composition of soybean seed. USDA Tech. Bull. No. 787, 66 pages.

Channon, H.J., and A.C. Chibnall. 1929. The ether-soluble substances of cabbage leaf cytoplasm. V. The isolation of n-nonacosane and di-n-tetradecyl ketone. Biochem. J. 23:168-175.

Chibnall, A.C., and S.H. Piper. 1934. The metabolism of plant and insect waxes. Biochem. J. 28:2209-2219.

_____, _____, A. Pollard, J.A.B. Smith, and E.F. Williams. 1931. The wax constituents of the apple cuticle. Biochem. J. 25:2095-2110.

_____, E.F. Williams, A.L. Latner, and S.H. Piper. 1933. The isolation of n-triacontanol from lucerne wax. Biochem. J. 27:1885-1888.

Conrad, C.M., and J.W. Neely. 1943. Heritable relation of wax content and green pigmentation of lint in upland cotton. J. Agric. Res. 66:307-312.

Dahlgren, B.E. 1933. The yield of the carnauba palm. Tropical Woods, No. 35, pp.3-5.

Doerschuk, A.P., and B.F. Daubert. 1948. Low temperature fractionation of corn oil and calculation of the glyceride structure. J. Am. Oil Chem. Soc. 25:425-433.

Emerson, R.A., G.W. Beadle, and A.C. Fraser. 1935. A summary of linkage studies in maize. Cornell Univ. Agric. Expt. St. Memoir No. 180, pp. 1-83.

Eyre, J.V. 1931. Notes on oil development in the seed of a growing plant. Biochem. J. 25:1902-1908.

Galston, A.W., and M.E. Hand. 1949. Adenine as a growth factor for etiolated peas and its relation to the thermal inactivation of growth. Arch. Biochem. 22:434-443.

Gardner, H.A., and G.G. Sward. 1942. Creosote bush resin. Natl. Paint, Varnish, and Lacquer Assoc., Inc., Circular No. 647, 12 pages.

Garner, W.W., H.A. Allard, and C.L. Foubert. 1914. Oil content of seeds as affected by the nutrition of the plant. J. Agric. Res. 3:227-249.

Gavin, G., and E.W. McHenry. 1941. The effects of biotin upon fat synthesis and metabolism. J. Biol. Chem. 141:619-625.

Ginger, L.G., and R.J. Anderson. 1944. The chemistry of the lipids of tubercle bacilli. LXXII. Fatty acids occurring in the wax prepared from tuberculin residues. Concerning mycocerosic acid. J. Biol. Chem. 157:203-211.

_____, and _____. 1944. The chemistry of the lipids of tubercle bacilli. LXXIII. Studies on phthiocerol. J. Biol. Chem. 157:213-219.

Groner, M.G. 1936. Respiration of green and chlorophyll-deficient types in maize. Am. J. Bot. 23:381-385.

Hilditch, T.P. 1947. The Chemical Constitution of Natural Fats. Wiley, New York.

_____. 1948. Recent advances in the study of component acids and component glycerides of natural fats. Fortschr. chem. organ. Naturstoffe 5:72-100.

Howes, F.N. 1936. Sources of vegetable wax. Royal Bot. Gardens, Misc. No. 10, pp. 503-526.

Jamieson, G.S. 1943. Vegetable Fats and Oils. Reinhold, New York.

Kramer, H.H., and R.L. Whistler. 1949. Quantitative effects of certain genes on the amylose content of corn endosperm starch. J. Am. Soc. Agron. 41:409-411.

Kreger, D.R. 1948. An x-ray study of waxy coatings from plants. Recueil des Travaux bot. neèlandais 41:603-736.

Kurtz, E.B. 1949. A study of the waxes of desert plants. unpublished manuscript.

_____. 1950. The relation of the characteristics and yield of wax to plant age. Pl. Physiol. 25:269-278.

Lardy, H.A., R.L. Potter, and C.A. Elvehjem. 1947. The role of biotin in bicarbonate utilization by bacteria. J. Biol. Chem. 169:452-453.

Lee, B., and J.H. Priestley. 1924. The plant cuticle. I. Its structure, distribution, and function. Ann. Bot. 38: 525-545.

Lipmann, F., N.O. Kaplan, G.D. Novelli, L.C. Tuttle, and B.M. Guirard. 1947. Coenzyme for acetylation, a pantothenic acid derivative. J. Biol. Chem. 167:869-870.

Liverman, J., and E.B. Kurtz. 1951. unpublished data.

Mangelsdorf, P.C. 1947. The inheritance of amylaceous sugary endosperm and its derivatives in maize. Genetics 32: 448-458.

McNair, J.B. 1929. The taxonomic and climatic distribution of oils, fats, and waxes in plants. Am. J. Bot. 16:832-841.

_____. 1930. The taxonomic and climatic distribution of oil and starch in seeds in relation to the physical and chemical properties of both substances. Am. J. Bot. 17: 662-668.

_____. 1931. Some properties of plant waxes in relation to climate of habitat. Am. J. Bot. 18:518-525.

_____. 1945. Plant fats in relation to environment and evolution. Bot. Rev. 11:1-59.

Meyer, M. 1938. Die submikroskopische Structur der Kutinisierten Zellmembranen. Protoplasma 29:552-586.

Miller, E.C. 1910. A physiological study of the germination of Helianthus annuus. Ann. Bot. 24:693-726.

Miller, P.A. 1951. Use of chromosomal interchange for investigating the inheritance of oil in the corn kernel. J. Am. Soc. Agron. 43:229-234.

Milner, H.W. 1951. Possibilities in photosynthetic methods for production of oils and proteins. J. Am. Oil Chem. Soc. 28:363-367.

Newcomer, E.H. 1951. Mitochondria in plants. II. Bot. Rev. 17:53-89.

Nord, F.F., J.V. Fiore, G. Kreitman, and S. Weiss. 1949. On the mechanism of enzyme action. XL. The interaction of solanine, riboflavin, and nicotinic acid in the carbohydrate-fat conversion by certain Fusaria. Arch. Biochem. 23:480-494.

Nyman, M.A., and E. Chargaff. 1949. On the lipoprotein particles of yeast cells. J. Biol. Chem. 180:741-746.

Pack, D.A. 1925. Dispersion of lipoids. Bot. Gaz. 79: 334-338.

Painter, E.G., and L.L. Nesbitt. 1943. Thiocyanogen absorption of linseed oils. Ind. Eng. Chem., Anal. Ed. 15:123-128.

Pearson, L.K., and H.S. Raper. 1927. The influence of temperature on the nature of the fat formed by living organisms. Biochem. J. 21:875-879.

Pohl, F. 1928. Uber die physikalische Beschaffenheit des Wachses bei seinem Erscheinen auf die Epidermis. Planta 6:526-534.

Pollard, A., A.C. Chibnall, and S.H. Piper. 1931. The wax constituents of forage grass. I. Cocksfoot and perennial ryegrass. Biochem. J. 25:2111-2122.

_____, _____, and _____. 1933. The isolation of n-octacosanol from wheat wax. Biochem. J. 27:1889-1893.

Potter, R.L., and C.A. Elvehjem. 1948. Biotin and the metabolism of Lactobacillus arabinosus. J. Biol. Chem. 172: 531-537.

Rabinowitch, E.I. 1945. Photosynthesis I. Interscience, New York.

Rittenberg, D., and K. Bloch. 1944. The utilization of acetic acid for fatty acid synthesis. J. Biol. Chem. 154: 311-312.

Rittenberg, D., and K. Bloch. 1945. The utilization of acetic acid for the synthesis of fatty acids. J. Biol. Chem. 160: 417-424.

Rogers, J.S. 1950. The inheritance of photoperiodic response and tillering in maize-teosinte hybrids. Genetics 35: 513-540.

Runyon, E.H. 1934. The organization of the creosote bush with respect to drought. Ecology 15:128-138.

Sherman, H. 1950. Pyridoxine and fat metabolism. Vitamines and Hormones 8:55-68.

Shields, L.M. 1950. Leaf xeromorphy as related to physiological and structural influences. Bot. Rev. 16:399-447.

Shive, W., and L.L. Rogers. 1947. Involvement of biotin in the biosynthesis of oxalacetic and alpha-ketoglutaric acids. J. Biol. Chem. 169:453-454.

Skoss, J. 1951. personal communication.

Smedley-Maclean, Ida, and D. Hoffert. 1924. The carbohydrate and fat metabolism of yeast. II. The influence of phosphates on the storage of fat and carbohydrate in the cell. Biochem. J. 18:1273-1278.

Smith, J.H.C., and V.M. Koski. 1951. Genetic aspects of chlorophyll formation. Abstract, AAAS meeting, Western Section.

Sprague, G.F. 1946. The experimental basis for hybrid maize. Biol. Rev. 21:101-120.

_____, B. Brimhall, and R.M. Hixon. 1943. Some effects of the waxy gene in corn on properties of the endosperm starch. J. Am. Soc. Agron. 35:817-822.

Stadtman, E.R., and H.A. Barker. 1949. Fatty acid synthesis by enzyme preparations of Clostridium kluyveri. I. Preparation of cell-free extracts that catalyze the conversion of ethanol and acetate to butyrate and caproate. J. Biol. Chem. 180:1085-1093.

_____, and _____. 1949. II. The aerobic oxidation of ethanol and butyrate with the formation of acetyl phosphate. J. Biol. Chem. 180:1095-1115.

Stadtman, E.R., and H.A. Barker. 1949. III. The activation of molecular hydrogen and the conversion of acetyl phosphate and acetate to butyrate. J. Biol. Chem. 180: 1117-1124.

_____, and _____, 1949. IV. The phosphoroclastic decomposition of acetoacetate to acetyl phosphate and acetate. J. Biol. Chem. 180:1169-1186.

_____, and _____. 1950. VI. Reactions of acyl phosphates. J. Biol. Chem. 184:769-793.

_____, T.C. Stadtman, and H.A. Barker. 1949. Tracer experiments on the mechanism of synthesis of valeric and caproic acids by Clostridium kluyveri. J. Biol. Chem. 178:677-682.

Stotz, E. 1949. Biological synthesis of fatty acids. J. Am. Oil Chem. Soc. 26:341-345.

Teas, H.J., and E.G. Anderson. 1950. Blue fluorescent, a new mutant in maize. Genetics 35:696.

Velick, S.F. 1944. The chemistry of the lipids of tubercle bacilli. LXVI. Concerning the structure of tuberculostearic acid. J. Biol. Chem. 154:497-502.

Warth, A.H. 1947. The Chemistry and Technology of Waxes. Reinhold, New York.

Wellensiek, S.J. 1928. Preliminary note on the genetics of wax in Pisum. Am. Natl. 62:94-96.

Went, F.W. 1950. The Earhart Plant Research Laboratory. Chronica Botanica 12:93-108.

White, A.G. C., and C.H. Werkman. 1947. Assimilation of acetate by yeast. Arch. Biochem. 13:27-32.

Williams, V.R., and E.A. Fieger. 1946. Oleic acid as a growth stimulant for Lactobacillus casei. J. Biol. Chem. 166: 335-343.

Williams, W.L., H.P. Broquist, and E.E. Snell. 1947. Oleic acid and related compounds as growth factors for lactic acid bacteria. J. Biol. Chem. 170:619-630.

Winter, F.L. 1929. The mean and variability as affected by continuous selection for composition in corn. J. Agric. Res. 39:451-476.

Wood, H.G., R.W. Brown, and C.H. Werkman. 1945. Mechanism of the butyl alcohol fermentation with heavy carbon acetic and butyric acids and acetone. Arch. Biochem. 6:243-260.

Woodworth, C.M. 1948. High-low chemical strains of corn still being continued. Ill. Agric. Expt. St. Nine-Year Report, 1937-47:27-28.

Yasuda, M. 1931. The determination of the iodine number of lipids. J. Biol. Chem. 94:401-409.

Zarudnaya, K.L. 1951. The genetic control of anthocyanin synthesis. Abstract, AAAS meeting, Western Section.