A T H E S I S

THE COLORING MATTER IN FAT FROM COW'S MILK

by

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INTRODUCTION.

The great variety of coloring matters that exist throughout the entire plant and animal kingdoms have long been of interest to scientists. From the standpoint of both the chemist and the physiologist a great deal of investigation has been carried on to determine the chemical constitution, the cause, and the function of these various pigments. To greatly increase the interest in this work it has been discovered within the last few years that a definite relationship exists between a number of the animal and vegetable pigments.

To the practical dairyman and especially to the creamery man the color of the butter is of vital importance. The public demands that marketable butter shall be of a certain depth of yellow. It is known of course that during the spring and summer seasons of the year milk fat is much richer in color than during the other seasons. The creamery is thus forced,at least throughout part of the year, to use artificial coloring matters in its butter to meet the demands of the public. A great many farmers who sell small amounts do not artificially color their butter and in the minds of a great many people the distinction between creamery and so called "country" butter lies

in the fact that the former is yellow and the latter sometimes almost as white as lard, when in reality both butters, with the exception of the color, would be scored equally high by both the chemist and the butter judge.

As previously stated, the natural color of the butter depends on the season of the year. This is due of course to the fact that the cows are then on pasture. Green feeds of all kinds, especially fresh grass or green alfalfa hay, greatly increase the color of butter. Other feeds are said to have the same effect, such as carrots, while others decrease the color to a marked degree.

The breed of the cow has something to do with it also. For instance Jersey cows are known to give higher colored butter fat than Holsteins, altho both may be on the same ration.

There is also some difference among individual cows. Some Jerseys always give higher colored butter fat than others of the same breed.

The color of butter fat then offers many interesting and important problems for investigation to the chemist, the physiologist, and also to the practical dairyman. Some of these problems may be stated as; (1) the chemical constitution of the butter pigment; (2) the physiological and biological

importance of the pigment; (3) the physiology of the production of the pigment; (4) the chemical relationship between the pigment and the various feeds that affect its production; (5) the chemical relationship between the pigment and the other animal and vegetable pigments; (6) the cause of the difference of intensity of color as exhibited by different breeds of cows and also by different cows of the same breed; (7) the question whether the intensity of the color* of the butter fat is due to different amounts of pigment or to different degrees of intensity of the pigment itself; (8) the question whether it may be possible to increase the natural color of the fat by means of feeds so that the cow may produce highly colored butter fat thruout the entire year and artificial coloring matters thus be dispensed with.

The present investigation naturally could not include all of these problems. The scope of this work was confined to a thoro search of the literature in regard to the subject, to the collection of some data which would throw light on the chemical constitution of the pigment and if possible to the

*Note:- A word of explanation here may make my meaning clearer. There is at the most such a small quantity of the pigment present in the fat (the total crude pigment seldom exceeding .5%) that it seems difficult to explain the very high color that is some times seen merely on the ground of the amount of pigment.

assigning of at least an impirical formula to it.

Research is now in progress by means of which it is hoped that data may be secured that will, at least in some measure, answer all of the problems that have been stated.

PREVIOUS INVESTIGATIONS.

An examination of the literature discloses a great deal of investigation in regard to plant and animal pigments. Aside from the earlier work on plant pigments by Grew in 1682, and by Rouelle, Meyer, Fourcroy, Berthellot, Senebier, Proust, and Vanquelin during the eighteenth century and in the early years of the nineteenth, which pigments were named chlorophyll by Caventon in 1817, Thudicum[51] seems to have been the first one to call attention to the general distribution of these bodies, especially those of yellow color. He says, "Various parts of animals and plants contain a yellow crystalizable substance which has hitherto not been defined, and which I call lutein." "It occurs in the corpora lutea of the ovaries of mammals, in the serum of the blood, in the cells of the adipose tissue, in butter, in the yolks of eggs of oviparous animals, in seeds such as maize, in husks and pulps of fruits such as anatto, in roots such as carrots, in leaves such as those of the colens, and in the stamina and petals of a great many flowers."

Thudicum describes the properties of these luteins as being soluble in alcohol, ether, chloroform,

albuminous liquids and blood serum, but insoluble in water. The luteins show a characteristic spectrum, the red, green and yellow parts being markedly brilliant. The spectrum is further characterized by three absorption bands in the blue, indigo and violet, the positions of the bands varying a little with different solvents.

Thudicum claims to have obtained the luteins in crystaline forms. He describes them as, "Rhombic plates of which two or more are always superimposed in a curious manner," the peculiar appearance being due to the fact that, " they may be rhombohedra imperfectly developed on four of their edges." He says further that the crystals are, "microscopic, yellow when thin, orange or red when thick, and have no resemblance to any other known animal or vegetable substance."

Some very interesting reactions are described. The luteins are precipitated as a yellow deposit with mercury acetate and mercury nitrate, the latter precipitate turning white on standing. Concentrated nitric acid when poured over the crystals gives a blue color which immediately passes into yellow. This color reaction is transient when the luteins are dissolved in acetic acid, and does not appear

at all when they are dissolved in alcohol, chloroform or ether.

The luteins show a great affinity for fats, according to Thudicum. He says in this connection, "They exist in granules in egg yolk and in the corpora lutea and when extracted from these are always mixed with a considerable amount of an oily fat which contains cerebrine, and nautral fats, amongst which is one containing phosphorous, like cerebrine." "They are also found dissolved in butter fat."

Thudicum's observation in regard to the solubility of luteins in fat is correct. The fact that they are found with fat after extraction is, however, due to the fact that the solvents used are also fat solvents. The phosphorous containing neutral fat is undoubtedly lecithin or one of its decomposition products as Kirsten later showed, which work will be referred to again.

In 1879 Blythe published an article(3 in which is described the preparation of a body which he called Lactochrom. This lactochrom is said by Oliver (Milk, Cheese and Butter) to cause the color of butter and whey. Oliver says that it is associated with a peptone called albumenous, and, "Is probably

derived from the hematin of the blood." Blythe obtained the body from the yellow whey of milk from which both the casein and albumen had been separated. It is obtained first from the yellow whey by precipitating with mercury nitrate. Blythe says, "This precipitate by nitrate of mercury has long been known, and was considered to denote the existence of a third albumenoid, called lacto-protein." Blythe says further, "Lacto-protein as a single simple substance has no existence, the precipitate by nitrate of mercury being a mixture of galactin, lactochrome, any trace of albumen which may still remain in solution, and similarly any trace of urea which the milk may have contained, all united with mercury."

Blythe effects the separation of the two principal constitutents, galactin and lactochrome, by decomposing the mercury salt with hydrogen sulphide, filtering off the mercury sulphide and precipitating the galactin by lead acetate, the lactochrome failing to form an insolu ble lead salt. To obtain the lactochrome, the lead salt of galactin is filtered off, the excess of lead acetate in the filtrate decomposed by hydrogen sulphide, the resulting lead sulphide filtered off and the lactochrome again precipitated as its mercury salt by the addition of

mercury nitrate. Blythe calls the lactochrome an alkaloidal coloring matter and assigns to it the formula $HgO\,C_6H_{18}NO_6$. Blythe says further, "Lactochrome itself may be obtained by the careful decomposition of the mercury compound." "As obtained by evaporating its solution in water or alcohol, it is in the form of bright red-orange resin-like masses, softening at 100° C., very soluble in hot alcohol, but partially separating as the liquid cools; it is freely soluble in water." "Concentrated solutions give a simple spectrum, allowing most of the red and yellow rays to pass thru." "No bands were discovered."

I have never found any reference to this very interesting work of Blythe's in any of the literature on plant or animal coloring matters so that its direct bearing upon the subject is not evident. I repeated Blythe's work, with the exception of the determination of the composition of the body, and confirmed it in every particular. Some of the results that were obtained will be discussed later.

About this time a number of independent investigations began in regard to animal and plant pigments. On the one hand Sorby was studying vegetable coloring

matters, which work extended from 1867 to 1873, the reference to which may be found in his classic paper, "Comparative Vegetable Chromotology"[47]. This was followed immediately by the work of Schwalbe[44], Capranica[49] and Kühne.[23]

Schwalbe and Capranica worked on the means of identification of this class of pigments by the use of color reactions with concentrated acids and were the first to make use of the iodine reaction.

Kühne isolated three pigments from the retina of the eyes of certain birds, and from egg lutein and other bodies. He called them collectively chromophans and respectively-rhodophan, chlorophan and xanthophan, and he showed how to distinguish them from each other and how to recognize them when met with elsewhere. For this purpose he made use of the color reactions of Schwalbe and Capranica and by directing renewed attention to them showed how important they are. These reactions may be mentioned here for they will be referred to a number of times during the course of this paper. They may be stated as follows: When in the solid state the pigments are colored blue-green to blue with concentrated nitric, sulphuric and hydrocholric acids, and generally blue-green with a solution of iodine in

potassium iodide.

Basing his work on the researches of Kühne, Krukenberg commenced a series of researches which extended from 1880 to 1886, the most important of which appeared in his, "Vergleichende Physiologishe Studien"[19], and especially in the paper, " Grundzüge einer vergleichenden Physiologie der Farbstoff und der Farben"[20] which appeared in 1884. Krukenberg made an exhaustive study of what had been done on animal pigmentation and included under one head all those pigments which had previously been known as luteins, carrotin, zoonerythrin (tetronerythrin) and Kühne's chromophans and called them lipochromes and assigned to them the following properties. They are bodies which are solu ble in alcohol, ether, chloroform, benzol, carbon bisulphide, petroleum ether, etc. They are colored blue-green to blue by concentrated nitric and sulphuric acids and generally blue-green with iodine in potassium iodide, when in the solid state. They show two and some times three absorption bands in the blue and violet end of the spectrum. In the solid state they are usually red or yellow, and their solutions are yellow. Later work by other investigators assigned other properties to the lipochromes.

To show more fully the properties and relations of the lipochromes as worked out by Krukenberg I quote from his paper, "Grundzüge einer vergleichenden Physiologie der Farbstoff und der Farben." Krukenberg says, "The lipochromes are characteristically similar in their solubilities, in their indestructibleness by saponification with dilute boiling alcoholic caustic alkali, in their blue coloration which they show thru contact in the warm with concentrated sulphuric or nitric acids, in their sensibility towards the light, in the similarity of their bleach products which are cholesterin- or cholesterin-like bodies, in their chemical composition (i.e. composed only of carbon, hydrogen and oxygen) and finally in their color (greenish yellow, yellow, orange and red)."

Credit is assigned for this work in some of the notes accompanying the paper. Wittich in 1863 first noticed the blue coloration with sulphuric acid and Buchholtz noticed it with a fat pigment from a Gangleen cell of an invertebrate animal, and Marquart's flower yellow (Anthrozanthine) is colored dark indigo blue blue to purple red with sulphuric acid, the color disappearing with water. Piccolo[40] found a similar change in lutein and Filhol[6], Städeler[48]

and Thudicum[50] reported that concentrated hydrochloric acid gives the same color reaction. Schwalbe first showed the iodine reaction, i.e. green changing to blue green and then to blue. The absence of nitrogen in these bodies is shown in the investigations of the pigment. Carotin. The absence of nitrogen in animal lipochromes was shown by Göbel in 1823 in his investigations of bird skins and Crustaceans and the investigations of Maly and Kühne confirmed this. Krukenberg says of Kühne's work, "Kühne's pioneer work on chromophans showed how to separate the lipochromes from other pigments and fat, and from other members of pigments of the same class and how to characterize them thru their spectroscopic absorption and their different sensibilities towards light." In regard to crystaline forms among lipochromes Krukenberg says, "Wittich seems to have obtained a rhodophan-like pigment in 1863 from Euglenia sanguiruba and a chlorophan-like one was obtained in 1876 by Pouchet[41] from lobsters, both in crystaline form." "Crystaline luteins were first represented by Piccolo and Leben[40], later by Thudicum, and Kühne succeeded in crystalizing Ellochrin and Lecithinochrin, and Hansen[12

obtained chlorophyll yellow in crystaline form."

To quote further from Krukenberg's paper in regard to lipochromes, he says, "A sharp distinction between the lipochromes is not possible, because they are sometimes difficultly distinguished in the spectroscope and because the lipochromoids which are difficultly or almost insulu. ble in the lipochrome solvents readily go over into Melanoids." "Furthermore, at other times, as Kühne has shown, a true lipochrome, namely rhodophan, after purification will no longer give the blue coloration with concentrated hydrochloric acid." "The lipochromes have this characteristic, namely that while in one case they are colored blue-green by moistening with iodine in potassium iodide, in another case they give this reaction very indefinitely." "The spectroscopic absorption of certain lipochromes in many cases differ considerably; for example, the solutions of xanthaphan and rhodophan are indicated by one absorption band and those of chlorophan by two and three." "The absorption bands of the same lipochromes are even different in different solutions." "In alcohol or ether they lie mostly in the violet end of the spectrum and in carbon bisulphide in the red end, while in chloroform and fatty oils they

lie between these extremes." "Experiment has shown that this difference is not due to the specific gravity of the solvent but to the broken force of the solvent, and it may be stated in general that the greater the dispersion of the solvent for the blue part of the spectrum, that much further do the absorption bands advance towards the more refrangible side of the spectrum."

"The color intensity of the lipochromes is an extraordinary strong one, and the transposition into cholesterin-like matter which the lipochromes under go thru oxidation in the light (this action is much slower in the dark) is correspondingly rapid, so that it is very difficult to obtain large quantities in the pure state."

Krukenberg says further, "In regard to the establishment of the chemical composition of lipochromes, it may be stated that up to this time every one has depended entirely upon Carotin, which is recognized as the best known representative of this class of coloring matters." "Carotin ($C_{18}H_{24}O$) is the coloring matter of the cultivated carrot ((Daucus Carota L)." "Its brown-red striped crystals of rhombic plate form melt at 168° C. and show direct resemblance to Hydrocarotin ($C_{18}H_{30}O$) which is close to it in the carrots and stands close to cholesterin."

Krukenberg says in regard to the origin of lipochromes that, " It is probable that in most cases they originate from fatty substances, for frequently, if not without exception, they occur in company with fat and allow themselves to easily go over into cholesterin-like bodies." "In the same way their occurrence in green plants is explained, which, as is well known, is so general that until the meretorious work of Hansen[11] it could not be considered as an individual substance notwithstanding the innumberable discussions in regard to the association of the green and yellow chlorophyll coloring matter." "But doubtless the lipochromes occur in other ways, i.e. from lipochromogens or pigments which have no direct relation to the lipochromes; for example from cyano crystals, the blue crystaline coloring matter that occurs in the coats of many Crustaceans, and which are changed into lipochromes by the slightest interference."

Krukenberg says that the wide distribution of lipochromes is of great interest and that, "Collectively the yellow flower petals, yellow and red lymphatic fluids, and numerous secretions of vertebrate and invertebrate animals, the colored fat globules in the uvulva of vertebrate animal retinas, the corpora lutea, the egg yolk of many different animal species,

the yellow,green, orange and red membranes of Arthropodes and vertebrates (from fish to birds) which have been investigated, owe their color without a single exception to either slightly or diffusly deposited lipochromes; on the other hand the lipochromes have no share in the coloration of shells of birds' eggs and they appear also to be absent in many protoplasmic and unicellular creatures." Krukenberg says that the intensity of the lipochromatic colors, especially among fatty bodies, exhibit very striking constant differences in different species, and says further that, "It is very surprising that the lipochromes occur only in traces in snakes, while the ver^ied orange colors of birds, amphibians, fish and of many reptiles are saturated with lipochromatic solutions."

Krukenberg says that a very fruitful investigation could be carried out which would accurately classify the many yellow, orange, red and brown pigments of plants such as are now designated as Bixin, Polychroit, Safflower, Carthamine, Luteolin and Draconium, as to whether or not they are lipochromes, and he says further that, "It would be especially useful to learn whether sharp chemical differences occur between the spectroscopically so distinguished chlorophan, xanthophan and rhodophan, and whether they perhaps are

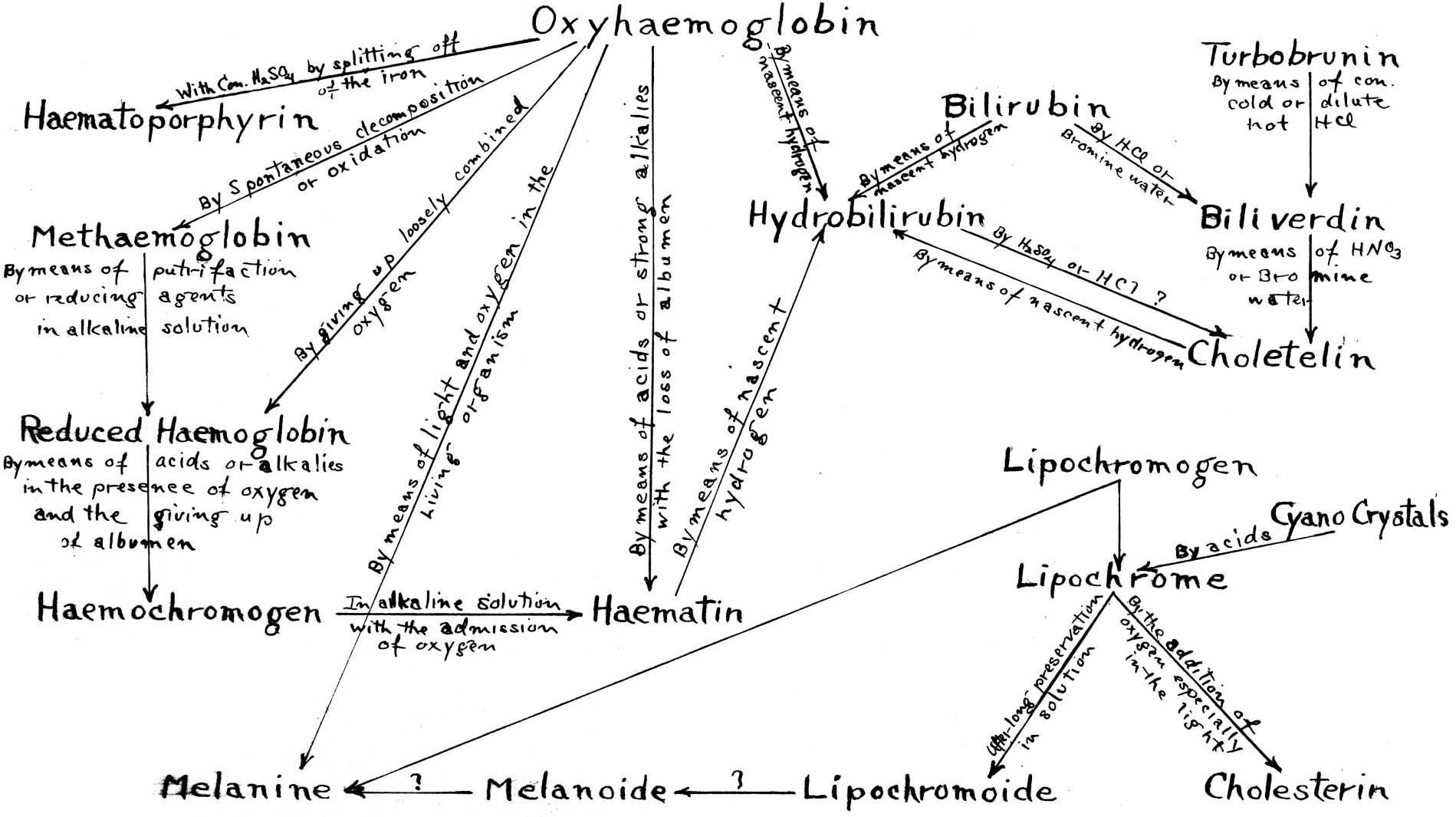
not derived from one another as homologues or as anhydrous compounds."

Krukenberg devotes a large part of the remainder of his paper in attempting to show a relation between the haemoglobins, i.e., the blood pigments, the gall pigments, the melanoids and the lipochromes, and in this connection presents the very interesting chart shown in table I.

I will not attempt to discuss the results as later investigators have not given much weight to them.

That possibly there is some relation between haemoglobin and lipochrome and thus between lipochrome and chlorophyll is indicated by Marchlewski's work in 1903.[32] Marchlewski used Pechmann's very interesting work as the basis of his own. Pechmann's[39] work is the first and probably the only work which throws any light on the possible structure of the lipochromes and yet it seems to have been almost unnoticed. He condensed maleic acid anhydride with methane by the presence of aluminium chloride, forming ketone acids, which under the influence of a water free agent, go over into a coloring matter which shows great similarity to the lipochromes. It shows the blue color with concentrated sulphuric acid, and also shows similar spectrum bands. By way of parenthesis

Table No. 1


Oxyhaemoglobin
With Con. H_2SO_4 by splitting off of the iron
Haematoporphyrin
By Spontaneous decomposition or oxidation
Methaemoglobin
By means of reducing agents in alkaline solution
putrifaction
By giving up loosely combined oxygen
Reduced Haemoglobin
By means of acids or alkalies in the presence of oxygen and the giving up of albumen
By means of light and oxygen in the Living organism
By means of acids or strong alkalies with the loss of albumen
By means of nascent hydrogen
Haemochromogen
In alkaline solution with the admission of oxygen
Haematin
By means of nascent hydrogen
Hydrobilirubin
Bilirubin
By means of nascent hydrogen
By HCl or Bromine water
Turbobrunin
By means of cold or hot con. dilute HCl
Biliverdin
By means of HNO_3 or Bromine water
By H_2SO_4 or HCl ?
By means of nascent hydrogen
Choletelin
Lipochromogen
Cyano Crystals
By acids
Lipochrome
By long preservation in solution
By the addition of oxygen especially in the light
Melanine
?
Melanoide
?
Lipochromoide
Cholesterin

it may be said that at present there seems to be no doubt that chlorophyll and haemoglobin are intimately related. Schunck[42], Marchlewski[34], Willstätter[54], Nenki[37] and Zaleski and many others have firmly established this fact. It must be noted, however, that chlorophyll, as referred to here, is the green constitutent of that body. Returning to Marchlewski's paper, he says, "Both haemoglobin and chlorophyll break down to haemopyrrol which is 3methyl 4 n propyl-pyrrol, a reaction which, according to Nenki[37] occurs also in living organisms." "Further Küster[22] showed that haemopyrrol can be oxidized to methyl n propyl maleic acid anhydride." Marchlewski thinks that, "The latter bodies which are not different principally from those studied by Pechmann can enter into condensations within the animal and plant organisms, and that the products obtained go over further into coloring matters which are identical with the lipochromes." Marchlewski thinks that these reactions are a proof of a relation between chlorophyll, haemoglobin and lipochrome. He condensed methyl-ethyl maleic anhydride with methane, the anhydride, he says, "Being one in close relationship to haematinic acid," and reports that he was at that time occupied, "With the study of the action of

the water free agent on the resulting ketonic acids, as also with the clearing up of the constitution of the simple coloring matter of Pechmann," and adds that he intends to take up methyl n propyl maleic anhydride as well as other homologues of maleic anhydride during his investigations. As far as can be learned Marchlewski has never reported the results of his further work.

It may perhaps be well to state here that the relation that has been established between haemoglobin and green plant chlorophyll is not that these bodies have similar functions. It has been shown however that the disintegration products of haematin and chlorophyll are strikingly similar. The more likely conclusion then is that the relationship between the bodies is explained by the fact that haematin is formed from both chlorophyll and haemoglobin, altho unfortunately no one has been able to offer positive proof of this.

Continuing the review of work on lipochromes, we find that Halliburton(9 reports that he extracted a yellow lipochrome from the blood serum of the pigeon, hen, dove, and tortoise by means of alcohol. The body was solu ble in alcohol, ether, petroleum ether, chloroform, benzine, carbon bisulphide and light

petroleum, but was insolu ble in water and turpentine. It was readily bleached by sunlight, but Halliburton claims that this was neither an oxidation or a reduction. It gave a spectrum band just over the F line extending from λ 475 to λ 500. Krukenberg had obtained a yellow lipochrome from the blood serum of the ox[21] but it differed spectroscopically and in its solubilities from the one of Halliburton. The latter pigment gave a green color with fuming nitric acid but the reaction was momentary. Concentrated sulphuric acid and iodine gave no reaction but together produced a violet color. Halliburton reports an identical pigment in the body fat of these same animals.

The next investigator of note along the line of animal pigmentation was McMunn. Some of his most interesting work was in regard to a pigment named by him Enterochlorophyll[26]. This pigment was found in the "bile" of invertebrates and it derived its name from its great similarity to plant chlorophyll. Hansen[11-12], by saponfying plant chlorophyll obtained crystals of "chlorophyll green" and "chlorophyll yellow" and claimed that chlorophyll is not decomposed by the treatment. McMunn repeated the work, using fresh green grass, and he gives evidence to show that

the chlorophyll is decomposed by saponification, finding that the spectrum bands of the solution after saponification are in an entirely different position than they were before saponfication. McMunn separated the constitutents of plant chlorophyll by Hansen's method and obtained the "chlorophyll green" and "chlorophyll yellow", the latter in some cases in yellow needles, altho McMunn states that he does not think it proved whether or not these needles may belong to a fatty acid whose crystals are stained by the pigment. The yellow crystals gave the color reactions of the lipochromes and were soluble in the lipochrome solvents. McMunn found that entero-chlorophyll was altered by saponification. With some invertebrate livers, he separated and obtained crystals of a green and yellow constitutent, the latter being radiating needles. The solid yellow pigment gave the lipochrome color reactions with the exception of the iodine reaction, which was doubtful, and were soluble in the lipochrome solvents.

McMunn concludes from these results that the yellow constituent of both plant chlorophyll and entero-chlorophyll are lipochromes. Hansen drew the same conclusion in regard to plant chlorophyll. That they are, however, not the same lipochrome is shown by the fact that their spectrum absorption was different and that

only the plant lipochrome gave the iodine reaction. Of the six absorption bands that plant chlorophyll shows, McMunn believed Nos. 1 to 4 were due to the "green constitutent", and Nos. 5 and 6 to the "yellow constitutent." He found that the first four bands of enterochlorophyll corresponded to those of plant chlorophyll but Nos. 5 and 6 of the latter were replaced by one and some times two, occupying somewhat different positions, in the enterochlorophyll.

In a paper published that same year McMunn[27] says, "Just as there are several haemoglobins (Hoppe Seyler, Physiol. Chem. p 397) so there are many haematins, haematoporphyrins, chlorophylls, lipochromes, including xanthaphylls and so on." "There are in fact, classes of coloring matters which present bands similar but not always identical in position, but each class has well defined characteristics, such as solubility in certain media, behavior towards alkalies and acids, bands in the blue or red end of the spectrum, and reactions of the solid residue." "To any one practiced in this kind of work, the recognition of the identity or of the relationship of these pigments, is not a matter of much difficulty." "There is one fact, however, that has forced itself on my attention, namely that one may meet with coloring matters identical in all respects with decomposition

products of haemoglobin, but are not derived from it." "And this is not to be wondered at when one considers that physiological complexity may progressively increase as we ascend the scale, just as morphological complexity increases." "It is possible that the study of such gradually increasing complexity may in time enable one to understand the synthesis of very complex molecules." "At the same time it must not be lost sight of that pigments may be degraded and in the process of disappearance as Moseley[36] points out, the animal to which they belong, having, owing to changed modes of existence, no longer any use for them, and, it may be added, for the coloring matters of which they are the metabolites."

In a paper published two years later McMunn[29] was lead to believe from observing that in sponges lipochromes so often accompany chlorophyll and some times replace it, that the step between a chlorophyll and a lipochrome is not a great one. "It is highly probable, "he says,"that these pigments (lipochromes) are concerned in the formation of the fatty matters from waste carbonic anhydride given off during the katabolic changes in the tissues, and from the water in which they are bathed." This corresponds with the view that chlorophyll is a respiratory pigment altho

probably a carbonic acid carrier rather than an oxygen carrier.

McMunn's greatest contribution to animal chromotology was in 1899[30]. The pigments of a great many marine animals, Crustaceans, worms and sponges were examined and classified. Lipochromes and enterochlorophyll were found abundantly, McMunn drawing a distinction as to whether the lipochrome was a rhodophan or a chlorophan-like lipochrome. This distinction is mainly one of color, the former being a red lipochrome and the latter a yellow lipochrome.

In the concluding remarks McMunn calls attention to the fact that a great many pigments formerly designated tetronerythrins, are in reality lipochromes and lipochromogens and therefore disputes the results of Merejkowsky who claimed that all tetronerythins were respiratory pigments. McMunn says, "There has never been any valid reason given for considering any of the lipochromes included under the name tetronerythin, as respiratory pigments." "And for this reason, that they fail to respond to the test used in determining whether any pigment is respiratory or not, i.e. change of color and spectrum under the influence of reducing agents."

In regard to some of the properties of the lipochromes McMunn states, as did Krukenberg, that

they are sensitive to light, both in the solid state and in solution, and yield in many cases cholesterin-like substances, "An observation", he says, "which gains additional significance when we compare an animal lipochrome with one of the most typical of that series among plants, namely carotin, which is accompanied by a cholesterin." McMunn gives as the general properties those that have already been stated in connection with Krukenberg's paper, altho he says that he has often found that animal lipochromes fail to respond to the iodine tests. McMunn also takes exception again, as in a former paper, to Krukenberg's statement that the lipochromes are not decomposed by saponification, and thinks too that Kühne's failure to obtain the nitric acid color reaction with rhodophan, after purification, was due to the fact that purification in that case meant decomposition.

Krukenberg's remarks in regard to carotin are shown to be erroneous. The formulas $C_{18}H_{24}O$ for carotin and $C_{18}H_{30}O$ for hydrocarotin, which are due to Hussmann[15], were shown by Arnaud[1] to be wrong. Arnaud showed that carotin is identical with the orange-red crystaline substance that can be obtained from green leaves, and which exists in many fruits,

especially the tomato. It has the formual $C_{26}H_{38}$, and is therefore not an oxygen compound but an unsaturated hydrocarbon. Hydrocarotin is merely an impure cholesterin. Arnaud isolated the pure cholesterin from carrots and assigned the formula $C_{26}H_{44}O$ to it. Carotin itself was obtained by Arnaud in rhombic plate crystals, which had a metallic lustre, blue by reflected and orange by transmitted light. McMunn showed, however, that carotin is a genuine lipochrome for he carefully examined a coloring matter which Hartsen called chrysophyll, and Bougarel called erythrophyll and which according to Arnaud are identical with carotin.

McMunn says of this pigment, "It was crystalized in beautiful plates of a fine orange or red color, by transmitted light, and under the microscope the crystals appeared reddish yellow." "Dissolved in ether, they formed an orange solution, which in deep layer transmitted the red and a little green, but the solution had to be diluted to a pale yellow color before the bands could be seen." "The first of these began at λ 496 and extended to λ 471, the second from about λ 462 to λ 444." "It formed a fine red color in carbon bisulphide, which in a suitable depth showed the following bands;

first λ 535 to λ 506; second λ496 to λ475." "There may have been a third feeble band near the violet." "It was also solu ble in alcohol, but not so easily as in the above solvents, and here it had like spectroscopic characters." "The residue from the solutions became a fine blue and green with iodine in potassium iodide, and a fine dark blue and perhaps deep violet with concentrated sulphuric acid, and a blue which rapidly faded passing into a violet tint with nitric acid."

McMunn concludes by stating that altho, "No animal lipochrome has yet been obtained pure enough for an analysis, that can be depended upon, still the spectroscopic and chemical charac ters of animal and plant lipochromes are so similar, that there can not be a great difference between them."

The next investigator of note was C. A. Schunck. He confined his attentions to chlorophyll and its constitutents. He first showed (42 that chrysophyll and carotin gave three distinct absorption bands of the spectrum, as McMunn had suggested might be the case. He next reported that his observations led him to believe that the yellow constitutent of chlorophyll is a mixture of coloring matters, the chief constitutent of which is chrysophyll, the only member

that so far may be obtained in crystaline form.

Schunck later reports an exceedingly interesting investigation of the coloring matters which accompany the chrysophyll, which he groups under the name xanthophylls. He says of this, "The group considered comprises those coloring matters occurring in flowers, leaves, fruit, etc., which accompany the crystaline chrysophyll and which are insolu ble in water, but are solu ble in alcohol, ether, carbon bisulphide and other organic solvents, and which are unattacked i.e. unsaponified by alkalies." "There is another group that sometimes accompanies the xanthophyll group that are solu ble in boiling water, alcohol, but sparingly solu ble in ether and insolu ble in carbon bisulphide and react towards alkalies."

Schunck found three constitutents in the xanthophyll group and he calls them respectively L. B. and Y. xanthophyll. He was not able to isolate, purify or crystalize any of the constitutents but depended entirely upon their color reactions and absorption spectra for their identification. He found that the xanthophylls were all characterized by giving the same color reactions in the dry state with concentrated sulphuric and nitric acids as are given by the lipochromes, and similar absorption bands in the

violet region of the spectrum. The three xanthophylls, however, do not appear to be identical and Schunck reports the very interesting fact that on comparison of the xanthophylls with the pigment of egg yolk and fowl serum, the bright yellow alcoholic solution of the latter show an identical spectrum with that of L. xanthophyll. He says further that the action of acids upon the spectra of the egg yolk and fowl serum pigments is identical with that upon L. xanthophyll, and the color reactions with hydrochloric, sulphuric and nitric acids are the same, altho perhaps not so brilliant, due no doubt to the presence of fats. In his experiments with egg yolk, Schunck obtained a few red crystals on evaporation of an ether solution, but the quantity was so small that he could not decide whether they were the pigments itself or some other substance colored by it.

Since Schunck's investigation seems to indicate that his so-called L. xanthophyll and the lipochromes from egg yolk and fowl serum are identical, it might be well to report their properties more in detail. Schunck's description is as follows; "The absorption bands of these three pigments are three in number and lie between the solar lines F and H; the line S being just between the two heavier bands, the light band

lying near F." "The ultra violet rays are transmitted to a considerable extent." "Hydrochloric acid has no immediate effect on the bands." "They fade after a time, however, with an indication of a fourth more refrangible one, the solution becoming by degrees paler and assuming a slight green tinge before becoming colorless." "Nitric acid rapidly affects the coloring matter, a fourth more refrangible pronounced band being formed, the first and second by degrees disappearing, and the third becoming faint, the solution in a short time assuming a green tinge and finally becoming colorless." "The same reactions take place with sulphuric acid and hydrogen peroxide and nascent hydrogen, but the action is slower." "No color reaction is produced in alcoholic solutions of L. xanthophylls with hydrochloric acid." "In the dry state, however, it turns a Prussian blue color with a drop of nitric acid, which is evanescent, and an indigo blue with concentrated sulphuric acid, which is more lasting."

Schunck concludes,"Whether the lipochromes from other sources will also prove to consist of the same coloring matter, opportunity of investigation has not so far been afforded, but from the spectroscopic properties of the lipochromes in the above cases, they appear to be identical with L. xanthophyll, and as

they thus appear to be present along with both chlorophyll and haemoglobin, an interesting speculation is presented whether this coloring matter too is of physiological importance."

M. Henze[13] reports some very interesting lipochrome properties in his article on, "A cholesterin like body in a sponge and its relation to lipochrome." Henze states that an ether solution of the lipochrome from which the cholesterin had been separated, when evaporated left a strong reddish yellow salve like body which had a pleasant odor which disappeared on warming. The lipochrome could not be crystalized. In a solution of suitable concentration it showed one absorption band over line C. Henze noticed that the lipochrome under went some changes in the sunlight. While no crystalization was at any time observed, there was a slow and complete decoloration. Henze says in regard to this, "The slow decoloration of the pigment in the sunlight manifestly appears to be due to an oxidation change." "We know that fat and a great many other organic compounds take on ozone like properties in the light and approach auto-oxidation." "It is very probable that lipochrome, a fatty body, is analegous." "The suppostion that the decoloration or decomposition of the lipochrome lies close to an oxidation change, will appear probable in

the light of the investigation."

"An ether solution of the lipochrome was sopped up with blotting paper." "After the evaporation of the ether the strips of paper with the lipochrome on them were rolled up and put into little bottles which contained respectively; (1) air; (2) oxygen; (3) carbon dioxide; (4) hydrogen." "The tubes were put out in the direct sunlight." The results were very interesting. "After six months tubes (3) and (4), which contained the carbon dioxide and hydrogen, were almost as colored as at the beginning." "Tube (2) was decolorized in twenty minutes." "After that time tube (1) still retained a little yellowish red color, manifestly because the oxygen of the air was soon used up and the tube consequently did not reach full oxidation." Henze found too that solutions of the lipochrome in organic solvents were unstable in the light but much more slowly decolorized in the dark, altho/if in the latter case the atmosphere was full of ozone the decoloration was very rapid. Henze found that the bleached lipochromes did not give the usual lipochrome color reaction, but instead an intense brown with concentrated sulphuric acid, which is the only reaction reported.

To return briefly to butter fat, its coloring matter is said to be present in what is usually known

as the unsaponified matter. Kirsten[16] reports that it appears there along with cholesterin and a phosophorous containing body, which is probably lecithin or more likely a decomposition product of lecithin. Lewkowitz (Oils, Fats and Waxes) also states that the butter pigment is separated in the unsaponified matter. This would seem to indicate that it is a body which is unsaponified, and if the butter pigment is a lipochrome we have here a disputed point.

In reviewing the rather voluminous amount of literature we are led to conclude without any further experimentation that the coloring matter in butter fat belongs to the class of pigments known as lipochromes.

To summarize briefly, it may be said that the lipochromes are fat like pigments of yellow or reddish yellow color, that occur very widely distributed in both the plant and animal kingdoms. The plant lipochromes, of which carotin is a typical example, are crystaline, while the animal lipochromes , of which almost any example can be taken as typical, are amorphous, salve like bodies. It seems probable, however, that lipochromes do occur in plants which are identical in every respect with animal lipochromes. The properties of the lipochromes need not be given as they have been mentioned a number of times. Briefly, however, their characteristics are their solubility in fat solvents,

their color reactions with acids and iodine, their absorption bands and the readiness with which they oxidize in the sunlight. As stated before their reaction toward alkalies is a disputed point. The most recent work seems to indicate that the lipochromes are unsaturated hydrocarbons corresponding to the empirical formula $C_{26}H_{38}$.

RESULTS OF INVESTIGATIONS.

Our own investigations may be reported briefly as follows.

It was not attempted to confirm all the lipochrome reactions as applied to butter pigment. We found, however, that its solubilities were those of lipochrome, that it gave the acid color reactions and was readily bleached in the sunlight both in the solid state and in ether solution when in contact with the air. The crude pigment had present with it cholesterin and also a phosphorus containing body. No test for nitrogen or sulphur were obtained, the usual qualitative test for these elements being made. The crude color was non-crystaline and had a deep yellow orange color and a peculiar pleasant odor. No attempts were made to purify the crude pigment.

Blythe's experiment, in which he obtained his so called lactochrome, was repeated, as previously stated, in order to see if the lactochrome was in any way related to or corresponded to the pigment extracted from the butter fat itself. No relation between these bodies could be detected. In fact it was very difficult to see how these bodies could be

the same. In one lactochrome extraction the casein and albumen precipitates were saved and the butter fat extracted from them by warming on the water bath. This fat had a high yellow color which quite equalled that of the fat churned from a sample of milk taken from the same cow the following milking after that used for the lactochrome extraction. This indicated that while lactochrome may be the coloring matter in the fat, not by any means all of it is extracted in the way proposed by Blythe. Also the extreme solubility of lactochrome in water and its insulubility in ether practically proves that it is an entirely different body. What this interesting body is, that is found in milk, is a problem worthy of further consideration.

The first attempt made to extract the coloring matter from the butter fat itself gave some very interesting results, which altho subsequent experiments failed to confirm them, are worthy of record. About 5 grams of pure, highly colored butter fat were saponified with alcoholic potash in the usual way. On addition of ether to the cool alcoholic solution of the soap, a white precipitate came down. Ether was added until no more precipitate formed. The precipitate was filtered off and the filtrate,

which contained all the color that the original alcoholic solution had contained, was washed thoroughly with water in a separatory funnel to remove the excess alkali and alcohol. The yellow colored ether was then evaporated. The residue was very small. It was a deep yellow oily liquid with a melting point just above 0° C., and which had a peculiar valer-ate odor. It was solu ble in hot alcohol and ether, insolu ble in water, and readily lost both its odor and its color when exposed to the air. It gave a bright red cherry coloration, which was trans ient, on addition of alkali to its alcoholic solution. Ammonium hydroxide gave the same result, the coloration being more stable but disappearing in 24 hours. We were led to believe that we had here the body which gives the color reaction of milk with sodium or potassium hydroxide or ammonium hydroxide, which is described by Gautier and Morel[17] and by Krüger[18]. The latter found that milk which had been extracted with ether, i.e. fat free, did not give the reaction, indicating that it might be something in the fat which caused the coloration.

As previously stated no subsequent yields of the crude pigment gave these color reactions, so that their significance of cause is not readily seen.

It is commonly observed in the determination of the Reichert Meissl Number, that the yellow color of the aqueous solution of the soaps goes into the fatty acids that are liberated by acidification with sulphuric acid. After the volatile acids have been distilled off the fatty acids still retain this color and an attempt was made to extract it. For this purpose the residues from a large number of Reichert Meissl determinations were saved. All attempts prove fruitless, which seems to be a point in favor of the view that saponification does alter the fat pigment. In this case, at any rate, where the saponification takes place at a high temperature the pigment was altered to such an extent as to render it insolu ble in ether altho the color was still retained.

Up to this time all extractions of the pigment had necessarily been very small and an attempt to obtain a large amount by extracting the alcoholic potash saponification with ether would have involved a long and tedious manipulation, on account of the ether and alcohol being so miscible, and the subsequent precipitation so voluminous. It seemed reasonable to suppose that if saponification could be carried out in a solution whose solvent did not mix with ether, much more satisfactory results could be obtained.

To this end about 75 grams of fat were saponified in the cold in an aqueous solution of potassium hydroxide. Several days, with constant shaking of the flask, were required for the saponification. The solution was then warmed to allow traces of unsaponified fat to rise to the top so that they could be removed. The clear, high colored soap solution was then extracted with ether in a large separatory funnel. The ether took up all the color leaving the soap solution colorless. The ether was washed thoroughly to remove excess alkali, dried over fused calcium chloride, filtered thru a dry filter paper and evaporated. The residue was a deep orange yellow, amorphous, salve like body having the peculiar odor described before. It differed, however, from the first yield in that it was solid at ordinary temperature. This is due no doubt to the fact that it contained more cholesterin since no alcohol was used, from which solvent cholesterin tends to precipitate in the cold. Altho the yield by this method is not so pure, it was much larger, it being possible to extract the pigment from 300 to 500 grams of fat at one time. The method has one objection, namely, that the excess alkali required for cold saponification in water solution makes the ether extracting difficult, as the alkali and water readily forms a temporary emulsion with the ether.

A method of extracting the color is at present in the process of perfection whereby it is hoped that both the above objectionable feature and also the presence of large amounts of cholesterin in the crude extract may be avoided.

A very interesting experiment showing the effect of different feeds on the color of the butter fat has recently been carried on by the Government Co-operative Laboratory at the University of Missouri. The experiment involved the effect of the feeding of cottonseed hulls and cottonseed meal on the composition of the milk and butter. The data secured showed very markedly the relative effects of green grass, green alfalfa hay, cottonseed hulls and cottonseed meal on the color of the butter fat. The color determinations were made with the Lovibond Tintometer, the color being expressed in units of yellow and red as given by the standard glasses, comparing them with a one inch strata of melted butter fat. The results are shown in the following table, numbered 2.

The experiment shows that green grass and green alfalfa hay give equally high colored butter, while cottonseed seed hulls and cottonseed meal greatly decrease the amount of pigment in the fat.

Table No. 2.

Feed	COLOR Yellow	Red
Grass alone - - - - - - - - - - - - - - -	66.5	2.3
Alfalfa Hay and grain - - - - - - - - -	66.5	2.3
Cottonseed hulls and grain and alfalfa	44.5	2.1
Cottonseed hulls and cottonseed meal and alfalfa - - - - - - - - - - - - -	40.5	1.5
Cottonseed hulls and cottonseed meal -	9.8	1.3
Cottonseed hulls and grain - - - - - -	8.8	1.3
Alfalfa hay and grain - - - - - - - - - -	34.8	1.3

SUMMARY

With the questions in mind that were presented for solution at the beginning of the paper, this problem of the coloring matter in fat from cow's milk may be summarized as follows:

1. The pigment belongs to a class of coloring matters known as lipochromes which exist in both the animal and the vegetable kingdoms.

2. If it is the same body as that known as carotin, which exists in many places thruout the vegetable kingdom, it corresponds to the formula $C_{26}H_{38}$ and is an unsaturated hydrocarbon. This, however, seems doubtful if Schunck's work on the xanthrophylls is to be accepted.

3. The questions in regard to its chemical relation to the coloring matters of feeds and to other animal pigments rest on the clearing up of the physiology of its production in the fat and the physiological and biological importance of the pigment, which questions have not yet been answered.

4. That there seems to be some relationship between the constitution of the fat and its color presents an interesting problem, further data in regard to which may thro some light on the cause for the varying intensity of the color and methods that can be employed to increase its production, as well as the causes for its variation in different animals.

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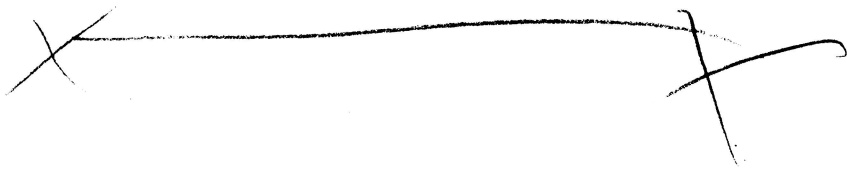

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