RESEARCH BULLETIN NO. 13

UNIVERSITY OF MISSOURI

COLLEGE OF AGRICULTURE

AGRICULTURAL EXPERIMENT STATION

LACTOCHROME—THE YELLOW PIGMENT OF MILK WHEY

Its Probable Identity With Urochrome, the Specific Yellow Pigment of Normal Urine.

COLUMBIA, MISSOURI
April, 1914

UNIVERSITY OF MISSOURI

COLLEGE OF AGRICULTURE

Agricultural Experiment Station

OFFICERS OF THE STATION.

THE PRESIDENT OF THE UNIVERSITY.
F. B. MUMFORD, M. S., Director, Animal Husbandry.

J. W. Connaway, D.V.S., M.D., Veterinary Science.
Frederick Dunlap, F.E., Forestry.
C. H. Eckles, M.S., Dairy Husbandry.
W. L. Howard, Ph.D., Horticulture.
C. B. Hutchison, M.S.A., Agronomy.
M. F. Miller, M.S.A., Agronomy.
G. M. Reed, Ph.D., Botany.
E. A. Trowbridge, B.S.A., Animal Husbandry.
P. F. Trowbridge, Ph.D., Agricultural Chemistry.
J. C. Whitten, Ph.D., Horticulture.
H. O. Allison, M.S.A., Animal Husbandry.
H. L. Kempster, B.S.A., Poultry Husbandry.
A. J. Meyer, Assistant to Director.
L. S. Backus, D.V.M., Veterinary Science.
T. R. Douglass, B.S.A., Agronomy.
J. B. Gingery, D.V.M., Veterinary Science.
Howard Hackedorn, B.S.A., Animal Husbandry.
J. C. Hackleman, M.A., Agronomy.
L. D. Haigh, Ph.D., Agricultural Chemistry.
Leonard Haseman, Ph.D., Entomology.
O. R. Johnson, M.A., Farm Management.
H. F. Major, B.S.A., Landscape Gardening.
C. R. Moulton, Ph.D., Agricultural Chemistry.
L. S. Palmer, Ph.D., Dairy Chemistry.
L. G. Rinkle, M. S. A., Dairy Husbandry.
L. A. Weaver, B.S.A., Animal Husbandry.
P. L. Gainey, M.S., Assistant, Botany.
R. R. Hudelson, B.S.A., Assistant, Agronomy.
C. A. LeClair, M.S., Assistant, Agronomy.
E. C. Pegg, M.S., Assistant, Forestry.
R. S. Besse, B.S.A., Assistant, Farm Management.
C. E. Brashear, B.S.A., Assistant, Animal Husbandry.
C. E. Deardorff, B.S.A., Assistant, Soil Survey.
A. J. Durant, B.S.A., Research Assistant, Veterinary Science.
A. R. Evans, B.S.A., Assistant, Agronomy.
W. E. Foard, B.S.A., Assistant, Farm Management.
J. F. Hamilton, Assistant, Veterinary Science.
Elmer H. Hughes, B.S.A., Assistant, Animal Husbandry.
F. Z. Hutton, (1) B.S.A., Assistant, So Survey.
M. A. R. Kelley, B.S. in M.E., Assistant, Agronomy.
E. W. Knobel, B.S.A., Assistant in Soil Survey.
H. H. Krusekopf, B.S.A., Assistant in Soil Survey.
T. C. Reed, B.S.A., Assistant, Dairy Husbandry.
W. Regan, B.S.A., Assistant, Dairy Husbandry.
Helman Rosenthal, B. A., Assistant, Agricultural Chemistry.
O. C. Smith, A.B., Assistant, Agricultural Chemistry.
A. C. Stanton, B.S.A., Assistant, Dairy Husbandry.
E. R. Spence, B.S.A., Assistant, Veterinary Science.
A. T. Sweet, (1) A.B., Assistant, Soil Survey.
Boleslaus Szymoniak, B.S.A., Assistant, Horticulture.
Thomas J. Talbert, B.S.A., Assistant, Entomology.
B. W. Tillman, (1) B.S.A., Assistant, Soil Survey.
T. T. Tucker, B.S.A., Assistant, Veterinary Science.
E. E. Vanatta, M.S.A., Assistant, Agricultural Chemistry.
E. S. Vanatta, (1) B.S.A., Assistant, Soil Survey.
W. I. Watkins, B.S.A., Assistant, Soil Survey.
C. C. Wiggans, A.M., Assistant, Horticulture.
C. A. Webster, B.S.A., Assistant, Poultry Husbandry.
George Reeder, (1) Dir. Weather Bureau.
Flora G. Ernst, A.M., (1) Seed Testing Laboratory.
J. G. Babb, M.A., Secretary.
R. B. Price, B.S., Treasurer.
R. H. Gray, Accountant.
Harriet Bixby, Librarian.
T. D. Stanford, Clerk.
Vallye Boyce, A.B., Stenographer.
J. F. Barham, Photographer.
Arthur Rhys, Herdsman, Animal Husbandry.
C. M. Pollock, Herdsman, Dairy Husbandry.

(1) In the service of the U. S. Department of Agriculture.

TABLE OF CONTENTS.

CO-OPERATION.

The investigation here reported was conducted by the Missouri Agricultural Experiment station in co-operation with the Dairy Division of the United States Department of Agriculture, Washington, D. C.

LACTOCHROME—THE YELLOW PIGMENT OF MILK WHEY

Its Probable Identity With Urochrome, the Specific Yellow Pigment of Normal Urine.

LEROY S. PALMER AND LESLIE H. COOLEDGE.

The natural yellow color of cows' milk is caused by two entirely different kinds of pigments. The principal one of the two pigments is found in the milk fat and causes the more or less yellow color of cream and butter. This pigment has recently been found to be identical with carotin,* the widely distributed orange-red hydro-carbon vegetable pigment found in the carrot and which is also found accompanying chlorophyll in all green plants.

The secondary or minor pigment of milk has not heretofore been identified. Its presence in the milk is largely masked by the white color of the caseinogen and is only seen in the whey which remains after coagulation of the caseinogen. The pigment is then seen imparting the usual greenish-yellow color to the resulting whey. The same greenish-yellow color also characterizes the so-called rennet whey and is a familiar phenomenon to the cheese maker.

The object of the present investigation was to study the importance of the whey pigment in the pigmentation of milk and if possible to establish its relation to other well known animal pigments of similar character.

HISTORICAL

The yellow color of milk whey was observed as long ago as 1784 by Schoepff,[1] "Liquidem colore diluti citrinum." Nearly one hundred years later Blyth[2] in studying the so-called lacto-protein which Millon and Cammille[3] isolated from cows' milk, separated it into a protein which he called galactin and a pigment which he called lactochrome.[4]

*"Carotin—The Principal Natural Yellow Pigment of Milk Fat," by L. S. Palmer and C. H. Eckles. Missouri Agricultural Experiment Station Research Bulletin No. 10; Jour. Biol. Chem. 17, p. 191 (1914).

1. Schoepff, "Specimen Inaugurale chemico-medicum de Varus Lactis Buli Salibus alusque Substantius in ejusdem parte Aquosa Contentis, etc." Quoted from Blyth, "Foods, Their Composition and Analysis."
2. Trans. Jour., London Chem. Soc. p. 530, 1879.
3. Comp. Rend. 59, p. 301 (1864).
4. The method of isolation is given on page 455.

Blyth obtained the pigment as a mercury salt and finally in the free state. "As obtained in this way it was in the form of a bright red-orange, resin-like mass, softening at 100° C, very soluble in hot alcohol but partially separating out on cooling. The concentrated solution gave a simple spectrum allowing most of the red and yellow rays to pass through, and no bands were observed." Blyth analyzed the mercury salt and gave it the formula $HgOC_6H_{18}NO_6$. It seems very probable that Blyth was dealing with the specific milk whey pigment.

Reference to the pigment lactochrome is occasionally found in textbooks but in most cases erroneously in connection with the color of the milk fat. Blyth [1] himself says, "Milk fat under the form of butter is constantly tinted more or less yellow from dissolved lactochrome."

Lewkowitsch [2] states that, "Pure butter fat —— contains cholesterol and some natural coloring matters (lactochromes)." Oliver [3] says, "Lactochrome is probably derived from the haematin of the blood, is of a resinous character, of a bright orange color, and is soluble in water. This causes the color of butter and of whey. It is present in very small proportions and varies much in that respect according to the action of the alveolus cells." Wing [4] in his latest text book states that lactochrome gives to milk its characteristic color, and that it is associated principally with the palmitin of the butterfat. He also states that the amount of lactochrome in the milk varies with the breed of the animal and the character of the food.

Desmoulière and Gautrelet [5] recently studied a greenish-yellow pigment which they isolated from milk whey and from their investigation felt justified in concluding that it was identical with urobilin.[6] According to these authors if the slightly cloudy greenish-yellow milk whey is acidifed with sulphuric acid and saturated with ammonium sulphate the greenish-yellow color is entirely precipitated along with the proteins; and the precipitate will yield to 90 per cent alcohol a yellow pigment with a greenish fluorescence, which, after acidifying,

1. Text "Foods, Their Composition and Analysis," 1896, 4th edition, p. 239.
2. Oils, Fats and Waxes, Vol. II, p. 667 (1909 edition).
3. "Milk, Cheese and Butter," p. 44.
4. "Milk and Its Products," 1912 edition.
5. Compt. rend. soc. Biol. 55, p. 632 (1903); cited in "The chemistry of Milk," by Kastle and Roberts, Hygienic Laboratory, Bul. No. 56, p. 319.
6. This is the pigment that is found in small quantities in normal urine and which gives to urine its characteristic spectrum of a single absorption band in the neighborhood of the F line.

will show the characteristic absorption band of urobilin. In addition the pigment is claimed to have been isolated in the free state as rusty colored flakes, difficultly soluble in water.

In order to show that the pigment which they isolated pre-existed in the milk and was not converted into urobilin during the process of isolation, the investigators saturated a volume of milk with ammonium sulphate and added one half its volume of 90 per cent alcohol. The solution was shaken and let stand until a clear separation was obtained. The alcoholic layer had extracted the greenish-yellow color of the whey. The authors do not state that the yellow extract thus obtained showed the absorption band of urobilin. The conclusion that the pigment extracted in this way was urobilin seems to have been based on the grounds that this extraction test is a characteristic one for urobilin. The authors, in addition, emphasize the fact that the isolated pigment did not give the characteristic color reactions of a lipochrome with concentrated sulphuric and nitric acids. They accordingly conclude that no lipochromes exist in milk. The absurdity of the conclusion is apparent. It seems likely that these investigators as well as Blyth were dealing with the specific milk whey pigment.

The two investigations reported above, i. e. the one by Blyth and the one by Desmoulière and Gautrelet, are the only ones which the literature report in connection with the whey pigment. It is clear that both investigations concern only a pigment of the whey, notwithstanding the erroneous allusion in one case to the pigment of butter and in the other to lipochromes. In addition it is readily seen that the question is far from being satisfactorily settled. If Blyth was dealing with the true pigment, his work is unsatisfactory in that no attempt was made to show its relation to any of the known animal pigments. If the results of Desmoulière and Gautrelet are correct and the whey pigment is urobilin then both investigations would seem to require repetition to see whether they were dealing with entirely different bodies, and if so to determine which is the specific whey pigment. At the same time the apparently contradictory results of the two investigations could be satisfactorily explained.

THE ISOLATION OF BLYTH'S LACTOCHROME

Lactochrome was isolated from milk according to Blyth's method as follows: 1000 cubic centimeters of separated milk were divided into three portions. One of these portions was diluted with water to about four times its original volume and acidified with dilute acetic acid until the casein coagulated. The whey was then syphoned off

into a second portion of the milk and more acid added if necessary. The resulting whey was in its turn syphoned into the third portion of the milk. More acid, if necessary, was added and the casein filtered out. This solution, free from casein, was now raised to the boiling point and gently boiled for several minutes. The albumin and globulin coagulated and were easily filtered off. The greenish-yellow whey was then precipitated with acid mercuric nitrate solution.* The dense precipitate which formed was washed by decantation and suspended in a small amount of water. It was then decomposed with hydrogen sulphide gas, after acidifying slightly with either nitric or hydrochloric acid. The resulting mercuric sulphide was filtered off and the excess hydrogen sulphide removed from the filtrate with the aid of heat. An excess of lead acetate was added, which threw down the dirty white precipitate which Blyth called galactin. This was filtered off and the excess of lead acetate in the filtrate decomposed with hydrogen sulphide. The resulting lead sulphide was filtered off, the filtrate being more or less yellow in color depending upon the amount of lactochrome in solution. The lactochrome in this solution was purified by reprecipitation several times with mercuric nitrate, decomposing the final mercury salt with hydrogen sulphide, evaporating the aqueous solution to dryness, and dissolving the residue in hot 98 per cent alcohol. This alcoholic solution was considered to be practically pure lactochrome.

The maximum yield obtained by this method was about 0.04 gram per liter of milk. Some of the pigment was lost with each purification. About 0.5 gram in all was obtained for the following study.

The lactochrome, as obtained, was an amorphous orange colored substance with a peculiar odor. It conformed in every particular to the description given by Blyth. It showed no spectroscopic absorption bands either in aqueous or alcoholic solution when the solutions were neutral or after they had been acidified with hydrochloric acid.

The pigment was precipitated as a yellow salt from both its alcoholic and aqueous solutions by phosphotungstic and phosphomolybdic acids, and by mercuric nitrate, silver nitrate and lead acetate, the lead salt being soluble in an excess of lead acetate. Lactochrome was not precipitated by lead acetate from its solution in the presence of either

*Note:—The mercuric nitrate solution was made by dissolving 100 gms. of pure mercury in one liter of concentrated HNO_3, a further quantity of acid was added until no red fumes were evolved; the solution was evaporated to a syrup and after adding enough HNO_3 to prevent the formation of a basic salt the solution was made up to 1400 c.c. with distilled water.

nitric or hydrochloric acid which is the principle upon which is based the separation of the "galactin" from the lactochrome by lead acetate.

Lactochrome gave a greenish colored copper salt on the addition of a neutral solution of copper acetate. This salt was readily soluble in NH_4OH. The lead and mercury salts were readily soluble in ammonium acetate solution. Very little ammonium acetate was necessary to prevent the precipitation with lead acetate; it required more, however, to prevent the precipitation of the mercury salt.

The yellowish-brown residue which was left on evaporation of the alcoholic solution, imparted a faint color to chloroform which seemed to increase on the addition of a drop of concentrated NH_4OH. Alcoholic zinc chloride when added caused no green fluorescence. Amyl alcohol seemed to extract a slight amount of color from the dried residue, the amount of color absorbed seeming to increase on standing. This was probably due to absorption of water from the air,—the pigment being very soluble in water. The amyl alcohol solution showed no absorption bands after acidifying with HCl when viewed in front of the spectrometer through a 10 mm. layer, although the solution showed a good yellow color at this depth.

Lactochrome was only very slightly precipitated on saturation of its aqueous solution with ammonium sulphate. A scant brown precipitate was thrown down in this way but the filtrate had apparently retained all its former color. On addition of an equal volume of absolute alcohol to this filtrate and mixing thoroughly, the alcohol rose to the top with practically all the color.

Lactochrome was found to be readily soluble in milk whey, a small amount appreciably increasing the original color of the whey.

THE ISOLATION OF UROBILIN FROM MILK ACCORDING TO THE METHOD OF DESMOULIERE AND GAUTRELET

In order to demonstrate the presence of urobilin in milk, six liters of skim milk were treated as follows according to the method of Desmoulière and Gautrelet: The casein and fat were precipitated by acidifying with acetic acid at a temperature of 50°C. After filtering off the casein, the slightly cloudy serum had a pronounced yellow color. The acetic acid in the serum was neutralized with NH_4OH and the serum made acid with H_2SO_4 and saturated with solid ammonium sulphate. After standing for several hours the heavy precipitate was filtered off.

Contrary to the observations of Desmoulière and Gautrelet the filtrate was not colorless but was apparently as high colored as before

saturation with ammonium sulphate. That the precipitate contained some pigment, however, was shown by the fact that after careful washing with a saturated solution of ammonium sulphate and drying in the steam oven, it gave up a greenish-yellow extract to 90 per cent alcohol. On evaporating off the alcohol from this extract, leaving the pigment in aqueous ammonium sulphate solution, only a small part of the pigment was re-precipitated, the bulk of the coloring matter being again found in the filtrate. An alcoholic solution of the portion of the pigment which had been precipitated the second time was obtained as before by washing with saturated ammonium sulphate solution, drying, and extracting with 90 per cent alcohol. The golden-yellow green-fluorescent solution of about 25 c.c. volume, which was obtained in this way, was tested for urobilin properties. The solution, however, showed no absorption band in either neutral or acid (HCl) solution. On evaporation of the solution the residue was somewhat soluble in chloroform giving a yellow solution with a slight green fluorescence. The addition of ammoniacal alcoholic zinc chloride to the chloroform solution gave no indication of the beautiful green fluorescence of Wirsing's urobilin reaction, and the solution showed no absorption bands. The coloring matter was completely precipitated from both aqueous and alcoholic solutions by mercuric nitrate and silver nitrate.

The result of this experiment, the principal features of which were confirmed by numerous other investigations of the same nature, showed that the claims of Desmoulière and Gautrelet in regard to the presence of urobilin in milk cannot be confirmed. In addition, they led to an investigation of the pigment which in such large measure remained in solution on saturation with ammonium sulphate. As was stated above, Desmoulière and Gautrelet apparently based their conclusion that urobilin is present in milk partly on the ground that alcohol will extract a yellow color from whole milk which has been saturated with ammonium sulphate. Attention was accordingly directed to the extraction by this method of the pigment which we found was not precipitated on saturating the acid whey with ammonium sulphate. It was found on adding an equal volume of absolute or 98 per cent alcohol to the highly colored ammonium sulphate saturated serum under investigation above, that after thorough mixing, the alcohol rose to the top in a clear greenish-yellow layer, carrying with it practically all the pigment that was in the serum. Numerous attempts to make the pigment of this extract exhibit spectroscopic absorption properties resulted in failure. On the other hand the pigment exhibited all the properties which had been found to be characteristic of lactochrome. In this

connection attention was especially drawn to the facts that neither pigment was precipitated to any extent on saturation of its aqueous solution with ammonium sulphate, and that both pigments on isolation failed to show any spectroscopic properties. (The latter was also true of the small portion which was precipitated from the fresh whey with ammonium sulphate.)

On studying the above mentioned properties of the whey pigment isolated as either lactochrome or by the alcohol extraction method, there seemed to be a remarkable similarity to the properties of urochrome, the specific urinary pigment. Hammarsten [1], summarizing the properties of urochrome, describes the isolated pigment as, "Amorphous, brown, readily soluble in water and ordinary alcohol, but less soluble in absolute alcohol. It dissolves but slightly in acetic ether, amyl alcohol, and acetone, while it is insoluble in ether, chloroform, and benzene. Urochrome is precipitated by lead acetate, silver nitrate, mercuric acetate, phosphotungstic and phosphomolybdic acids. On saturating the urine with ammonium sulphate a great part of the urochrome remains in solution. It does not show any absorption bands and does not fluoresce after the addition of ammonia and zinc chloride."

In addition Hammarsten states that, "Urochrome can be prepared according to a rather complicated method which is based upon the fact that the substance remains in great part in solution on saturating the urine with ammonium sulphate. If the proper quantity of alcohol is added to the filtrate, a clear, yellow alcoholic layer forms on the salt solution, which contains the urochrome and which can be used for the further preparation of the latter."

It is especially striking that the whey pigment lends itself to isolation in exactly the same way. It might be stated here that the property of being extracted by alcohol from a solution saturated with ammonium sulphate is a urochrome characteristic and not a urobilin characteristic as Desmoulière and Gautrelet believed in connection with their work on "Urobilin" in milk.

Up to this point it was concluded that it has been satisfactorily demonstrated that the whey pigment is not urobilin, that the whey owes its characteristic color to the pigment which Blyth called lactochrome; and that the pigment could be isolated by means of a method identical with the one which was used by Garrod [2] to isolate urochrome from

1. Hammarsten-Mandel, "Text book of Physiological Chemistry," p. 703, 6th American edition 1911.
2. Proc. Roy. Soc. 55, p. 394 (1904).

urine. In addition the pigment which for the present may be called lactochrome, was characterized by a large number of properties identical with those of the pigment urochrome. In fact the resemblance was so strong that the investigation of the lactochrome was further pursued with a view of establishing its relation to the specific urinary pigment.

THE STUDY OF THE UROCHROME PROPERTIES OF THE WHEY PIGMENT

It became necessary before proceeding with the investigation, to study more closely the properties of urochrome in order to select some properties which could be applied to the pigment lactochrome, the result of which would be to demonstrate beyond a doubt the identity of the two pigments.

The most desirable means of identification would have been a study of the elementary composition. This was abandoned, however, partly because of the great difficulty attending the isolation of the pigment in a degree of purity sufficient for elementary analysis, but chiefly on account of the fact that none of the investigations of the elementary analysis of urochrome which have already been made have been found to agree. For instance, Dombrowski [1] found 11.15 per cent nitrogen, Holweg [2] found 9.89 per cent nitrogen, while Klemperer [3] found only 4.2 per cent nitrogen. Again Dombrowski,[4] basing his method upon the fact that urochrome readily forms a copper salt with copper acetate solution, isolated a product containing varying amount of sulphur up to six per cent, while Holweg [5] using a method which did not involve the use of any metallic precipitants, found that urochrome was free from sulphur. Salomonsen,[6] using Holweg's method, found that the product contained plenty of sulphur and in addition contained, "Considerable iron which could not be removed by repeated solution in alcohol and precipitation with ether." Mancini [7], also using Holweg's method, found that the substance contained sulphur but that its union with the urochrome molecule was very unstable.

1. Zeit. f. Physiol. Chem. 54, p. 188 (1908).
2. Biochem. Zeit. 13, p. 199 (1908).
3. Ber Klin. Wochensch. 40, No. 14 (1903).
4. Loc. cit.
5. Loc. cit. Holweg evaporated the urine to a syrup, extracted the urochrome with animal charcoal and after washing, extracted the pigment from the animal charcoal with glacial acetic acid. The urochrome was obtained in the free state from its solution in glacial acetic acid by precipitation with ether or by evaporation of the acetic acid in vacuum.
6. Biochem. Zeit. 13, p. 205 (1908).
7. Biochem. Zeit. 13, p. 208 (1908).

Garrod [1], studying urochrome in relation to other animal pigments, found that an alcoholic urochrome solution after treatment with "active" acetaldehyde exhibited a great many of the characteristic properties of urobilin including the characteristic urobilin absorption band and the production of the usual green fluorescence on the addition of ammoniacal zinc chloride solution. At the same time, the "artificial" absorption band showed the position which characterizes the ordinary urobilin band after the same treatment. According to Garrod, this reaction is a very delicate one, "A solution of one part of urochrome in 30,000 yielding the reaction quite definitely, although such a solution appears colorless in depths of two or three centimeters."

Dombrowski [2] failed to confirm the action of "active" aldehyde on the urochrome prepared by him, no bands whatever being formed. He considered his aldehyde solution "active," however, because it restored the optical properties of a urobilin solution whose absorption band had been destroyed by evaporation of its aqueous solution in the presence of ether,[3] or by oxidation with potassium permanganate.[4] In these cases, however, the position of the band was not in the same place in which it was found before.

Holweg also tried to apply Garrod's aldehyde test to the urochrome isolated by him, with the result that the urochrome solution, "After a short treatment with pure acetaldehyde in the warm and subsequent addition of ammoniacal zinc chloride solution, showed an extraordinary strong green fluorescence, not at once, but after standing forty-eight hours in the air. At the same time no characteristic urobilin band occurred in the blue but only a diffuse absorption of the stronger refracted rays of the spectrum."

One of the few tests for urochrome which all investigators of the pigment have confirmed is the fact that it gives the reaction for pyrrol when heated with zinc dust or KOH.

Lately Salomonsen [5] and also especially Mancini [6] have shown that urochrome will give a bromine derivative which they claim is a very characteristic compound and which may be used for the identification of the pigment.

1. Jour. Physiol. 21, p. 190 (1897); 29, p. 335 (1903).
2. Loc. cit.
3. Method of Hopkins and Garrod—Jour. Physiol. 29, p. 339 (1903).
4. Method of Riva and Chiodera—Arch. Ital. d. chem. med. 35, p. 505 (1896).
5. Loc. cit.
6. Loc. cit.

After a careful review of the above investigations especially the ones concerning the aldehyde reaction and the bromine compound, it was concluded that they were sufficiently characteristic to apply them to the whey pigment and thus definitely establish its relation to urochrome. It will not be necessary to discuss the pyrrol reaction or the formation of the bromo-urochrome further at this point. In view, however, of the contradictory results obtained by Dombrowski and Holweg with the "active" aldehyde reaction, this reaction seems to deserve some consideration.

A close study of Garrod's paper setting forth the action of "active" aldehyde on alcoholic urochrome solutions, will lead to the conclusion that Garrod's results cannot well be doubted. Certainly the possibility of the presence of urobilinogen in his solutions is precluded, on the ground of the method of preparation and also on the ground of the existence of a number of characteristic properties which accompany the reaction. The method of preparation itself would preclude urobilinogen if we are to believe the text book statements, that that inert body is precipitated on saturation with ammonium sulphate; and chief among the accompanying properties is to be considered the development of a second very characteristic absorption band in the blue on continuing the action of the aldehyde upon the urochrome, especially since no such band developed on allowing the aldehyde to act for a corresponding period of time upon a natural urobilin solution which already showed the normal urobilin band. Again, Garrod found that the urobilin which resulted from the action of the aldehyde resembled other artificial urobilin-like products in that the redward border of the band was less distinct than the natural urobilin band, and that the product did not give the E band spectrum which is given by natural urobilin on neutralizing or slightly acidifying an alkaline urobilin solution with sulphuric acid. On the other hand the artificial urobilin resembled natural urobilin in many respects. The action of the aldehyde changed the original yellow color of the urochrome to a deep orange tint and on dilution this color changed to the characteristic pink color of dilute urobilin solutions. Ammonia changed the deep orange color to a pale yellow with the disappearance of the absorption band. On addition of a little zinc chloride solution to the ammoniacal solution the characteristic brilliant green fluorescence of a natural urobilin solution appeared and also the shifted absorption band. Garrod gives the following measurements of the absorption bands.

	Neutral Alcoholic solution	Ditto with zinc chloride and NH_4OH
Product from urochrome	λ 5150-4860 shading to 4550	λ 5170-4990 shading to 4770
Urobilin from urine	λ 5110-4810 shading to 5590	λ 5170-4990 shading to 4770

The urochrome product further resembled natural urobilin in being precipitated on saturation of its aqueous solutions with ammonium sulphate.

On allowing the action of the aldehyde to continue upon the urochrome, Garrod found that the color of the solution changed to a deep red brown and a second absorption band appeared.

Garrod measured the two bands at this stage as follows:

Band A λ 5130-4910

Band B λ 4720-4570

The substance which gave the second band was extracted along with the urobilin-like band with chloroform, but after repeated washings with water the chloroform showed only Band B while the washings showed Band A. After evaporation of the resulting chloroform solution, an alcoholic solution of the residue showed Band B with great distinctness.

As a final proof of Garrod's results is to be mentioned the fact that similar results were obtained with a urochrome solution which had been obtained by an entirely different method,[1] namely by the method devised by Kramm.[2] This method was similar to the one used by Holweg[3] and consisted in extracting the urochrome from the urine by animal charcoal and, after washing and drying in vacuum, extracting the pigment from the animal charcoal by means of a phenol-alcohol solution. The above especial consideration of Garrod's "active" aldehyde reaction has been given because, as previously stated, the work seemed to us of such high character and the reaction so characteristic that it would certainly lend itself, if rightly carried out, to an ultimate solution of the identity of the milk whey pigment. That this proved to be the case will be seen on consideration of the results obtained.

1. Garrod—Jour. Physiol. 21, p. 191 (1897).
2. Deut. med. Wochenschr. 22, pp. 25 and 42 (1896).
3. Loc. cit.

THE ACTION OF "ACTIVE" ACETALDEHYDE ON UROCHROME AND LACTOCHROME

For the study of the action of "active" acetaldehyde, fresh quantities of urochrome and lactochrome were prepared as follows: The preparation of the "active" aldehyde is also described.

PREPARATION OF UROCHROME

The urochrome was isolated according to Garrod's [1] method as follows: About eight liters of well colored normal human urine were concentrated to about 250 c.c. on the steam bath, and the syrupy fluid filtered. The filtrate was saturated with ammonium sulphate in substance and the resulting precipitate filtered off. The filtrate was clear. It had a deep red color, but a small portion of it on dilution passed through a golden to a pale greenish-yellow color.

An equal volume of 98 per cent alcohol was now added to the filtrate, precipitating considerable ammonium sulphate. After mixing thoroughly and letting stand for a few minutes, the alcohol layer rose to the top as a highly colored solution. The alcohol layer was separated and fresh alcohol added to the lower aqueous layer. More pigment was extracted. The alcohol extracts were combined and the resulting solution of about 300 c.c. volume poured into 500 c.c. of water. The alcohol layer was made to separate from this solution by adding ammonium sulphate with the aid of gentle warmth. The alcohol extract obtained in this way was poured upon some solid ammonium sulphate and gently warmed. Two layers again formed, and the alcohol layer again separated.

The final alcohol extract was now evaporated nearly to dryness at a low temperature keeping the reaction of the solution alkaline with NH_4OH, and the evaporation finally was carried to dryness in vacuum. A brown-red residue remained which softened on warming but was nearly solid when cold. It was washed twice with acetic ether to remove indoxyl sulphate and was then allowed to soak over night in absolute alcohol, in a glass stoppered bottle. The alcoholic extract was filtered off and the residue treated with fresh absolute alcohol. The combined extracts were poured into several volumes of anhydrous ether, precipitating most of the pigment as a sticky brown-colored substance which clung to the bottom and sides of the beaker. The alcohol-ether solution was poured off and the precipitated pigment

1. Proc. Roy. Soc., 55, p. 394 (1894).

dried in the vacuum oven. It was then allowed to stand under chloroform for about one hour which extracted a little color; and after draining off as much of the chloroform as possible the remainder was driven off in vacuum.

Three extracts were obtained from the residue, an absolute alcohol extract, a 98 per cent alcohol extract from the residue left after the absolute alcohol extraction, and an aqueous solution of the residue left after the two alcohol extractions. These were all considered to be fairly pure urochrome solutions and the three extracts were used in the studies to be reported later.

ISOLATION OF THE MILK WHEY PIGMENT

The isolation of the milk whey pigment was found to be rather difficult on account of the fact that the pigment so readily decomposes in aqueous solution, especially in its solution in the serum.* A beautiful greenish-yellow casein and albumin-free serum will sometimes almost entirely bleach out on standing over night. The alcoholic solutions are much more stable, however, and in this respect are identical with urochrome. The conditions surrounding the decomposition of the whey pigment are not well defined, the bleaching occuring as well in neutral as in acid solution. Again it has been found possible to carry out the entire tedious procedure of isolation with no indication of the bleaching. These remarks seem pertinent at this point because they emphasize the fact that ordinarily the isolation required that it be carried out with the utmost speed to the point where the pigment is brought into alcoholic solution.

The method which proved fairly successful and which was used in the isolation of the pigment for some of the following studies was a combination of Blyth's method for the isolation of lactochrome and Garrod's method for the isolation of urochrome.

Skim milk, which a preliminary test indicated would yield a well colored serum, was acidified with sufficient acetic acid to coagulate the casein and, after filtering, the cloudy acid whey brought to boiling and boiled for several minutes. The clear, hot filtrate from this coagulation was now neutralized with sodium hydroxide solution and the further coagulation which occurred removed by filtration. Mercuric nitrate was now added in excess and the cream colored

*Freudenreich (Land. Jahrb. Schweiz. 14, p. 51, 1900), noticed that milk serum containing 5 per cent lactic acid had a higher yellow color than the same milk containing 1 per cent lactic acid.

precipitate which formed removed by filtering on a Büchner funnel, using suction. The precipitate on the filter was suspended in water and thoroughly washed and then filtered off again on the Büchner. This treatment was repeated several times, thereby removing all water soluble impurities including the lactose, excess mercuric nitrate, etc. After the final washing and filtering, the precipitate was sucked quite dry and then removed from the funnel and rubbed up with water in a mortar to a fine suspension and decomposed with a stream of hydrogen sulphide. The mercury sulphide which formed was separated by acidifying with HCl and boiling, and then filtering off on the Büchner, using suction, through heavy pads of filter paper to remove finely divided sulphur and mercuric sulphide. The filtrate was now a clear, beautiful greenish-yellow liquid, whose appearance was identical with freshly prepared casein and albumin-free whey. It was made slightly alkaline with NH_4OH, then acid with H_2SO_4, and saturated with ammonium sulphate in substance. After standing for a few hours the precipitate which had formed was filtered off, and an equal volume of absolute alcohol added to the greenish yellow filtrate. After mixing, the alcohol rose to the top with all the pigment. This alcoholic extract was saturated with ammonium sulphate at a temperature of 60-70° C. The concentrated alcoholic layer which formed contained all the pigment. It was removed, concentrated to low volume and cooled in a brine-ice mixture. Some ammonium sulphate crystallized out and was filtered off. The golden-yellow alcoholic filtrate was used for the aldehyde reactions described below.

PREPARATION OF "ACTIVE" ALDEHYDE

According to Garrod, the activity of the acetaldehyde used in his investigations depended upon the production of some decomposition or polymerization product which forms in the aldehyde after standing for some time in the warm sunlight. Fortunately this investigation was conducted during the months from May to October when plenty of warm sunlight was available.

Two samples of acetaldehyde were prepared, one of which was a crude solution distilled from the oxidation of alcohol with potassium dichromate and collected in a receiver cooled in ice water; the other was prepared by diluting some c.p. Merck's acetaldehyde with sufficient absolute alcohol to prevent its rapid volatilization. Both samples were set out in the direct sunlight, in stoppered bottles, for several weeks. Their activity was tested from time to time by adding a few drops to 5 c.c. of dilute urochrome solution (which exhibited no

absorption bands) and allowing the test tube containing the solution to stand in the hot sunlight or on the steam bath for a few minutes and then examining the solution for absorption bands.

The aldehyde solutions first exhibited signs of "activity" by causing the color changes in the urochrome solutions which are described by Garrod i.e. yellow to orange to brown-red on continued heating. At this stage it was not possible to detect the development of any absorption bands. The aldehyde preparations were allowed to continue in the warm sunlight, however, with a result that they eventually became "active" in their ability to produce the absorption bands described by Garrod.*

ACTION OF "ACTIVE" ALDEHYDE ON UROCHROME

None of the urochrome preparations prepared above exhibited any absorption bands either in neutral solution or after acidifying with HCl. The solutions were also non-fluorescent. On adding about 10 per cent of either of the aldehyde preparations to any of the alcoholic urochrome solutions and allowing the mixture to stand in the warm sunlight or on the steam bath, the light yellow color of the solutions changed to a golden and finally to a deep brown-red color. When examined in the spectrometer at the proper dilution the golden yellow solutions exhibited a beautiful absorption band in an identical position with the normal "acid" urobilin band. On making the solution alkaline with NH_4OH the color of the solution became much fainter and the band disappeared. On adding a few drops of alcoholic zinc chloride solution, a beautiful green fluorescence appeared and the absorption band reappeared, considerably broader and slightly shifted toward the red end of the spectrum. On dilution with alcohol these solutions showed the characteristic pink color of a similar urobilin solution without, however, destroying the green fluorescence. After allowing the action of the aldehyde to continue until the solutions had become red colored, the solutions showed a clear second band in the blue as described by Garrod in connection with his study of "active" aldehyde on urochrome.

These spectroscopic observations were made with an ordinary spectrometer of narrow dispersion using an 80 candle power Mazda lamp for the source of light. The lenses and prism were crown glass.

*Note:—Aldehyde solutions retain their "activity" for a long time. The sample prepared from Merck's c.p. acetaldehyde was still very active after it had stood in the author's laboratory for twelve months.

The absorption bands were so clear and characteristic that they were photographed. The photographs were made on Cramer's spectrum plates using an Adam Hilger spectrocopic [1] with photographic attachment. They are shown in the accompanying plates. Crown glass lenses and prism were used. An 80 candle power Mazda lamp was used for the source of light, it being arranged in a special box so that only the spectrometer slit and the cell containing the solution were illuminated. A photograph of an electric spark in hydrogen atmosphere was made on each plate so that a few solar lines could be had for comparison, especially the F. line. It was not possible to standardize the concentration of the pigments in the solutions to be photographed. It was attempted, however, to use solutions of such concentration that the absorption bands to be photographed were all clear and as distinct as possible when viewed in 10 mm. layer by the observation spectrometer of narrow dispersion.

Where photographs of the normal urobilin bands were made for comparison the urobilin solutions were prepared as follows: A volume of normal urine whose urobilinogen had been transformed by allowing the urine to stand exposed to the air in the presence of HCl, was shaken gently in a separatory funnel with an equal volume of ether. The ether extract was separated, evaporated to dryness, and the residue dissolved in a few cubic centimeters of alcohol. The alcoholic solution exhibited the characteristic urobilin band and other urobilin properties.

Plate 1, Figures II and III show respectively the normal (acid) urobilin band and the band produced by "active" aldehyde on alcoholic urochrome solutions. Plate 2, Figures II and III show the same bands after being treated with ammonia and zinc chloride. As stated above these solutions showed the beautiful green fluorescence characteristic of "urobilin zinc" solutions.

Plate 3, Figure II again shows the result of "active" aldehyde on urochrome solutions. Figures III and IV of the same plate are two different exposures of the same solution after the reaction of the aldehyde had been allowed to continue until the second band had developed. This is plainly visible in the two photographs.

These photographs very strikingly confirm the observations of Garrod. One can only suppose that the failure of Dombrowski and Holweg to confirm the action of aldehyde on urochrome, was due

1. The authors are indebted to Dr. O. M. Stewart, chairman of the Physics Department, University of Missouri, for the use of this instrument and assistance in making the photographs.

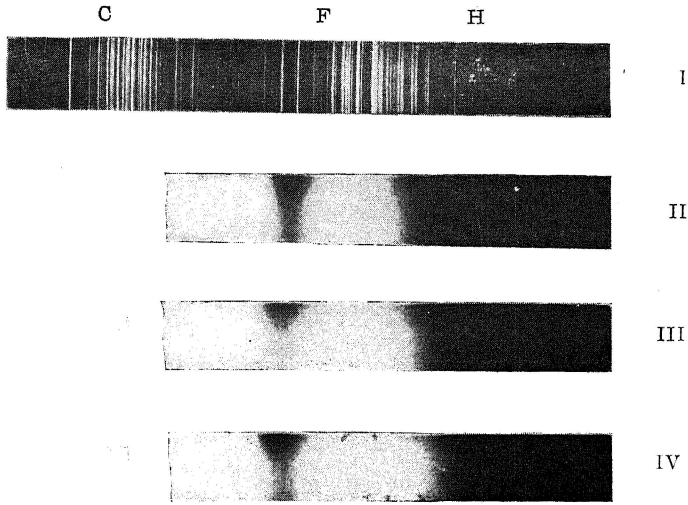

Plate No. 1

I. Spectrum of electric spark in hydrogen.
II. Absorption band of urobilin in acid solution.
III. Band produced by "active" aldehyde on urochrome.
IV. Band produced by "active" aldehyde on lactochrome.

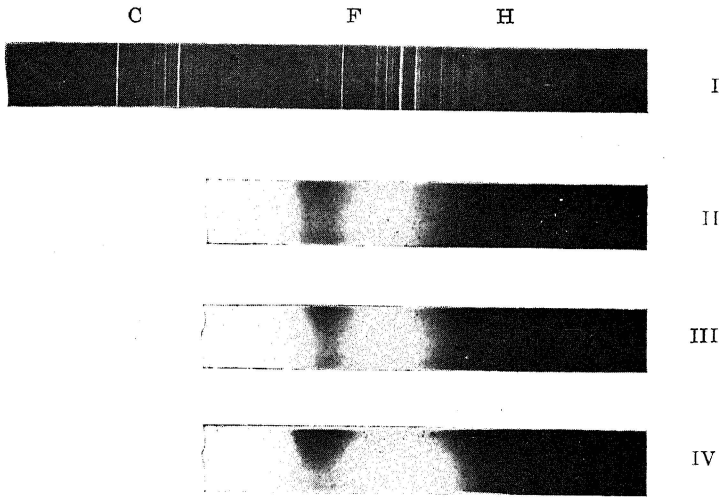

Plate No. 2

I. Spectrum of electric spark in hydrogen.
II. Normal absorption band of "urobilin zinc."
III. Band produced from urochrome by aldehyde after treatment with NH_4OH and $Zn\ Cl_2$.
IV. Band produced from lactochrome by aldehyde after treatment with NH_4OH and $Zn\ Cl_2$.

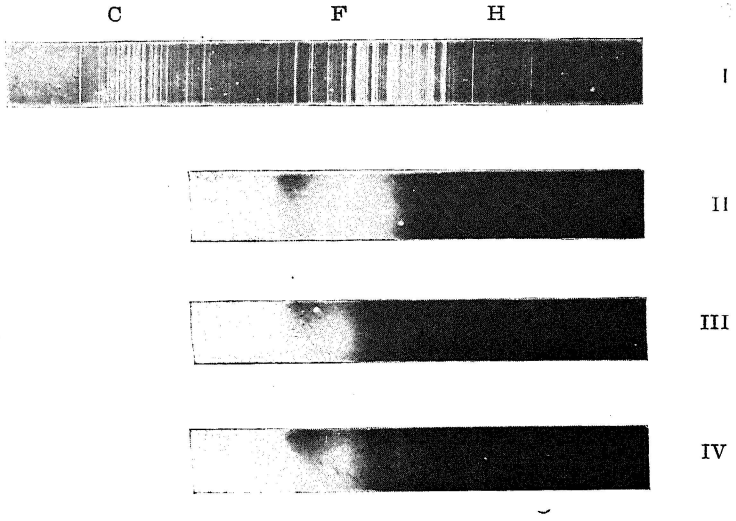

Plate No. 3

I. Hydrogen spectrum.
II. Absorption band produced by aldehyde on urochrome.
III and IV. Two different exposures of urochrome solution in which action of aldehyde had reached the double band stage.

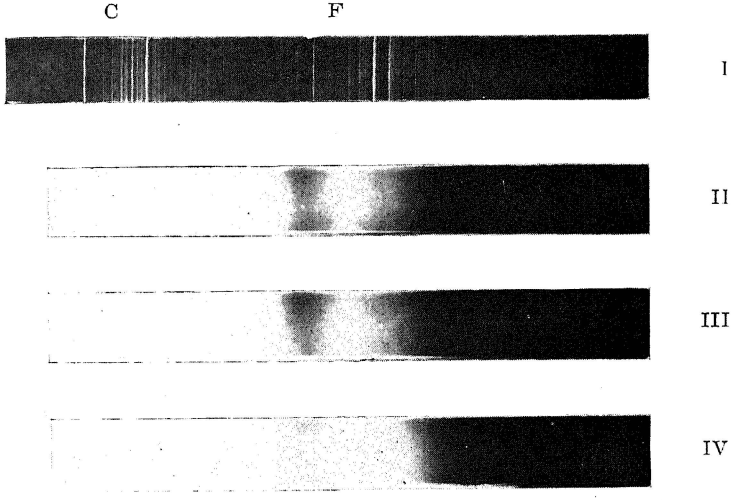

Plate No. 4

I. Hydrogen spectrum.
II, III and IV. Three exposures of a lactochrome solution showing the development of the second band.

probably to the fact that the aldehyde used by them was not really "active." This seems especially probable in the case of Holweg in the light of observations made at this station in regard to the difficulty in obtaining a truly "active" aldehyde. In the case of Dombrowski's work it is possible that his urochrome preparations were so "pure" that they were in reality decomposed.

THE ACTION OF "ACTIVE" ALDEHYDE ON LACTOCHROME

The alcoholic solution of lactochrome, whose preparation was described above, was spectroscopically inert in both neutral and acid solution. The action of the "active" aldehydes upon the solution, however, was in every respect identical with the action of the aldehydes upon the urochrome solutions. On warming about 10 c.c. of the alcoholic lactochrome solutions to which one cubic centimeter of "active" aldehyde had been added, or allowing the same to stand in the warm bright sunlight the yellow color of the solution deepened to an orange tint and a single beautiful absorption band appeared in an identical position with the normal (acid) urobilin band. A photograph of this band is shown on Plate 1, Figure IV in comparison with the normal urobilin band and the similar band produced by the "active" aldehyde upon the urochrome solutions.

Upon addition of a drop of concentrated NH_4OH to the orange colored solution, the color was changed to a light yellow. The absorption band disappeared entirely. Upon addition of a few drops of alcoholic zinc chloride to this ammoniacal solution a broader band appeared, shifted slightly toward the red end of the spectrum from the original band. This band is shown in Plate 2, Figure IV in comparison with the normal "urobilin zinc" band and the band produced by a similar action upon urochrome solutions. The three bands are seen to be identical. In conjunction with the appearance of this band the solution took on beautiful green fluorescence, and on dilution the color of the solution changed to a dull pink.

Continued action of the "active" aldehyde upon the alcoholic lactochrome solutions caused the color to change to a deep brown-red color with the appearance of a second absorption band.

Plate 4, Figures II, III and IV are three different exposures of the same lactochrome solution showing both absorption bands.

The product which resulted when the transformation of the lactochrome was allowed to proceed only to the one band stage, resembled natural urobilin in that it was almost completely precipitated as reddish colored flakes on saturation with ammonium sulphate. The

precipitate was readily soluble in ordinary alcohol, and in chloroform, and could not be extracted from the latter solution by water. On evaporation of the chloroform solution into alcohol, the resulting alcoholic solution showed the characteristic urobilin absorption band. A little "active" aldehyde was added to this solution and on continued heating on the steam bath a faint second band appeared without any visible change in the color of the solution. When more aldehyde was added and the solution placed in the strong sunlight the pigment precipitated and would not again dissolve on addition of either water or alcohol.

"Active" aldehyde was found to produce results identical with the above when acting merely on an impure alcoholic extract of casein and albumin-free milk serum which had been saturated with ammonium sulphate. The reaction also proceeded normally with an alcoholic extract of milk whey which had been produced by coagulation of the protein with rennet, and saturation of the filtrate with ammonium sulphate. These results greatly facilitated the spectrographic work. Some of the photographs of the bands produced by aldehyde on lactochrome were made with solutions prepared in this simple fashion.

"Active" aldehyde was found to have a very characteristic action on the greenish-yellow milk serum from which the casein, fat and coaguable proteins had been removed. On standing in the hot bright sunlight or warming on the steam bath, after the addition of about 10 per cent of "active" aldehyde, small volumes of serum which themselves were practically colorless and exhibited no absorption bands either before or after acidifying with HCl took on a light rose color which finally changed to a lavender. At the same time one and finally two characteristic absorption bands appeared. Sometimes the bands were in the normal positions, and again they were considerably shifted toward the red end of the spectrum, Band A extending almost into the yellow. The addition of NH_4OH destroyed the bands and changed the color of the solution to a faint pink.

This result seems to be contradictory to the statement made by Garrod that "active" aldehyde had no action on aqueous solutions of urochrome. An explanation might be found, however, in the fact that the aldehyde preparations used in these studies contained considerable alcohol. The cause of the shifting of the bands does not seem to be so readily explained. It may have been due to the percentage of alcohol present, for it was found that some samples of serum exhibited identical properties on treatment with "active" alde-

hyde without, however, the shifting of the bands. More alcohol might have been present in these cases. The point to be emphasized is that a purely alcoholic solution of lactochrome was not required for the manifestation of the "activity" of the aldehyde, equally good results having been obtained when only small quantities of alcohol were present.

THE ACTION OF "ACTIVE" ALDEHYDE ON THE PIGMENT OF THE WHEY OF MILK FROM ANIMALS OTHER THAN COWS

It was hoped to study the whey pigment from milk other than cows' milk, extending the study to include a number of other animals. Unfortunately, however, it was possible to make a thorough study of only sheep's milk. In addition one sample of human milk was examined.

The striking characteristic of all the sheep's milk examined was the unusually high color of the whey. Some figures will be given later in connection with some colorimetric studies of milk whey showing how much higher colored the whey of sheep's milk is than the whey of cows' milk. (See Table I.)

The pigment of the sheep's milk whey was found to be identical in every respect with the lactochrome of cows' milk. "Active" aldehyde acting on alcoholic extracts of the ammonium sulphate saturated whey, produced the urobilin-like pigment and its accompanying properties in even more striking manner than when acting on similar solutions from cows' milk. The development of the second band on continued action of the aldehyde was especially pronounced. The solutions were spectroscopically inert before being treated with the active aldehyde.

In the case of the sample of human milk, the well known difficulty of obtaining an acid coagulation of the casein interfered with the observation of the color of the serum. An alcohol soluble yellow pigment giving the reaction with "active" aldehyde was obtained, however, by the following method: A pinch of NaCl and a little $(NH_4)_2SO_4$ was added to about 350 cc. of human milk and the milk brought to a boil after acidifying with acetic acid. The precipitate was filtered off and washed with a little 95 per cent alcohol and then extracted until colorless with ether and hot 95 per cent alcohol. The ether-alcohol extract was evaporated to dryness and the residue freed from fat with ether. An orange-yellow ether insoluble residue was left which readily dissolved in ordinary alcohol. This solution gave the properties of urobilin after treatment with "active"

aldehyde, turning to a deep red in the warm sunlight and showing a beautiful absorption band. $ZnCl_2$ and NH_4OH caused the usual green fluorescence, the solution at the same time showing the absorption band of urobilin zinc.

Only one conclusion is possible from these results, namely, that sheep's milk and human milk are characterized by the same whey pigment found in cows' milk.

ACTION OF "ACTIVE" ALDEHYDE ON UROBILIN

A few observations were made in connection with these studies on the action of the aldehyde on solutions of natural urobilin. An alcoholic solution of urobilin was prepared by acidifying 100 cc. of fresh urine with HCl, letting stand in the sunlight to transform urobilinogen to urobilin, and extracting with about 50 cc. of chloroform. The chloroform extract was filtered and the filtrate evaporated to dryness at a low temperature. The red colored residue was dissolved in a few cc. of 96 per cent alcohol.

The golden-yellow solution which resulted showed a beautiful absorption band. On adding a little "active" aldehyde to this solution it at once showed a beautiful green fluorescence. After warming for some time on the steam bath the solution when examined in the spectroscope showed a broad band which appeared identical with the ordinary band of "urobilin zinc:" The solution, however, was acid to litmus paper. The appearance of the band differed from the normal band of "urobilin zinc" in that it had the appearance of the normal acid urobilin band laid upon a broader but fainter one. Continued heating caused the wide band to become uniformly intense. No second band appeared and no color change was exhibited other than the production of the green fluorescence already noted. After a time the band disappeared entirely.

These results studied in connection with the photographic plates make it clear that some substance other than urobilin is the cause of the characteristic reactions which urochrome and lactochrome give with "active" aldehyde. The reaction which natural urobilin gave with the aldehyde in the main confirmed the results obtained by Garrod, who found that aldehyde had no action on preparations of urobilin already showing the urobilin absorption properties.

THE BROMINE COMPOUND OF UROCHROME AND LACTOCHROME

Following the method of Salomonsen [1] and Mancini,[2] bromine was added a drop at a time to a portion of the crude aqueous solution of urochrome prepared as described above. When it was considered that an excess had been added the solution was set aside in the ice box. After twelve hours a yellow precipitate had come down which became granular on rubbing up with water in a mortar. The compound was not examined very closely, but in general answered to the description given by Salomonsen. It was soluble in glacial acetic acid, only slightly soluble in alcohol and insoluble in cold water.

In a similar way bromine was added to a concentrated aqueous solution of lactochrome which had been prepared from four liters of highly colored skim milk whey in the following manner: The acetic acid whey was boiled and filtered. Concentrated NaOH solution was added to the hot filtrate until another coagulation took place. The reaction was still acid. In this way a perfectly clear, beautiful, deep greenish-yellow solution was obtained. Neutral $Hg(NO_3)_2$ solution was added to the cold solution until no more precipitate came down, and the heavy cream colored precipitate removed by filtration on a Büchner funnel, using suction. The precipitate was washed a number of times by suspending it in distilled water and refiltering. It was finally decomposed with H_2S. The black emulsified solution was heated to boiling and concentrated HCl added a little at a time until the HgS came down. This was filtered off, thoroughly washed with water, resuspended in water and more H_2S passed in. The emulsion was treated as before. The filtrate contained quite a little additional pigment. The combined filtrates of about 800 cc. volume had a golden-greenish-yellow color like that of concentrated milk serum. It was saturated with $(NH_4)_2SO_4$ and the pigment extracted with absolute alcohol in the usual way. Several extractions were required to obtain all the color. The combined extracts of about 800 cc. volume were warmed for some time at almost boiling temperature with a considerable quantity of solid $(NH_4)_2SO_4$. About 300 cc. of water were thus removed. After cooling, the solution was placed in an ice-salt bath for several hours. The $(NH_4)_2SO_4$ which crystallized out was removed by quick filtration. This operation was re-

1. Loc. cit.
2. Loc. cit.

peated until no more crystallization occurred. The alcohol solution was now treated with an excess of $CaCO_3$ to neutralise free acid, thereby strongly reducing the acid reaction of the solution. It was still acid to litmus paper, however. The solution was now concentrated in vacuum at 50° C. to about 50 cc. volume, and was then treated with bromine.

The immediate result of bromination was the precipitation of a brownish-yellow granular substance. When the addition of bromine caused no more of this substance to come down the solution was set aside in the ice box over night. In the morning the precipitate was filtered off on a hardened filter paper and thoroughly washed with cold water, in which it was not soluble.

The Precipitate: The precipitate, after washing and drying, had a brownish-yellow color and granular appearance. In many respects it closely resembled the bromine compound of urochrome described by Salomonsen and Mancini. It was only slightly soluble in hot water and hot absolute alcohol and then only after long digestion on the steam bath. It was, however, readily soluble in both water and alcohol in the presence of a very little alkali (NaOH, KOH, or NH_4OH) giving yellow to brown solutions. Like bromo-urochrome it was precipitated from its slightly alkaline solutions as a green precipitate by $CuSO_4$, as a red precipitate by $FeCl_3$, and as a faintly yellow precipitate by $AgNO_3$. The silver salt was readily soluble in an excess of NH_4OH and was reprecipitated on addition of HNO_3. The latter precipitate became granular on heating and darkened to a brownish color in the sunlight.

The substance gave a strong flame test for halogen, but did not give up its bromine on heating with strong KOH. When heated with zinc dust, the substance gave a strong pyrrol reaction to a pine splinter moistened with HCl.

Contrary to true bromo-urochrome the substance was not readily soluble in glacial acetic acid or 60 per cent alcohol. It was slightly soluble in the glacial acetic acid on boiling and was precipitated from this solution, after cooling, on addition of ether.

On treating the pale yellow, neutral, or slightly acid alcoholic solution with "active" aldehyde and placing it in the strong hot sunlight, the solution turned to a dark brownish-red and showed the absorption band of urobilin. NH_4OH and $ZnCl_2$ changed the color to a golden-yellow and gave the solution a beautiful green fluorescence, the solution at the same time showing the broad absorption band of "urobilin zinc."

The Filtrate: The filtrate from the bromine precipitate had a deep red bromine color but it did not give up this color on heating or in fact after evaporation to dryness. Instead it left a heavy red colored residue. This residue was only partly soluble in hot absolute alcohol. The absolute alcohol insoluble portion had a flaky appearance and a chocolate-brown color. It was readily soluble in water or dilute alcohol giving solutions of a dull brownish color. The absolute alcohol soluble portion left a reddish-brown gummy residue on evaporation. Its solutions had a brilliant golden-yellow color.

Both portions of the residue were rich in loosely combined bromine, giving it up in alkaline solution so that on neutralization it was readily thrown down by silver nitrate, the white granular precipitate in both cases rapidly turning purple in the sunlight. Neither of the residues gave a pyrrol reaction on heating with zinc dust.

Alcoholic solutions of the chocolate-brown residue gave a strong reaction with "active" aldehyde, quickly turning to a rose and then to a deep red color in the sunlight and showing the urobilin band and other urobilin properties. Solutions of the gummy residue also gave the urobilin properties on treatment with aldehyde, but not so clearly and with much greater difficulty.

THE PYRROL GROUP IN UROCHROME AND LACTOCHROME

An excess of aqueous copper acetate solution (neutral) was added to about 100 cc. of an alcoholic urochrome solution. A heavy flocculent greenish-brown precipitate came down. It was filtered off on a hardened filter paper and washed thoroughly with water and then with alcohol and ether. The dry precipitate was a greenish powder. On heating with zinc dust the heavy fumes which came off gave a beautiful pyrrol reaction to a pine splinter moistened with HCl.

In regard to a pyrrol group in lactochrome, it can only be said that a compound was obtained from milk serum which very closely resembled the bromine compound of urochrome and which gave a strong pyrrol reaction. It should be stated also that a compound was obtained from the same solution which was readily transformed into the urobilin-like pigment by "active" aldehyde but which did not give any reaction for pyrrol.

The significance of this result is not apparent. It is possible that the pyrrol group in the second compound had in some way or at some time during the isolation been broken down, thereby changing the appearance and some of the properties of the original body. A more detailed discussion of this result will be found below.

FACTORS INFLUENCING THE COLOR OF MILK WHEY

During the course of the investigations of the milk whey pigment a wide variation in the color of the whey from the milk of different animals was noticed. This was especially evident when the color of the whey from sheep's milk was compared with the color of the whey from cows' milk, and was also often evident when merely observing two samples of freshly prepared whey from the milk of two different cows. In order to establish a better standard of comparison and study the factors that might be influencing the amount of lactochrome in the milk the following procedure was adopted.

Freshly drawn milk was run through a cream separator at a uniform speed and the casein precipitated from the skim milk with the smallest amount of 10 per cent acetic acid necessary to cause a clear coagulation. A portion of the cloudy yellow filtrate was then boiled for a few minutes to bring down the coaguable proteins. After filtering, the yellow serum which resulted was in some instances cloudy. To get a clear solution in these cases the filtrate was neutralized with ammonium hydroxide, warmed and filtered. The filtrate was then always perfectly clear. The yellow serum obtained by either of these methods was then placed in a 10 cm. cell and its color compared with the standard color glasses of the Lovibond tintometer. The colors were readily matched. In some cases a few tenths of red were required.

Using the above procedure, color readings of the whey from the milk from 43 cows and 6 sheep were made. The color of the sheep's milk whey is given to show how very much higher colored it is in many cases than the whey from cows' milk. The figures for sheep's milk are given in Table I.

TABLE I.—COLOR OF WHEY FROM SHEEP'S MILK.

Sample No.	Color, 10 cm. layer	
	Yellow	Red
1.	7.0	0.9
2.	7.0	0.5
3.	15.5	0.5
4.	24.0	1.2
5.	3.5	0.2
6.	4.5	0.5

The samples of cows' milk represent four breeds and include 4 Ayrshires, 4 Shorthorns, 15 Holsteins and 20 Jerseys, all pure bred dairy animals. Their milk production varied from 4.2 to 47.4 pounds per day. The stage of the lactation period of the animals varied from one to thirteen months, and their ages from three to fifteen years. The results of the colorimetric studies have been arranged according to breed, stage of lactation, age of the animal and volume of milk production, the results being given in Tables 2, 3, 4 and 5.

INFLUENCE OF BREED UPON THE COLOR OF MILK WHEY

The results according to breed are given in Table II. The average tintometer reading for the Ayrshires was found to be 4.78 units of yellow; for the Jerseys, 3.59 units of yellow; for the Holsteins, 2.41 units of yellow; and for the Shorthorns, 2.15 units of yellow.

TABLE II.—INFLUENCE OF BREED.

Breed	Herd Number	Units of yellow	Breed	Herd number	Units of yellow
Ayrshire	305	4.5	Shorthorn	400	2.6
Ayrshire	306	4.0	Shorthorn	403	3.0
Ayrshire	307	5.0	Shorthorn	404	1.6
Ayrshire	301	5.5	Shorthorn	406	1.4
	Average	4.75		Average	2.15
Jersey	57	2.9			
Jersey	23	3.0	Holstein	223	1.5
Jersey	11	2.7	Holstein	217	1.7
Jersey	41	3.1	Holstein	208	2.2
Jersey	53	3.5	Holstein	213	2.2
Jersey	16	3.5	Holstein	209	3.0
Jersey	124	5.0	Holstein	204	3.0
Jersey	10	3.5	Holstein	219	2.2
Jersey	3	3.0	Holstein	222	3.6
Jersey	30	2.7	Holstein	227	2.7
Jersey	54	3.0	Holstein	224	2.6
Jersey	50	3.1	Holstein	220	1.3
Jersey	8	3.3	Holstein	210	2.2
Jersey	2	2.7	Holstein	215	3.0
Jersey	13	3.2	Holstein	226	2.3
Jersey	317	5.3	Holstein	211	2.7
Jersey	27	4.5		Average	2.41
Jersey	22	5.2			
Jersey	59	4.5			
Jersey	14	4.2			
	Average	3.59			

These colorimetric averages emphasize what was found by mere observation of even the cloudy yellow whey after removal of the casein, namely that the Ayrshire and Jersey milk is characterized by yielding much higher colored whey than Holstein and Shorthorn milk. In some cases it was possible to select the samples of Ayrshire milk from a mixed lot of Ayrshire, Jersey and Holstein milk, on account of its relatively higher colored whey. Only in a few cases was the Jersey whey as high colored as the Ayrshire whey.

These figures showing such a marked difference between the average color of the whey of the milk from the four different breeds, when taken into consideration with the fact that each breed represents widely different conditions of milk production, stage of lactation, etc., would indicate that the color of the milk whey is primarily a breed characteristic. Within narrower limits it also appears to be an individual characteristic.

TABLE III.—INFLUENCE OF STAGE OF LACTATION.

Herd number	Stage of lactation (Months).	Units of yellow.	Herd Number	Stage of lactation (Months).	Units of yellow.
Ayrshire			Jersey		
301	10	5.5	317	13	5.3
305	3	4.5	27	13	4.5
306	3	4.0	41	12	3.1
307	1	5.0	23	12	3.0
			22	12	5.2
			54	11	3.0
Holstein			30	10	2.7
222	10	3.6	57	8	2.9
223	10	1.5	14	7	4.2
209	8	3.0	16	7	3.5
215	8	3.0	3	6	3.5
211	8	2.7	124	5	5.0
224	8	2.6	53	5	3.5
227	7	2.7	10	4	3.5
217	7	1.7	8	3	3.3
226	4	2.3	59	1	4.5
213	4	2.2	2	1	2.7
219	4	2.2			
220	4	1.3			
208	3	2.2	Shorthorns		
204	2	3.0	403	8	3.0
			406	7	1.4
			404	1	1.6

INFLUENCE OF THE STAGE OF LACTATION UPON THE COLOR OF THE WHEY

The results are arranged in Table III according to the stage of lactation of the cows. The differences here are not very pronounced, and are hardly definite enough to warrant any conclusions. A calculation, however, shows that 78.5 per cent of the cows which had been in milk for less than five months gave a color reading slightly below the average of the breed.

INFLUENCE OF THE AGE OF THE ANIMAL UPON THE COLOR OF THE MILK WHEY

The results are arranged in Table IV according to the age of the animals. An examination of the table indicates that the age of the animal may slightly influence the color of the whey. Nearly all the cows over 7 years of age, especially the Jerseys, gave color readings above the average for their breed. Of the cows under 7 years of age, 70 per cent are below the average. This would indicate that the older the cow the higher the color of the milk whey. Three of the old cows, however, i. e. Nos. 317, 27 and 301 were also well advanced in lactation, which may cause the apparent influence of the age to lose some of its significance.

INFLUENCE OF THE VOLUME OF MILK PRODUCTION ON THE COLOR OF THE MILK WHEY

The results are arranged in Table V according to the volume of the milk produced by the animals.

The interpretation of Table III that fresh cows seem to have less color in their milk whey might seem to indicate that the variations in the color may be merely a dilution effect. If the average milk production and average color reading of the Holstein cows are compared with the average milk production and average whey color of the Jersey cows, the highest color evidently accompanies the lowest milk production. This does not hold good, however, when comparing either the Jerseys or the Holsteins with the Ayrshires. Similarly the relation of high color to low milk production does not hold good when comparing the Jerseys or Holsteins among themselves. For instance Jersey Cow No. 124, with the highest milk production, gave one of the highest colored wheys of the series while Jersey Cow No. 30, with a milk production less than one fourth as great gave the lowest colored

whey of the Jerseys. The high color of the whey from Cow No. 124 might be explained on the grounds of her age as seen in Table IV. The same explanation, however, could not be offered for the high color of Cow No. 59 whose age was only six years. These examples could easily be multiplied both among the Jerseys and the Holsteins, as a consultation of the table will show.

TABLE IV.—INFLUENCE OF AGE.

Herd number	Age (Years)	Units of yellow	Herd number	Age (Years)	Units of yellow
Ayrshire			Jersey		
301	9	5.5	317	15	5.3
305	5	4.5	124	15	5.0
306	4	4.0	16	13	3.5
307	4	5.0	27	10	4.5
			10	7	3.5
			59	6	4.5
Holstein			53	6	3.5
204	12	3.0	41	6	3.1
209	10	3.0	50	6	3.1
211	7	2.7	54	6	3.0
210	7	2.2	57	6	2.9
213	6	2.2	3	5	3.0
215	5	3.0	8	4	3.3
208	5	2.2	23	4	3.3
217	5	1.7	2	4	2.7
222	4	3.6	22	3	5.2
219	4	2.2	14	3	4.2
223	4	1.5	30	3	2.7
220	4	1.3			
227	3	2.7			
224	3	2.6	Shorthorn		
226	3	2.3	403	10	3.0
			404	4	1.6
			406	3	1.4

It must be concluded, then, that the differences in color of the whey among different cows, while perhaps influenced in some cases by the volume of the milk, is not primarily a dilution effect. If any interpretation at all can be put upon the figures given in the tables it seems that the variation in the color is primarily a breed characteristic, as stated before, with the milk yield, age of the animal and possibly the period of lactation as minor influencing factors.

TABLE V.—INFLUENCE OF THE VOLUME OF MILK PRODUCTION.

Herd No.	Milk produced	Color of whey	Herd No.	Milk produced	Color of whey
Holstein			Jerseys		
210	47.4	2.2	10	28.0	3.5
204	46.0	3.0	16	27.2	3.5
211	41.6	2.7	3	23.6	3.0
213	40.8	2.2	54	19.0	3.0
209	38.4	3.0	53	18.4	3.5
219	34.2	2.2	41	15.0	3.1
208	30.0	2.2	14	14.2	4.2
223	29.6	1.5	12	13.6	2.7
217	28.8	1.7	317	13.4	5.3
226	27.2	2.3	50	13.0	3.1
220	23.0	1.3	8	12.4	3.3
227	20.8	2.7	27	12.4	4.5
215	14.0	3.0	57	10.8	2.9
224	12.0	2.6	11	10.0	2.7
222	8.0	3.6	30	8.4	2.7
			22	8.0	5.2
	Av. 29.4	2.41	13	4.2	3.2
Shorthorn					
404	27.2	1.6		Av. 16.8	3.59
403	26.0	3.0	Ayrshire		
400	24.0	2.6	305	32.0	4.5
406	11.2	1.4	307	30.8	5.0
			306	25.0	4.0
	Av. 22.1	2.15	301	19.4	5.5
Jersey					
124	36.0	5.0		Av. 26.8	4.75
59	33.4	4.5			

INFLUENCE OF FEED UPON THE COLOR OF MILK WHEY

In addition to the foregoing studies it was considered advisable to study the influence of the food of the cow upon the color of the whey. This would seem to be especially important in view of the fact that in a contemporaneous [1] investigation the food was found to be the important factor in the pigmentation of the milk fat.

The color of the milk whey was observed with two different cows in two different succeeding periods in which the food had been varied primarily for the purpose of studying the effect upon the color of the milk fat. The results are given in Table VI.

1. Missouri Agricultural Experiment Station Research Bulletin No. 10 (1914); Jour. Biol. Chem. 17, p. 191 (1914).

TABLE VI.—INFLUENCE OF FEED UPON THE COLOR OF MILK WHEY.

Cow No.	Period	Feed	Color of Fat	Color of Whey
			1 inch layer Yellow	10 cm. layer Yellow
57	1	Bleached clover hay and white corn....	8.0	3.5
57	2	Green alfalfa hay and yellow corn.....	45.0	4.5
			Yellow	Yellow
301	1	Green alfalfa hay, corn silage and grain.	32.0	5.5
301	2	Bleached timothy hay and white corn...	1.3	3.0

The results of these two cases might seem to indicate that some feeds influence the color of the whey as well as the color of the milk fat. This is emphasized in the case of Cow No. 301 because the milk production in the two periods is known to have been the same. If there is an influence upon the color of the whey it certainly is not nearly so pronounced as it is upon the color of the milk fat. Besides, this apparent result is of no value whatever in explaining the wide difference between the color of the whey of the different breeds as shown in Table II for when those readings were taken all the cows were on the same ration consisting of alfalfa hay, corn silage and a grain mixture of corn, bran and oil meal.

It is to be concluded, then, that the feed has little or no influence upon the color of the milk whey. Certainly slight individual variations in the color of the whey are to be expected from time to time and it was probably merely accidental that the color of the whey was somewhat lower in each case in the period when the milk fat was very low colored, for there was a considerable lapse of time in both cases between periods 1 and 2. This was 12 days for Cow No. 57 and 8 weeks for Cow No. 301. This question was not considered of sufficient importance to pursue further.

DISCUSSION OF RESULTS

A study of the results of the chemical investigation of the whey pigment leaves no doubt of its very close relationship to urochrome, the specific pigment of normal urine. The general characteristics of the two pigments are identical. Both pigments are precipitated by the

same reagents, and in common they are not precipitated to any extent on saturation of their aqueous solutions with ammonium sulphate, but can be extracted from the latter solution by alcohol. Again, both pigments show the same solubilities, and their solutions are spectroscopically inert.

Solutions of both pigments give the same striking reaction with "active" acetaldehyde, being transformed thereby into a pigment whose spectroscopic and other properties are identical with urobilin. This is clearly shown in the spectrophotographic plates 1, 2, 3, and 4.

In addition to the above identical properties of the pigments a bromine compound was obtained from a concentrated solution of the whey pigment, whose properties were identical in almost every respect with the bromine compound of urochrome which has recently been made by Salomonsen and Mancini. Solutions of the bromine compound showed the very interesting property of still reacting with the "active" aldehyde and yielding a solution showing the properties of urobilin, a result which might lead to the supposition that this reaction was due to some group or groups in the urochrome molecule or to some accompanying substance, rather than to the pigment itself.

It is clear, however, that the entire whey pigment was not recovered as the bromine compound which so closely resembled the bromine compound of urochrome. Two other bromine compounds were obtained whose properties were in many respects unlike the urochrome-like compound, being readily soluble in water and alcohol and giving no pyrrol reaction. One of the compounds was very reactive with "active" aldehyde, the other less so.

The most reasonable explanation of this result would seem to rest on the supposition that a part of the whey pigment had undergone decomposition at some time previous to bromination. There are several grounds for this supposition. In the first place urochrome is known to readily decompose, especially with mineral acids, which were undoubtedly present (as HCl) in the isolation under discussion. Again it is to be remembered that before any attempt at isolation, the whey pigment always showed the same uniform properties, leaving no doubt that the whey owes its color to but one pigment. Again the secondary substances which were obtained were also bromine compounds, altho clearly of a different character, and one of them i. e., the absolute alcohol insoluble one, was still very strongly reactive with aldehyde. Finally, the fact that the bromination of the whey pigment did not give a quantitative yield of the urochrome-like compound is not an unusual result in reactions of this kind. A complete trans-

formation of the pigment into its bromine compound could hardly be expected. Certainly Salomonsen and Mancini give no indication that their yield of bromo-urochrome was a quantitative one.

The general conclusion which might therefore be accepted is that the whey owes its color to the same specific pigment as normal urine; that this pigment is readily transformed by "active" aldehyde into a pigment with marked resemblance to urobilin; and that the pigment is readily decomposed and at some stage in the decomposition gives two or possibly three different bromine compounds, all of which may still be transformed into the urobilin pigment by "active" aldehyde.

Although the above interpretation of the results seem the most acceptable, it is admitted that an explanation of the phenomenon of the transformation into urobilin by aldehyde may rest on other grounds. Reference has already been made to this, namely that this property may have been due to some group or groups in the urochrome molecule or to some foreign substance accompanying the urochrome. This view would readily explain why the bromine compounds were still reactive with the aldehyde. Moreover the conclusion would still be justified that the whey pigment is very closely related to, if not identical with urochrome, and that the several additional bromine compounds obtained were due to some previous decomposition of the whey pigment.

The exact nature of the accompanying foreign substance which could be transformed into urobilin by aldehyde is not brought out by the present investigation. It has been definitely shown, however, that if this foreign substance exists, it is present as such in the milk whey and follows the pigment of the whey through its entire isolation by the methods used in this investigation. According to Dombrowski,[1] Garrod's preparations of urochrome which gave the reaction with aldehyde were contaminated with oxy and antoxy proteic acids which, according to the same author, are different steps in the breaking down of the protein molecule which ends in the pigment urochrome. It will be recalled that Dombrowski's preparations of urochrome which were presumably free from these proteic acids, did not give the reaction with a preparation of acetaldehyde which he considered to be "active." Dombrowski believed that Garrod's results were due to some chromogen accompanying the urochrome. If this view be correct, it must therefore be concluded that the same chromogen accompanies the urochrome in both the milk whey and urine.

The results of this investigation are of some importance from a physiological standpoint as they furnish additional proof that uro-

(1). Loc. cit.

chrome probably originates from the blood rather than from the bile. No conclusions can be drawn from this work, however, as to whether urochrome is a decomposition product of protein or of blood pigment. The constant presence of urochrome in the whey of cows' milk is in itself an interesting physiological phenomenon, especially since it was also found in some cases in relatively very large amounts in the whey of sheep's milk and in small amount even in human milk.

Some interesting observations were made during the course of the investigation relative to some factors influencing the amount of urochrome in the whey, with the result that the breed of the cow was found to be the most important factor although some difference was also found among individuals of the same breed. This difference in the pigmentation of the whey from the milk of cows of different breeds may be of considerable importance in giving to milk its characteristic yellow color. The so-called blue color of milk, especially skim milk, which is so commonly referred to among milk consumers is undoubtedly due to the absence of lactochrome in the whey. On the other hand some samples of milk never seem to lose their yellow appearance even after much skimming. This is especially true of Jersey milk and is usually associated in the popular mind with the "richness" of the milk. Numerous observations during the course of this investigation have shown that this phenomenon is due to the relatively high lactochrome content of Jersey milk rather than to the high fat content.

SUMMARY

1. Lactochrome, the yellow pigment of milk whey, is very closely related in chemical and physical properties to urochrome, the specific yellow pigment of normal urine, and is very probably identical with it.

2. Alcoholic solutions of lactochrome, or aqueous solutions containing a little alcohol, on treatment with "active" acetaldehyde and heat, are transformed into solutions whose spectroscopic and other properties are practically identical with those of urobilin. On continued action of the aldehyde a secondary pigment is formed with still different spectroscopic properties. In these two properties lactochrome is identical with similar solutions of urochrome.

3. A concentrated aqueous solution yields a yellow granular compound on bromination which gives a strong reaction for pyrrol, and in almost all of its other properties is identical with a similar compound obtained on bromination of a concentrated aqueous solution of urochrome.

4. The presence of lactochrome was found to be characteristic of the milk of all breeds of cows tested, i. e., Ayrshire, Jersey, Holstein, and Shorthorn. The amount of lactochrome appears to be largely a breed characteristic, with the Ayrshire and Jersey breeds ranking considerably above the Holstein and Shorthorn.

5. The presence of comparatively large amounts of lactochrome in the milk of some animals is of considerable importance in imparting to milk its characteristic yellow color.

6. Lactochrome was found in sheep's milk, often in much larger quantities than in cows' milk, and was also found in traces in human milk.

BIBLIOGRAPHY

1. Blyth: Jour. Chem. Soc. p. 530 (1879).
2. Blyth: Text, "Foods, Their Composition and Analysis," p. 239, 4th Edition, 1896.
3. Desmouliére and Gautrelet: Compt. Rend. Soc. Biol. 55, p. 632 (1903).
4. Dombrowski: Zeit. f. Physiol. Chem. 54, p. 188 (1908).
5. Freudenreich: Land. Jahrb. Schweiz. 14, p. 51 (1900).
6. Garrod: Jour. Physiol. 21, p. 190 (1897); 29, p. 335 (1903).
7. Garrod: Proc. Roy. Soc. 55, p. 394 (1904).
8. Hammarsten-Mandel: Text book of Physiological Chemistry, p. 703, 6th American Edition 1911.
9. Holweg: Biochem. Zeit. 13, p. 199 (1908).
10. Hopkins and Garrod: Jour. Physiol. 29, p. 339 (1903).
11. Klemperer: Ber. Klin. Wochensch. 40, No. 14 (1908).
12. Kramm: Deut. med. Wochensch. 22, pp. 25 and 42 (1896).
13. Lewkowitsch: Text, "Oils, Fat and Waxes." Vol. II, p. 667 1909 Edition.
14. Mancini: Biochem. Zeit. 13, p. 208 (1908).
15. Oliver: Text, "Milk, Cheese and Butter." p. 44.
16. Riva and Chiodera: Arch. Ital. d. Chem. Med. 35, p. 505 (1896).
17. Schoepff: Cited from Blyth's Text, etc.
18. Salomonsen: Biochem. Zeit. 13, p. 205 (1908).
19. Wing: Text, "Milk and Its Products." 1912 Edition.