STUDIES ON LIPOCHROMES.

III. THE QUANTITATIVE ESTIMATION OF CAROTIN IN BLOOD AND TISSUES.

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It has now been well established that carotin, one of the lipochrome series of pigments, is almost constantly present in the blood. Van den Bergh and Snapper (1), in 1913, distinguished the lipochrome of cattle serum from bilirubin, and showed that the pigment in the skin in the "xanthosis diabetica" of Von Noorden (2) was associated with a similar pigment in the blood plasma. Palmer (3) described the lipochrome of horse serum, and identified it as the carotin ingested with the food. Palmer and Eckles (4) also showed carotin to be the lipochrome of cow fat, milk, and blood, and found this same substance to be the natural yellow pigment of human milk. Another lipochrome, xanthophyll, was found by Palmer and Kempster (5) to be the principal pigment of chicken skin, blood, and egg yolk, and Palmer (6) has found both carotin and xanthophyll in human blood. Willstätter and Escher (7) identified the pigment of the corpus luteum of cattle as carotin, and Van den Bergh, Muller, and Broekmeyer (8) have extracted these pigments from human blood and tissues, and from the organs of various animals.

Carotinemia has been known since Van den Bergh and Snapper's paper, but Hess and Myers (9), in 1919, first applied that name to the condition in which an excess of lipochrome appeared in the blood as a direct result of a high vegetable diet. Umber (10) noted the same condition in 1916, and Bürger and Reinhart (11), and Salomon (12) showed the pigment to be of exogenous origin. A large number of cases were reported in Germany, in 1919, occurring in diabetics, in children under asylum conditions, or in adults upon the semistarvation diet during the war (13). Head and Johnson (14) have reported a single case of carotinemia in a diabetic, and Stoner (15), recently, another. Hashimoto (16) reported thirty-five cases in Japanese upon a heavy squash diet, and quoted Baelz as recording similar cases in 1896 under the term "aurantiasis cutis."

Palmer and Kempster showed that xanthophyll disappeared from the blood, skin, and egg yolk of chickens when fed a xanthophyll-free diet, and in the recorded cases of carotinemia the condition has disappeared upon change of diet to one containing less green and yellow vegetables. Dolly and Guthrie (17) found that certain nerve cells of chickens did not contain

lipochrome when none was present in the diet, and that, on the contrary, the pigment did appear in nerve cells when a lipochrome-rich diet was fed. It seems to be well established, therefore, also, that the lipochrome present in the animal body has been derived from the vegetable content of the food. It is noteworthy, however, that all cases recorded in which there has been an excess of pigment in the skin and blood have been among diabetics, or children or adults upon an otherwise deficient diet. It is also noteworthy that many diabetics have a yellowish coloration of the skin even when, by the use of insulin, they are not upon an excessively high vegetable diet (18).

Van den Bergh found that the carotin in the blood could not be directly extracted with petroleum ether, though the pigment is readily soluble in this substance. Palmer showed that the pigment is closely bound to the protein of the blood, and upon fractional precipitation it was found to come down with the euglobulin. It could be released from this union by the addition of 95 per cent alcohol to the plasma, after which it was easily extracted by lipoid solvents. But in a few cases of diabetes Palmer found that such a union did not exist; the carotin could be extracted directly with petroleum ether.

Willstätter and Stoll (19) proposed a test for carotin using 0.2 per cent potassium dichromate as a standard against carotin dissolved in petroleum ether. This standard is equal to a 5×10^{-5} molar solution of carotin equivalent to a 0.00268 per cent solution. This is not strictly quantitative, however, as they found 101 mm. of potassium dichromate solution in the colorimeter to equal 100 mm. of carotin solution, 41 mm. to equal 50 mm. of carotin, and 19 mm. to equal 25 mm. of carotin. This standard was used for the determination of the carotin content of leaves in which the pigment is relatively abundant. Palmer adapted this to the estimation of carotin in blood by treating the serum with an equal amount of 95 per cent alcohol, mixing with plaster of Paris, then adding petroleum ether. The method is useful for large amounts of blood, but cannot be used very well with the small amounts available in the clinical laboratory. Van den Bergh, Muller, and Broekmeyer used 1 to 2 cc. of serum, added an equal amount of alcohol, then extracted with petroleum ether. They used $\frac{1}{2}\frac{1}{4}$ per cent solution of potassium dichromate as a standard. They also estimated the amount of carotin and xanthophyll in the organs of various animals, but they do not record their method for the extraction of the latter.

While studying the reaction of animals when injected or fed upon carotin it became necessary to determine the carotin content of the blood and organs of these animals (20). I was, at the same time, attempting to distinguish the various pigments present in human organs (heart muscle, seminal vesicles, *etc.*) by histological methods, and as a check, a chemical examination of these organs was desirable. The carotin content of such tissues promised to

be exceedingly minute, and, as a consequence, an investigation of the standard used by Willstätter and Stoll seemed necessary.

It was first found that progressive dilution of the standard potassium dichromate solution did not equal an equivalent dilution of carotin. When the concentration was very low, a 0.2 per cent solution would no longer be accurate. So a series of tests was made with carotin freshly prepared according to a method previously described. The resultant carotin in petroleum ether showed typical absorption bands in the spectroscope. A solution containing 2.68 mg. per cent was made and compared with 0.2 per cent potassium dichromate solution. This was found to equal 103 per cent of the calculated amount from Willstätter and Stoll's figures. From this solution the range of colors with carotin in

TABLE I.

Relation of Carotin to Different Concentrations of Potassium Dichromate Solution, at 30 Mm. Depth, in the Colorimeter.

Potassium dichromate solution.	Carotin solution.
per cent	*per cent*
0.4	0.00462
0.2	0.00268
0.1	0.00119
0.05	0.00052
0.04	0.00033
0.025	0.00016
0.02	0.00010

various concentrations was determined as shown in Table I. It was found that the 0.02 per cent solution was the most generally useful.

Methods of Procedure and Results.

It was found (in about 60 per cent of the cases) that 2 cc. of blood serum would yield a visible pigment when extracted with petroleum ether after treatment with alcohol. In the other 40 per cent the pigment was either absent or present in an amount insufficient to measure. The use of whole blood reduced the proportion about half, and reduced also the yield per 100 cc., when present at all in measurable amount. It was found that 3 cc. of

plasma were the least amount which could be depended upon to yield a measurable amount of pigment. The test is simple: 3 cc. of 95 per cent alcohol are added to 3 cc. of plasma in a test-tube, the mixture shaken, and allowed to settle until solid. If excess alcohol rises to the top it may be poured off. 4 cc. of petroleum ether are added, the tube corked immediately, and shaken vigorously for a minute. It is allowed to settle until the petroleum ether rises to the top. This is poured into the colorimeter cup, calculated as 4 cc., and compared with 0.04 per cent or 0.02 per cent potassium dichromate solution equalling, respectively, 0.0003 per cent and 0.0001 per cent of carotin.

For the estimation of the pigment content of organs a much different procedure is necessary. Macerating the tissue in 95 per cent alcohol then extracting the crushed material, alcohol included, in a continuous Soxhlet extractor is effective but time-consuming. It was found that a quantitative extraction could be obtained by the following method. The weighed organ was placed in an excess of 20 per cent KOH solution in 70 per cent alcohol, and boiled until dissolved. The solution was then mixed with enough $CaSO_4$ to take up the water, and direct extraction with petroleum ether performed. The extract was washed with several portions of 85 per cent alcohol until no more color could be removed. The petroleum ether fraction was considered to be carotin, and the alcohol to contain xanthophyll, if present. The alcohol was mixed with ether (diethyl ether) after the method of Willstätter and Stoll, and the alcohol washed out with distilled water. The xanthophyll was thus transferred to the ether, and the pigment which remained in the alcohol-water fraction, if any, was discarded. The petroleum ether solution was made up to a definite amount and compared in the colorimeter against potassium dichromate solution. The xanthophyll was not determined quantitatively as it was present in extremely small amounts or not at all in all tissues examined except chicken fat. The results of these determinations and of blood under various conditions are as follows:

Blood.—Thirty-six specimens obtained from the Massachusetts State Wassermann laboratory, condition of patients unknown; serum: 0.02 to 0.11 mg. per cent. None present in twelve specimens.

Three specimens from normal adults; plasma: 0.04, 0.07 mg. per cent, and none.

Eighteen specimens from diabetics under the care of Dr. E. P. Joslin;[1] plasma: 0.05 to 0.16 mg. per cent. Three specimens contained too little to measure.

One specimen of normal plasma: 0.05 mg. per cent; plasma 2 hours after ingestion of 50 mg. of carotin in olive oil: 0.08 mg. per cent; 4 hours after ingestion of carotin: 0.04 mg. per cent.

Van den Bergh, Muller, and Broekmeyer found 0.4 to 1.34 mg. per cent in normal blood, and 0.45 to 1.9 mg. per cent in diabetic blood.

Organs.—Heart muscle freed from fat and blood: (1) From a 44 year old woman; very faint trace of carotin; (2) from a 79 year old man; heart weight, 280 gm.; no carotin; (3) from an 81 year old man; heart weight, 340 gm.; no carotin; (4) from a 3 month old infant; heart weight, 21 gm.; no carotin. Adrenals, from eight adults, various ages; carotin 4.25 to 15.6 mg. per cent. Adrenals, from a 3 month old infant; no carotin. Liver from four adults, various ages; carotin 1.0 to 6.0 mg. per cent. Liver from 3 month old infant; no carotin. Spleen from four adults; carotin, a trace to 2.1 mg. per cent. Spleen from infant; no carotin. Fat from an obese elderly woman; carotin 7.5 mg. per cent. Seminal vesicles from three adults, two quite old; no carotin. Testicle from 79 year old man; no carotin. Corpus luteum from a surgical specimen; carotin 4.1 mg. per cent; also contained an alcohol-soluble pigment, possibly xanthophyll.

Guinea Pig Tissues.—Heart, spleen, testes, blood of a normal animal; no carotin. Adrenal from a normal animal; carotin 0.4 mg. per cent. Liver from a normal animal; carotin, a trace. Blood, bile, and urine from an animal 5 hours after feeding carotin in olive oil; no trace of carotin. Blood and urine after the intraperitoneal injection of about 300 mg. of carotin; no trace of pigment. Blood, spleen, kidneys, testes, adrenals of an animal which had been on a carotin-free diet; no carotin. Feces of a guinea pig which had been on a synthetic diet for about 3 years, receiving 5 cc. of orange juice a day; carotin 0.01 mg. per cent. (A sample of orange juice contained 0.04 mg. per cent of petroleum ether-soluble material, calculated as carotin, and 0.14 mg. per cent of alcohol-soluble pigment, calculated as xanthophyll.) Feces of a normal guinea pig on a carrot diet; carotin 7.0 mg. per cent.

Other Tissues.—Normal rabbit: fat, blood, spleen, testes, none; liver, a trace; adrenals, from two animals; 0.2 and 0.3 mg. per cent. Pork fat, mutton fat, beef fat, none. Chicken fat contained considerable yellow pigment which was soluble in 85 per cent alcohol and was therefore considered to be xanthophyll.

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[1] Secured through the courtesy of Miss Hazel M. Hunt, Head of the Clinical Laboratory, Deaconess Hospital, Boston.

DISCUSSION.

It is generally asserted by text-books of pathology (MacCallum, Karsner, Wells) and in the literature on the subject (review by Oberndorfer (21)) that lipochromes are present in the adrenal cortex, corpus luteum, seminal vesicles, testes and epididymi, sympathetic ganglion cells, nerve cells of the central nervous system, liver, heart, muscle of the intestine, pigmented cells of the pineal body, kidney, spleen, fat, skin, sebaceous glands, perineural and perivascular sheaths. Most of the work supporting these reports has been based upon histological methods, and has been dependent upon the assumption that all pigments which are associated with fats, as determined by staining methods, are lipochromes. I have shown previously (22) that most of the methods heretofore used for the differentiation of these pigments cannot be depended upon; that, in fact, lipochrome does not stain with fat stains, and that other crystalline pigments may adsorb some of the fat stains on their surfaces, and so give the appearance of having been stained.

Most of the tissues enumerated above are present in the body in so small amount that they are not amenable to chemical analysis. But liver, heart, spleen, adrenal, fat, corpus luteum, and seminal vesicle tissues were obtained in comparative abundance. Of these only the adrenals, liver, and corpus luteum showed definite measurable amounts of lipochrome. The seminal vesicles, which in all instances contained a generous amount of brownish pigment, contained no carotin or xanthophyll, and likewise two hearts, both fairly typical examples of the condition known as brown atrophy, were negative. The amount of pigment found in the spleen was in no greater amount than could be expected to be present in the contained blood.

The results with normal blood show that this pigment is not present in measurable amount at all times. Here it may be explained that the figures given by Van den Bergh are not at great variance with those recorded above. This writer probably used $\frac{1}{24}$ per cent potassium dichromate solution to equal approximately 0.2 of Willstätter and Stoll's standard 0.00268 per cent carotin solution, or 0.0005 per cent, whereas I have shown that a 0.04 per cent solution of potassium dichromate equals not 0.0005 per

cent but 0.0003 per cent carotin. My figures, therefore, with use of a standard about half as strong as Van den Bergh used equal about half of his calculated results. It is quite possible, however, that more vegetables, and therefore more carotin, are eaten by the average person in Holland than in this country. It is shown that the carotin content of the blood rises slightly after the ingestion of carotin in olive oil.

The diabetic blood shows slightly but consistently higher figures than normal blood. Many of these cases were upon what may be regarded as essentially normal diets. In a few cases there was also a moderate lipemia, and it is possible that these two conditions, lipemia and hypercarotinemia, may be associated. It remains to be determined, however, how constant such an association may be.

SUMMARY AND CONCLUSIONS.

A method, essentially a modification of that used by Van den Bergh, Muller, and Broekmeyer, is proposed for the quantitative estimation of carotin in small amounts of blood. A similar method is devised for the estimation of carotin and xanthophyll in organs and tissues. Various concentrations of potassium dichromate were tested against known amounts of carotin for the purpose of securing accurate standards for comparison when extremely small amounts of pigment were present in blood or tissues. By the application of these methods it was found that carotin is frequently but not constantly present in normal blood; that it rises slightly in amount after the ingestion of carotin in olive oil; and that it is present in slightly greater amounts than usual in diabetic blood, sometimes associated in these cases with a lipemia.

Lipochromes are constantly present in the adrenals of adults, but not present in these or other organs of infants; they are present also in the corpus luteum, liver, and fat, but were not found in recognizable quantities in other organs except the spleen. The amount found in this last organ could be accounted for by that present in the contained blood. The seminal vesicles and heart, in all cases well colored, did not contain pigments demonstrable by the methods used. The adrenal glands of rabbits and guinea pigs contain the greatest amount of lipochrome in these animals; next to these organs the liver contains the most. There is no caro-

tin present in the blood of these animals even after the feeding or injection of comparatively large amounts.

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