THE IN VITRO PREPARATION AND HISTOCHEMICAL PROPERTIES OF SUBSTANCES RESEMBLING CEROID*

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Ceroid is the name which was given by Lillie, Ashburn, Sebrell, Daft, and Lowry (1) in 1942 to a golden yellow, lipoid pigment which occurs as coarse granules and globules in the cirrhotic livers of choline-deficient rats. This peculiar pigment had been reported almost simultaneously in the preceding year by four groups of investigators. Lillie, Daft, and Sebrell (2), Blumberg and McCollum (3), and György and Goldblatt (4) observed it in the cirrhotic livers of rats which had been fed low protein diets. Edwards and White (5) found it in the cirrhotic livers of rats which had been given carbon tetrachloride. Since then, there have been a number of reports regarding dietary and other factors influencing its formation (6–14).

Edwards and Dalton (15) noted a similar pigment in the cirrhotic livers of mice treated with carbon tetrachloride. Lee (16), however, has demonstrated some histochemical differences between this pigment and that found in rats. Deposits of ceroid have also been observed in other animal species and in other tissues and organs (1, 17–20).

The presence of ceroid in human tissues has not been demonstrated conclusively. Lillie, Ashburn, Sebrell, Daft, and Lowry (1), Popper, György, and Goldblatt (21), and Hartroft (22), were unable to find any. Wolf and Pappenheimer (23) and Pappenheimer and Victor (24) reported the presence in human tissues of an acid-fast pigment which they considered to be ceroid but whose identity has since been questioned (22).

Several investigations regarding the experimental *in vivo* and *in vitro* production of ceroid-like substances have been reported. Although published before ceroid was recognized, the *in vivo* studies of Hass concerning tissue reactions to natural oils (25), intercellular transformations of fatty acids and their esters (26, 27), and "membrane formation at lipoid-aqueous interfaces in tissues" (28, 29) should not be overlooked. Endicott (30) demonstrated the *in vivo* formation of ceroid-like substances following the subcutaneous or intraperitoneal injection of various unsaturated lipids. He was able to produce similar substances *in vitro* by oxidizing agar emulsions of the same lipids with dichromate. Hartroft (31, 32) found that a ceroid-like pigment was

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deposited in fatty, mesenteric hematomata experimentally produced in rats. *In vitro*, he was able to prepare ceroid-like material by incubating mixtures of cod liver oil and red blood cells.

Some of the chemical properties of a crude preparation of hepatic ceroid were reported by Endicott and Lillie who, in addition, made a detailed study of the histochemical and staining reactions given by ceroid in tissue sections (33). Its fluorescence was reported by Popper, György, and Goldblatt (21). The distinctive properties of ceroid by which it is most often identified in tissue sections include: (*a*) a golden yellow color when unstained, (*b*) insolubility in organic solvents, (*c*) sudanophilia (affinity for fat stains such as the sudan dyes) demonstrable even in paraffin sections, (*d*) acid fastness, (*e*) basophilia, and (*f*) fluorescence. Its intense coloration by pyronin-methyl green has been suggested by György (10) as another characteristic reaction. Usually, little or no iron can be detected histochemically. Lee (16) has reported that "in most cases the pigment was iron free, but some of the ceroid pigment granules did show a definite reaction, especially in the mouse."

The exact nature of ceroid is unknown. Blumberg and Grady (6) thought that it might be identical with hemofuscin but Endicott and Lillie (33) later were able clearly to differentiate between the two pigments. Edwards and White (5) considered that ceroid might be "a lipoid in conjugated form." György and Goldblatt (4), while attempting to explain its sudanophilia in paraffin sections, suggested that ceroid might be a lipoid "in some form of intimate combination with protein," a suggestion favored by Endicott and Lillie (33).

Lillie (35) has classified ceroid as one of the lipofuscins. Various investigators (11, 24, 36–38) have emphasized the similarity between ceroid and the lipofuscin pigment associated with vitamin E deficiency. Moore and Wang (39) have pointed out that the two pigments differ in their tissue distribution and fluorescence. Elftman, Kaunitz, and Slanetz (40) concluded that the uterine pigment in the vitamin E-deficient rats which they studied "differs from the published description of ceroid in its oxidation potential." Considering the problems associated with the use of reduction reactions to characterize pigments (41), however, they suggested that "further study of ceroid may establish a fundamental similarity." Mason (42), while discussing their paper, made the excellent suggestion that ceroid "may represent, for the most part, the oxidation products of fats abnormally mobilized and stored in the liver cells," while the pigment associated with vitamin E deficiency "may constitute oxidation products of intracellular lipids related to the normal functioning of smooth, skeletal, and cardiac muscle fibres."

The investigations of Hass (25–29), Endicott (30), and Hartroft (31, 32) have demonstrated that the histochemical properties of autoxidized, unsaturated fats and related compounds closely resemble those of ceroid. It is possible, therefore, that this pigment may be of a similar nature.

"Transitional" forms, differing in their coloration by oil red O in frozen sections and probably representing different stages in the formation of ceroid, have been described by Hartroft (31). The presence of large amounts of cod liver oil or other highly unsaturated fats, as well as deficiencies of choline and/or vitamin E in the diet favor hepatic deposition of ceroid (1, 9–14). Necrotic tissues (16) and blood cells (31, 32) may play a role in its formation.

The pathogenesis of ceroid remains to be fully elucidated. Assuming that the pigment is derived from autoxidized tissue lipids, theoretically, ceroid might be formed in the liver from unsaturated fats which have become pathologically accumulated there. The autoxidation might occur because of a relative or absolute lack of antioxidants. It might be catalyzed by tissue components to which the ceroid may adhere as it is formed. The degree of unsaturation of the tissue lipids may be influenced by the amounts of unsaturated fats provided by the diet. Abnormal accumulation of these lipids in the liver may be the result of toxic hepatic damage or a dietary deficiency of choline. Insufficient vitamin E in the diet may cause a decrease in the concentration of this important antioxidant in the tissues. Blood cells or products of necrosis may occur in the vicinity of the abnormally accumulated lipids as a result of other pathological changes in the liver. Most, if not all, of these factors theoretically favoring ceroid formation have been present in many of the experiments in which hepatic deposits of ceroid have been found.

Before the theory just stated concerning the formation of ceroid in the liver can be verified, the assumed identity of this pigment with the autoxidation products of tissue lipids must be proved. This problem has been approached through a comparative study of the histochemical properties of ceroid and the ceroid-like substances prepared *in vitro* from various unsaturated lipids. As a preliminary to that study, an investigation was made of the *in vitro* production of such substances and of some of the factors influencing their formation.

Methods of Preparation

The various procedures which were used while studying the *in vitro* production of ceroid-like substances and the factors influencing it are outlined below. The materials treated by each method and the results obtained are summarized in Table I.

Use of Tissue Blocks.—The formation of ceroid-like substances from various lipids in the presence of fresh tissues from adult Wistar rats was investigated by two procedures. In the first, after the animals had been sacrificed, approximately 5 mm. cubes of a variety of organs and tissues (see Table I) were excised and placed in small, stoppered tubes containing 3 to 5 ml. portions of the lipids tested. These mixtures were incubated at 37°C. for 10 days.

In the second procedure, each rat was sacrificed 20 minutes after being given an intraperitoneal injection of 1 ml. of heparin solution.[1] In order to perfuse most of the body tissues other than those of the hind limbs, the aorta was cannulated[2] as far caudad as possible and the inferior vena cava was severed at about the same level. Warm (35°C.) saline (0.85 per cent NaCl) was introduced through the cannula until the effluent from the cut vena cava was no longer colored by blood. The lipid to be tested was then passed through the tissues

[1] A saline solution of heparin containing 1000 units per ml. supplied by Connaught Medical Research Laboratories, Toronto, Ontario.

[2] Transflex tubing, size 24, obtained from Irvington Varnish and Insulator Company of Canada Ltd., Hamilton, Ontario.

in the same manner. Blocks of various perfused tissues and organs were incubated in portions of the lipid as described in the first procedure.

In all experiments, at the end of the incubation period, the tissue blocks were blotted on filter paper before fixation in Bouin's fluid for paraffin sections or in Baker's formal-calcium (43) for frozen ones.

Use of Blood Cells.—In the experiments with blood cells, saline-washed cells from heparinized blood of adult Wistar rats or adult mongrel dogs were used. Approximately 10 per cent suspensions of the packed cells were prepared using 1 ml. portions of various lipids. These mixtures were incubated in small, stoppered tubes except when α-tocopherol was added. In such cases, they were made up in small vaccine vials which were then evacuated and sealed. This effectively prevented the destruction of the antioxidant by atmospheric oxygen but, alone, did not inhibit the formation of ceroid-like substances. After being kept at 37° C. for as long as 10 days, during which time they were frequently shaken by hand, the mixtures were centrifuged, if necessary, and the supernatant lipid poured off. The residues of altered blood cells were washed four times with isopropyl alcohol and twice with xylene, smeared on albuminized slides, and fixed in formalin vapor before being tested histochemically. When paraffin sections were desired, however, the residue after extraction with the organic solvents was wrapped in lens paper,[3] and dehydrated and embedded in the routine manner.

Use of Tissue Derivatives.—Considering the activity of iron-containing compounds as catalysts for the autoxidation of fats (47), it was desired to compare the effects of erythrocytic stroma and hemoglobin upon the production of ceroid-like substances from various lipids. Because of Lee's (16) suggestion that necrotic tissue might play some role in ceroid formation, the effects of macerated liver were investigated.

Erythrocytic stroma was prepared by hemolyzing dog's blood with water, centrifuging the mixture, and repeatedly washing the small residue with distilled water until it no longer gave a positive benzidine reaction.

Hemoglobin was crystallized from a commercial preparation[4] according to the procedure of Marshall and Welker as described by Hawk, Oser, and Summerson (34).

Liver macerate was prepared by grinding the liver from an adult Wistar rat with a little saline in a mortar and incubating it at 37°C. for 3 hours to permit some autolysis. More saline was then added and the mixture filtered through four thicknesses of gauze. The filtrate was centrifuged and the residue repeatedly washed with saline.

Approximately 0.5 per cent suspensions of the packed stromal sediment or hemoglobin and 10 per cent suspensions of the liver macerate were prepared and treated in the same manner as the suspensions of blood cells outlined above.

Use of Emulsions.—Agar emulsions of various substances were prepared and oxidized according to the procedure employed by Endicott (30). Four per cent emulsions in 2 per cent agar were used. Test blocks of these were immersed in 5 per cent aqueous potassium dichromate and control blocks in distilled water. All were incubated at 37°C. for as long as 1 month. Paraffin sections were prepared from portions removed at weekly intervals.

Some of the lipids were tested as 10 per cent aqueous emulsions stabilized with 0.02 per cent mixed high molecular alkyl-dimethyl-benzyl ammonium chlorides.[5] These were incubated at 37°C. for as long as 1 month. Samples were removed at weekly intervals and extracted with isopropyl alcohol followed by xylene. Any insoluble residues were smeared on albuminized slides, fixed in formalin vapor, and tested histochemically.

Use of Inert Materials.—To investigate the formation of ceroid-like substances upon inert

[3] Obtained from H. Reeve Angel and Co., New York.

[4] Obtained from Difco Laboratories Inc., Detroit.

[5] "Zephiran chloride concentrate" obtained from Winthrop Chemical Co., Inc., Windsor, Ontario.

surfaces, small strips of filter paper,[6] fine glass capillaries, or 5 mm. cubes of agar[4] (2 per cent in water) or gelatin[4] (20 per cent in water) were immersed in portions of the lipids contained in small, stoppered tubes. These were incubated at 37°C. for up to 10 days and then extracted with isopropyl alcohol and xylene. Histochemical tests were applied to any residual material on the paper strips, glass, or agar or gelatin blocks.

Use of Lipids without Added Materials.—In many experiments, portions of the lipids tested were simply incubated in small, stoppered tubes. When α-tocopherol had been added, however, the mixtures were sealed in evacuated vaccine vials. For some tests, the lipids were painted on clean glass slides. As in the other procedures, most of the preparations were kept at 37°C. for as long as 10 days before extraction and subsequent histochemical testing. A few were left at room temperature (about 20°C.) or incubated at 45°C. or at 56°C.

Identification of Ceroid-Like Products.—The criteria which were used for the identification of ceroid-like products from experiments conducted according to the foregoing procedures were (*a*) insolubility in isopropyl alcohol and xylene, the solvents routinely employed in the preparation of paraffin sections in this laboratory, (*b*) sudanophilia persisting after extraction with these solvents, and (*c*) acid fastness. Controls, using some of the fresh test lipid, showed that extraction of the unaltered lipid from the experimental mixtures was complete every time. The procedures for demonstrating sudanophilia and acid-fastness are discussed in the section on histochemical methods below.

Observations on Production

Production of Ceroid-Like Substances.—As is shown in Table I, ceroid-like substances were obtained from unsaturated fats, fatty acids and their esters but from neither saturated ones nor hydrocarbons even after incubation for 6 weeks. The addition of tissues, blood cells, or their derivatives, or emulsification shortened the induction period for the appearance of ceroid-like substances from approximately 1 week to as little as 36 hours. In this regard, hemoglobin was more active than whole blood cells which, in turn, were more active than erythrocytic stroma. There were no marked differences between the activities of the various kinds of tissues and organs investigated. Ceroid-like substances were obtained sooner from the aqueous emulsions than from the plain lipids. In experiments in which no accelerating agent was present, the rate of appearance of ceroid-like substances was approximately proportional to the degree of unsaturation of the lipid being tested, to the amount of surface exposed to the air, and to the temperature of incubation.

The formation of ceroid-like substances from cod liver oil, rat liver lipids, linolenic acid or its methyl ester was effectively inhibited by the addition of an adequate amount of α-tocopherol or other antioxidant such as hydroquinone.

Sites of Formation of Ceroid-Like Substances.—Whenever blood cells, tissue blocks, pieces of filter paper, or other particulate matter was present in the mixtures, the ceroid-like substances appeared first as thin films on the surfaces in contact with the lipid and, in the cases of the perfused tissues or organs, also in the vascular spaces which had been filled with the lipid. The thickness of the films gradually increased as the incubation period was lengthened. In many

[6] Whatman No. 1 filter paper, supplied by W. and R. Balston, Ltd., England.

TABLE I

*Production of Ceroid-Like Substances in Vitro**

Substance tested	Tissues			Blood cells	Hemoglobin	Erythrocytic stroma	Emulsions		Inert surface	Pure substances
	Immersed‡	Perfused‡	Macerated§				Agar	Water		
Hydrogenated cottonseed oil‖	0			0	0	0	0	0	0	0
Paraffin oil	0			0	0		0		0	0
Stearic acid¶	0			0	0		0		0	0
Palmitic acid¶	0			0	0		0		0	0
Cottonseed oil	+			+	+	+	+	+	+	+
Cod liver oil	+	+	+	+	+	+	+	+	+	+
Corn oil		+	+	+			+		+	+
Linseed oil	+		+	+	+		+		+	+
Olive oil			+	+			+		+	+
Rat liver lipids**	+	+	+	+	+		+	+	+	+
Oleic acid¶			+	+			+		+	+
Linoleic acid¶			+	+			+	+	+	+
Linolenic acid¶	+		+	+	+	+	+	+	+	+
Methyl oleate¶	+		+	+			+		+	+
Methyl linoleate¶	+		+	+			+		+	+
Methyl linolenate¶	+	+	+	+	+	+	+	+	+	+

* Symbols: 0 = no ceroid-like substance was formed; + = ceroid-like substance was obtained.

‡ The following tissues were investigated: brain, heart, intestine, kidney, liver, lung, pancreas, skeletal muscle, spleen, testis.

§ Only liver was studied as a macerate.

‖ Specially prepared by Mr. H. W. Lemon, Ontario Research Foundation, Toronto, Ontario. Iodine value = 10.5.

¶ Obtained from Hormel Foundation, Austin, Minnesota.

** Kindly supplied by Drs. C. C. Lucas, J. H. Ridout, and J. M. Patterson of the Banting and Best Department of Medical Research, University of Toronto. Prepared by extracting rats' livers with acetone followed by hot ethanol and subsequent removal of the solvents by distillation *in vacuo*.

instances, the ceroid-like substances appeared to form also as rims within the cells and, sometimes, to fill them completely. As had been observed by Endicott (30), in the emulsions, ceroid-like material initially appeared as thin rims at the interfaces between the lipid droplets and the dispersing medium. Here, too, with the passage of time, the rims gradually thickened until apparently solid globules were formed.

Histochemical Methods

A variety of staining reactions and histochemical tests were applied to some of the experimental products and to the ceroid in sections of cirrhotic livers from choline-deficient rats. The methods which were employed are outlined below. The results are summarized in Table II in which they are compared with those reported by Elftman, Kaunitz, and Slanetz (40) for the "uterine pigment" in vitamin E-deficient rats.

Lipid and Lipoid Methods.—The sudanophilia of the experimental products and ceroid, and the presence of any free lipids in the extraction controls were demonstrated by staining with one of the fat-soluble dyes. Very satisfactory results were obtained with oil red O in the trichrome method described by Wilson (44), and with sudan black B in either Baker's (43) or Sheehan and Storey's (45) procedure. The method of Glavind, Granados, Hartmann, and Dam (46) was used to test for fat peroxides. Cholesterol and acetal phosphatides were sought for by the Shultz (35) and Feulgen plasmal (48) reactions respectively. Baker's acid hematein test (57, 58) was employed for the detection of phospholipids.

Protein Methods.—Some of the tests described by Serra (49) were employed to demonstrate the presence of chemical groupings suggestive of proteins. These included the Millon, Voisenet, biuret, nitroprusside, and xanthoproteic reactions.

Carbohydrate Methods.—The only two tests for carbohydrates and related substances which were applied were McManus' periodic acid–Schiff's reaction (50), and the toluidine blue method for metachromatism (35).

Iron and Hemoglobin Methods.—Iron was tested for by Gomori's variant of Perls' method (52) and by the Tirmann-Schmelzer reaction (35). These were applied either directly or after the materials to be tested had been treated with 2.5 per cent nitric acid in 95 per cent ethanol for as long as 48 hours at 37°C. Hemoglobin was detected by the Lepehne-Pickworth method (35).

Oxidation Methods.—The oxidation of the experimental ceroid-like substances and ceroid was studied with the aid of the diammine silver carbonate method of Foot and Menard (55), with alkaline potassium permanganate (0.33 per cent in 0.1 per cent KOH), and with McManus' periodic acid–Schiff's reaction referred to above.

Dissociation Methods.—Affinity for basic dyes, basophilia, was demonstrated most often by staining with the modified Hucker-Conn ammonium oxalate crystal violet solution described by Lillie (35). In addition, simple 0.1 per cent aqueous solutions of basic fuchsin (C.I. No. 677), crystal violet (C.I. No. 681), methyl green (C.I. No. 684), and safranin 0 (C.I. No. 841) were sometimes employed. To test for acidophilia, similar solutions of tartrazine (C.I. No. 640), eosin B (C.I. No. 771), and naphthol green B (C.I. No. 5) were used.

Acid-Fast Method.—Acid fastness was demonstrated by Lillie's carbolfuchsin–methylene blue method (35).

Miscellaneous Methods.—Other procedures which were applied to the experimental products and ceroid included Mallory's method for hemofuscin (53) and György's pyronin–methyl green stain (10).

Histochemical Observations

Lipid and Lipoid Reactions.—Intense coloration by fat-soluble dyes, even after treatment with organic solvents, is one of the outstanding characteristics of ceroid. This property was one of the criteria for the identification of ceroid-like substances among the experimental products. Controlled, preliminary ex-

TABLE II

*Histochemical Reactions of Experimental Ceroid-Like Substances Compared with Those of Ceroid and "Uterine Pigment"**

Histochemical test	Products from‡		Ceroid	Uterine pigment§
	CLO and RLL	LlA and MLl		
Sudanophilia	+	+	+	+
Cholesterol	0	0	0	0
Acetalphosphatides	0	0	0	0
Fat peroxides	+	+	±	—
Phospholipids	0	0	0	0
Xanthoproteic	‖	‖	‖	‖
Biuret	¶	¶	0	0
Millon	¶	¶	0	0
Voisenet	¶	¶	0	0
Sodium nitroprusside	¶	¶	0	0
Periodic acid–Schiff's reaction before and after amylase	+	+	+	+
Metachromatism	0	0	0	—
Diammine silver carbonate	+	+	+	+
Alkaline permanganate	+	+	+	+
Iron	¶	¶	0	0
Hemoglobin	¶	¶	0	—
Basophilia	+	+	+	+
Pyronin–methyl green	+	+	+	—
Hemofuscin	+	+	+	—
Acid-fast	+	+	+	+

* Symbols: + = positive reaction; 0 = negative reaction; ± = usually positive, occasionally negative reaction; — = not reported.

‡ CLO = cod liver oil; RLL = rat liver lipids; LlA = linolenic acid; MLl = methyl linolenate.

§ From Elftman, Kaunitz, and Slanetz (40).

‖ Yellow coloration by nitric acid not interpretable as a positive xanthoproteic reaction (see text).

¶ Positive reactions observed in (*a*) smears of some mixtures which contained blood cells or liver macerate and which had been incubated only 1 or 2 days, and (*b*) cells or tissues embedded in ceroid-like material, revealed in paraffin sections of such mixtures.

traction with fat solvents eliminated the possibility of false positive reactions due to retained, unaltered lipids. Fatty peroxides were demonstrable in the experimental products and some samples of ceroid. Negative reactions were obtained in all tests for cholesterol, acetal phosphatides, and phospholipids.

Protein Reactions.—The golden yellow color of the experimental products

and ceroid became somewhat deeper when they were treated with concentrated nitric acid. Considering that this change occurred even with the product from pure linolenic acid alone, it cannot be interpreted as a positive xanthoproteic reaction under these conditions. This conclusion is further supported by the observation that, in treated tissue sections, the color of the cells was a more intense and somewhat different shade of yellow.

Early tests with the Millon, Voisenet, biuret and nitroprusside reactions were uniformly negative except for a few smears of mixtures which contained blood cells or liver macerate and which had been incubated for only 1 or 2 days. Similar observations were made regarding the coloration of the blood cells or liver tissue by the light green in Wilson's trichrome method (see Figs. 1 to 3), and in the tests for iron and hemoglobin on mixtures containing blood cells. These findings suggested investigating the apparently negatively reacting blood cell- or tissue-containing mixtures by treating thin (4 micra) paraffin sections of them. As is shown in Figs. 1 to 3, when this was done, it was found that the cells or tissues embedded in the ceroid-like material still retained their native properties. They gave appropriately positive reactions with the protein tests and with tests for iron and hemoglobin, and were intensely stained by light green.

Carbohydrate Reactions.—Ceroid and the experimental products were colored a bright red by the periodic acid–Schiff's method, even after treatment with amylase which effectively removed the glycogen from the cells in sections of liver. They were not stained metachromatically by toluidine blue.

Iron and Hemoglobin Reactions.—Negative reactions for ferrous and ferric iron and for hemoglobin were obtained with ceroid and with most of the experimental products. The only exceptions among the latter, as mentioned in connection with the protein reactions, occurred with some smears of blood cell or liver macerate–containing mixtures which had been incubated only a few days and with paraffin sections of all such mixtures. In these, the Perls' reactions were positive after treatment with nitric acid in ethanol. Usually, the presence of hemoglobin could be readily demonstrated.

Oxidation Reactions.—Reduction of the diammine silver carbonate reagent by the experimental products was moderately evident after 30 minutes at 37° C., but was more marked after 24 hours at room temperature. Reduction by ceroid was just evident after 24 hours at room temperature. Both ceroid and the experimental products reduced the alkaline potassium permanganate solution, the former within 1 hour, the latter, usually within 30 minutes, at 10° C. As mentioned in the section regarding carbohydrate reactions, both ceroid and the ceroid-like substances were colored a bright red by the periodic acid–Schiff's reaction.

Dissociation Reactions.—Ceroid and products of the *in vitro* experiments were distinctly basophilic but showed no acidophilic properties whatever.

Acid-Fast Reaction.—Acid fastness is one of the distinctive properties of

ceroid. It was used as the other histochemical criterion for the identification of experimentally prepared ceroid-like substances.

Miscellaneous Reactions.—Ceroid and the experimental products were intensely colored by pyronin–methyl green and gave positive reactions when stained by Mallory's method for demonstrating hemofuscin.

DISCUSSION

The histochemical observations summarized in Table II show that all the reactions given by ceroid are similarly given by the lipoid materials experimentally produced *in vitro* by the autoxidation of unsaturated fats, fatty acids or their esters in the presence of various tissue elements and, with the exception of the occasionally observed positive test for iron, also by those produced in the absence of the tissue elements.

The reactions given by ceroid and the ceroid-like substances provide some insight into the probable nature of ceroid. Sudanophilia exhibited by material which is insoluble in fat solvents suggests that it may be constituted by lipoids which are highly polymerized, extensively substituted, or present as protein complexes. As far as the experimental products prepared solely from pure, straight-chain fatty acids are concerned, only polymerization is possible. This implies autoxidation which is also evidenced by the positive reactions for fatty peroxides. As was pointed out by Edwards and White (5), acid fastness does not imply that the lipoidal material is related to the wax responsible for this property of the tubercle bacillus, because ceroid is not soluble in chloroform whereas the wax is (56).

The possibility that in ceroid, the lipoids are closely bound to protein is important. The suggestion that the insolubility of ceroid in fat solvents is indicative of its being lipoprotein in nature is not convincing. Such insolubility of the experimental products from pure fatty acids indicates that ceroid need not contain or be linked to protein. Negative protein, iron, and hemoglobin reactions were to be expected for the products from simple fats and free fatty acids to which no cells or tissues had been added. On the other hand, such reactions are particularly interesting and significant in those preparations containing cells or tissues. In such instances, negative reactions suggest either that lipoid-protein or other complexes had been formed, completely masking the normal chemical reactivity of the protein- or iron-containing moiety; or that the polymerized lipids so effectively enveloped the cells or tissues that the reagents could not gain access to them. The latter suggestion is supported by the many observations that the ceroid-like material tends initially to coat foreign objects such as tissues and blood cells. It gains further support from the fact that, in paraffin sections, the enveloped cells or tissues could be shown to possess their normal reactivity. These observations emphasize that the negative reactions obtained when the tests were applied to ceroid do not justify asserting that

protein, hemoglobin, or iron is not present. They do provide a reasonable explanation of why iron has sometimes been found in ceroid—most likely, some blood cells were entrapped in the abnormally accumulated lipids and later were incompletely masked by the ceroid. The fate of the cytoplasm of those cells within which ceroid-like substances appear to form remains to be elucidated. There may be simple replacement by, or actual chemical combination with lipid. In the latter event, lipid-protein complexes would probably be formed.

The negative carbohydrate tests rule out the probability of glycogen or mucopolysaccharide esters being constituents of ceroid.

As was pointed out by König (41), the use of reduction reactions to characterize pigments is none too reliable and is controversial at best. In these experiments, the observations regarding the reduction of diammine silver carbonate by ceroid and the ceroid-like substances more closely resemble those of Elftman, Kaunitz, and Slanetz (40) for the uterine pigment in vitamin E-deficient rats than do observations of Endicott and Lillie (33) who reported that reduction by ceroid required from 48 to 96 hours. Apart from further demonstrating the reducing power of ceroid and the experimental products, reduction of alkaline potassium permanganate has little specific significance. Oxidation by periodic acid and subsequent reaction with Schiff's reagent are indicative of the presence of reactive amino, hydroxyl, or keto groups on adjacent carbon atoms. Positive results would be expected for the products from unsaturated fatty acids after at least initial peroxidation. Because the presence of polysaccharide esters and glycogen could not be demonstrated, the positive periodic acid-Schiff's reactions given by ceroid are most likely due to lipoids in it.

The other staining reactions exhibited by ceroid such as basophilia, coloration by pyronin-methyl green, and a positive hemofuscin reaction were also given by the experimental products. These results suggest that such reactions when given by ceroid may be attributable to the presence of autoxidized lipids.

These histochemical studies thus provide highly suggestive evidence of ceroid being constituted of the autoxidation products of unsaturated tissue lipids. That fats may be transformed into lipoid pigments has been demonstrated by several investigators:—

Pinkerton (54), Graef (55), and Endicott (30) have described the conversion of cod liver oil into amorphous, semisolid material in the pulmonic air passages of patients with lipoid pneumonia due to the aspiration of the cod liver oil. Hass (25–29) focussed attention upon the peroxidation and subsequent polymerization of unsaturated lipids in tissues. Endicott (30) suggested that such a mechanism was responsible for ceroid formation. He demonstrated that various unsaturated fats could be converted into ceroid-like substances *in vivo* and *in vitro*. Hartroft (31, 32) has shown that within 3 weeks after fat has been traumatically liberated from adipose cells in the presence of blood cells, ceroid-like material can be demonstrated at the site. He has also demonstrated the formation of ceroid-like substances *in vitro* from

unsaturated lipids in the presence of blood cells. Dam and Granados (37), amongst others, have suggested autoxidation as the mechanism for the formation of the pigment which appears in the adipose tissue of vitamin E-deficient rats and were able to demonstrate peroxides in it.

The experimental observations reported here, by extending the *in vitro* studies of Endicott (30) and Hartroft (31, 32), further emphasize the probable importance of unsaturated fats and autoxidation in ceroid formation. They provide a reasonable explanation for the apparently conflicting reports concerning the demonstration of iron in ceroid. They suggest that the role of blood cells and necrotic tissues may be a dual one of a catalyst and a surface upon which the ceroid may be deposited. The retardation of ceroid formation by vitamin E (11, 13, 14) appears to be related to its antioxidant properties *in vivo* and *in vitro*. The importance of dietary unsaturated fatty acids in the formation of ceroid is implied by the fact that ceroid-like substances could be prepared only from such compounds. All available evidence from these investigations into the *in vitro* production and properties of ceroid-like substances, therefore, supports the theory that ceroid may be the product of the autoxidation of unsaturated lipids which have become pathologically accumulated in tissues with a resultant relative, if not concurrent absolute, deficiency of biological antioxidants. In addition, the fundamental similarity between ceroid and the lipofuscin pigment associated with vitamin E deficiency which was anticipated by Elftman, Kaunitz, and Slanetz (40) is emphasized by these studies.

SUMMARY

Substances possessing the same histochemical properties as the ceroid in cirrhotic livers of rats fed choline-deficient diets have been prepared from various unsaturated fats, fatty acids and their esters by autoxidation but could not be obtained from hydrocarbons, or saturated fats or fatty acids.

The formation of ceroid-like substances occurred first on surfaces or at interfaces in the reaction mixtures. It was inhibited by antioxidants and was accelerated by the addition of tissues, blood cells, erythrocytic stroma, or hemoglobin, by emulsification, by increasing the surface exposed to the air, and by increasing the temperature.

Histochemical studies provided much evidence that the following properties of ceroid might be attributed to the products of the autoxidation of unsaturated lipids: insolubility in organic solvents, sudanophilia, yellowing by concentrated nitric acid, positive periodic acid–Schiff's reaction, basophilia, acid fastness, positive hemofuscin reaction, and reduction of diammine silver carbonate and alkaline potassium permanganate.

The normal reactivity of cells or tissues embedded in ceroid was effectively masked by the pigment, apparently, initially at least, by preventing the reagents' gaining access to them. It is suggested that the iron sometimes demon-

strated in ceroid may be that of blood cells or tissue fragments incompletely masked by the ceroid.

It is concluded that whenever conditions are such that unsaturated fats accumulate in tissues to such an extent that a relative lack of biological antioxidants results, autoxidation of the fats and their conversion to ceroid pigment are favored, and that ceroid and the lipofuscin pigment of vitamin E deficiency may be fundamentally similar.

It is a pleasure to acknowledge the inspiration and guidance of Professor C. H. Best, and the many valuable suggestions and kindly criticisms of Dr. W. S. Hartroft throughout this investigation. The histochemical studies were made with the technical assistance of Mr. W. Wilson and Mr. O. George.

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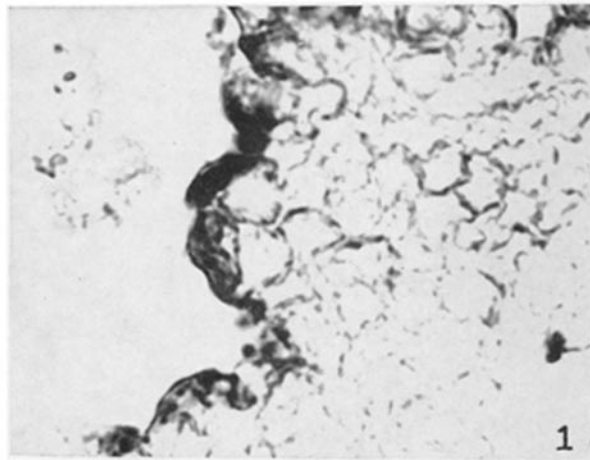
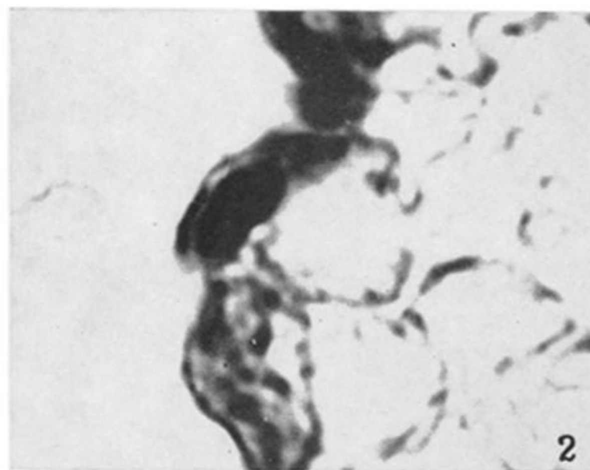
EXPLANATION OF PLATE 39

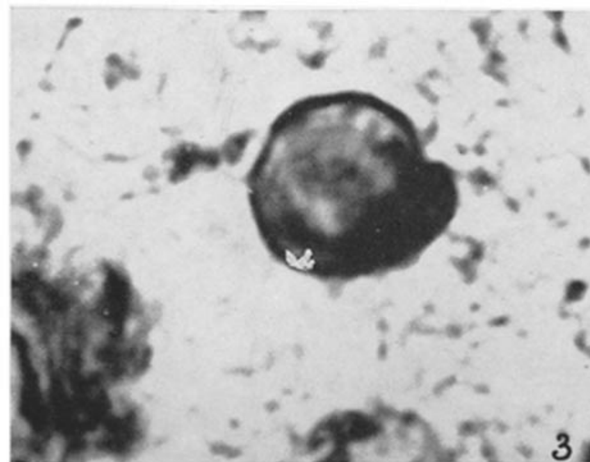
The photomicrographs were taken by Mr. W. Wilson.

FIGS. 1 to 3. Rat's blood cells were incubated with linolenic acid for 10 days at 37° C. before extraction with isopropyl alcohol and xylene. In smears of the mixture, the erythrocytes could not be stained. In paraffin sections, however, ceroid-like substance (black) was found to surround the cells or cell clumps completely (Fig. 1). In some instances, particularly after longer incubation, ceroid-like substance appeared within the cells, partially (Fig. 2) or almost completely (Fig. 3) filling them.

(Paraffin sections (4 micra) stained with oil red O, hematoxylin and light green; G and H filters; oil immersion; Fig. 1, × 1100; Figs. 2 and 3, × 2500 approximately.)







(Casselman: Substances resembling ceroid)