THE HISTOLOGICAL DEMONSTRATION OF LIPOIDS

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In connection with work on the interstitial cells of the ovary and the cortex of the suprarenal gland, I had occasion to carry out some experiments on the technique of fat demonstration and preservation and, as they supplement largely the careful observations of Bell¹ published in this Journal, it occurred to me that the results although not extensive or peculiarly original might nevertheless be of interest to other workers, who might in turn offer suggestions from their experience. The satisfactory preservation of lipoids is difficult for obvious reasons and is a neglected side of cytological work that will soon require more attention if we may judge by the suggestions of their importance that have been made in the field of cell physiology (lecithin in relation to nuclein synthesis, and *vice versa*; the form in which lipoids exist in the cytoplasm, masked fat, etc.; Overton's and Loeb's work). The usual cell picture generally neglects them and is clearly inadequate. The histological technique of lipoids is rendered more difficult by the fact that they exist in intimate mixtures and the probability might be suggested that the more intimate the 'mixture' the greater their importance in protoplasm.

The fat globules of the suprarenal and ovary are unusually labile as compared with 'body fats' (adipose tissue), as is of course well recognized,—this aside apparently from the difference in size of the globules. The nature of the suprarenal fat does not seem to have been sufficiently determined. Doubly refracting 'myelin' globules are present but the chemical composition of these is in dispute. It has been analyzed recently (*e.g.*, by White²) as cholesterin and oleic acid in physical or chemical combination. In the interstitial cells of the ovary no analysis has as far as I know been made. I have so far failed to secure evidence of the existence of the double refraction indicative of myelin (cholesterin and oleic acid); in all probability the granules are largely oleic acid. Doubtless many of the intracellular fat globules are of this character and cer-

¹ Bell, E. T.: The Anat. Record, vol. 4, no. 5, 1910.

² White, Chas. P.: The Journ. Pathol. and Bacteriol. vol. 13, pp. 11-13. 1908.

tainly much of the fat appearing under pathological conditions (*vide* Adami; principles of pathology).

Bell has pointed out that the group of selective fat dyes which owe their staining value to their solubility in fats,—Sudan III, Scharlach R., Indophenol, etc., give the most definite and delicate reaction for fat. He finds Herxheimer's alkaline (alcoholic) stain most efficient. The necessity of using the freezing microtome and mounting in glycerin or glycerin jelly renders them less serviceable for use in cases in which it is desirable to study the cell structure as well or employ serial sectioning. Personally I have employed them merely as a control,—counter-check to other methods. Since they color indiscriminately all free lipoids, including lecithin, cholesterin, and myeline, they afford no analysis of the lipoids present.

Osmic acid (osmium tetroxid). This well known reagent for the demonstration of fat usually requires little precaution to ensure satisfactory results;—the employment of thin pieces of tissue (1 mm.), the avoidance of absolute alcohol, the use of thick cedarwood oil or chloroform as a clearer before paraffin imbedding. With easily soluble fats such as those in the suprarenal and ovary, however, it is necessary to employ further precautions. I found carbol-xylene (1:3) or chloroform as a clearer before the paraffin gave best results but there was nevertheless usually more or less solution of the 'osmicated' fat particularly in the less superficial tissue, and it was different in different specimens. The reason for this difference is not quite clear. It is not due to absolute lack of penetration since the fat was blackened, though it seems to be associated with a less perfect oxidation of the fat. Duration of fixation or period of time in the alcohols had no appreciable effect. Celloidin, however, was found to be the best imbedding material and by employing 95% alcohol instead of absolute alcohol in making up the solutions, the blackened fat granules may be retained quite perfectly. It was found necessary however to mount without a coverglass as with chrome-silver impregnation preparations or employ very thick balsam. After the solvent of the balsam has largely dried out, it may be melted by heat and a coverglass added. Safranin was employed as a stain which will retain its brilliancy for sometime without a coverglass.

Tests carried out at my suggestion in the laboratory indicate that this is the best technique for permanency in the application of the Marchi method, depending as it does on the preservation (in situ) of osmicated fat granules (probably oleic acid?).

Chloroform or benzene balsam had no advantages as a mounting medium as compared with xylene balsam or natural balsam.

By the employment of celloidin and observing the above mentioned precautions in mounting, even small granules of fat that blacken with osmic acid may be preserved.

Altmann³ and Starcke⁴ have shown that it is only the unsaturated fats and fatty acids (such as oleic acid) which are able to reduce the osmium tetroxid, upon which reduction the staining reaction depends. Starcke added the significant point that in the case of the saturated fats (*e. g.* palmatin and stearin) blackening subsequently appears if the tissue is immersed in alcohol. A satisfactory explanation of this has not been vouchsafed. The two groups may of course be distinguished by making examination by the freezing microtome or otherwise without submitting the tissue to the action of alcohol and the presence of unsaturated fats determined (as in case the of the ovary and suprarenal). Since the reaction depends on the presence of a reduction of the osmium tetroxid it must of course be borne in mind that there are other reducing substances in the body in addition to the unsaturated fats, and R. Heidenhain's dictum that "all that blackens is not fat" be given due consideration.

That cytoplasm usually blackens to a certain degree is a matter of general observation. This is due to the presence of reducing substance in the protoplasm, whether masked lipid cannot be said at present.

The reducing power of fatty-acid-containing complexes such as 'myelin' may be markedly different from that of the fatty acid alone. Thus it is a common laboratory observation that myelin blackens readily and intensely with osmic acid used alone or as in Hermann's fluid but when combined with other oxidizing substances such as chromic acid (as in Flemming's fluid) the myelin blackens little or not at all. The histological analysis of intracellular fats may often be facilitated in this way; thus it becomes doubtful if Smith and Mair (*vid. seq.*) are correct in their argument that the oleic acid in myelin is in the form of loose combination with the cholesterin since (granting the correctness of the determination) the cholesterin oleic acid combination in the suprarenal cortex stains readily with osmium tetroxid in the presence of chromic acid. If not chemical, at least the physical conditions of the combination are markedly different. Fixation in Flemming's fluid for differentiating free fat from myelin has been used instead of the Marchi method with marked success in the case of small brains and peripheral nerves (*e.g.*, Myers⁵).

³ Altmann, R.: *Die Elementarorganismen*. Leipzig, 1894.

⁴ Starcke, J.: *Arch. f. Physiol.* 1895.

⁵ Myers, B. D.: *Zeitschr. f. Morphol. and Anthropol.* Bd. 3, pp. 183-207, 1900.

The Weigert method. The use of the Weigert hematoxylin method for other purposes than the staining of myelinic sheaths has already been described by Schiefferdecker who employed it as a stain for skin, lymphatic tissue and blood; by Harvey⁶ as a stain for zymogen granules, etc. It has also been used for the specific staining of fats and fatty acids (Fischler,⁷ Benda⁸).

Smith and Mair⁹ have recently published experiments on the chemistry of the Weigert method of staining medullated nerves which are of considerable interest to the histologist. The conclusion reached by them was that the mordantage by means of the dichromate on which the stain depends was determined by the ability of the myelin to reduce the potassium dichromate to the trioxid (?) held in combination in the myelin. The unsaturated fatty acids and their fats (*e.g.*, oleic acid and olein) were able to reduce the dichromates and stained in the hematoxylin; the saturated fats and fatty acids (*e.g.*, stearin and palmatin) gave negative results as did also pure cholesterin. Impure and combined cholesterin, however gave positive results even if combined with a saturated fat. Commercial lecithin (Kahlbaum's) stained, but purification rendered it less easily stained. The authors are inclined therefore to believe that pure lecithin like cholesterin would give negative results; that it is not the lecithin in myelin but a cholesterin-oleic acid combination which reduces the dichromate. While keeping in mind the questionableness of the last conclusion and the possibility that physical factors (fluidity, etc.) may be of fundamental importance in the reaction, there would seem to be no doubt as to the correctness of the main result,—that the Wiegert hematoxylin method rests upon the same basis as the osmic acid method,—namely, the presence of reducing substances. Alcohols were also found by them to stain. It would have been an interesting point to have ascertained whether as is the case with osmic acid according to Stareke's results the saturated fats might not have stained after treatment with ethyl alcohol.

A second study of the microchemical and staining reactions of lipoids of importance to the morphologist is one by Faure-Fremiet, Mayer and Schaeffer.¹⁰ In this they not only present many chemical facts in explana-

⁶ Harvey, B. C. H.: *Amer. Journ. of Anat.* vol. 6, pp. 207-243. 1906.

⁷ Fischler: *Centralbl. Allg. Pathol.* Bd. 15, 1904.

⁸ Benda: *Arch. f. pathol. Anat.*, Bd. 161, 1900.

⁹ Smith, J. L., Mair, W., and Thorpe, J. F.: *The Journ. Pathol. and Bacteriol.* vol. 13, pp. 14-27, 1908-1909.

¹⁰ Fauré-Fremiet, E., Mayer, A., and Schaeffer G. *Arch. d'Anat. Microscopique.* vol. 12, pp. 19-102, 1910.

tion of the fixation and staining of fats, myelin, etc., but deal as well with the chemical nature of the mitochondria, and offer a suggestion as to the basis of the chrome-silver reaction. Their main results are gathered together in the form of a table (7, p. 80). Their work supports that of Smith and Mair but is more comprehensive in its scope, not being limited to the Weigert hematoxylin method. They seem to find however that osmic and chromic acid oxidize the saturated fats, but with difficulty.

I found that the Weigert method gave very useful results with the lipid containing cells of the ovary and suprarenal. The procedure that was found most satisfactory was to fix two days in Zenker's fluid following it up by a mordantage in Muller's fluid for 10-14 days at 38°C (in an incubator). A longer stay in dichromate solutions in the cold did not seem as satisfactory even though prolonged (one month or more). The paraffin imbedding method was employed, iodine in the alcohol being used to remove any precipitate that might be present. The sections were stained on the slide by the 1885 Weigert hematoxylin method which seemed preferable to the Pal-Weigert method which was also tried. The preliminary fixation by means of the Zenker's fluid prevented the partial solution of the chromatin and loss of nuclear structure which the potassium dichromate would otherwise have caused. The combination of the dichromate with mercuric chlorid and acetic acid was found to give better results than the mixture of potassium dichromate and formalin (Orth's fluid) or a mixture of dichromate, mercuric chlorid and formaldehyde such as Helly's fluid, a small amount of acid seeming to facilitate the mordantage and sharpen the differential staining. Usually the amount of acetic acid in the Zenker's fluid was reduced to 1 percent. In the differentiation by means of the potassium ferricyanide and borax bleacher, the solution was diluted 3-4 times and permitted to act more slowly. Iron hematoxylin methods were used as well as copper hematoxylin methods but did not give, it seemed to me, as sharp a differentiation.

Peripheral myelinic nerve fibers, the fat containing cells and erythrocytes were sharply differentiated. The lutein cells of corpora lutea are strongly stained. Mitochondria are preserved and intensely stained, though not quite as well apparently as by the Benda method or Meves modification. In the case of the four first mentioned, the selective stain is undoubtedly associated with the presence of the lipid, and since the evidence is fairly strong that the so-called mitochondria or chondriosomes possess a lipid element in their composition (10), their preservation and staining may be explained on the same basis. The above technique is, in fact, essentially mitochondrial, and by using a minimal

amount of acetic acid ($\frac{1}{2}$ –1 per cent) gives usually good results. The Benda method of fixation, while it affords a sharper and a stronger stain of the mitochondria, gives generally inferior results with the cells containing free fat, the treatment with the pyroligneous acid being apparently largely responsible. Benda's fluid (modification of Flemming's fluid) employed by itself gives an excellent osmic acid stain of free fat and (after bleaching with hydrogen peroxid) admits good staining of the mitochondria. The copper hematoxylin gives a richer and more differential stain for mitochondria than does the iron hematoxylin more frequently employed for this purpose.

The intracellular globules of free lipoid themselves were not individually preserved by this technique and the intense staining was peripheral, either marking out a distinct surface layer of the globule or staining the interstitial cytoplasm often revealing an apparent granular structure (mitochondria?). The interpretations of this might be (a) that the reduction upon which the staining reaction depends took place only at the surface of the globule and the bulk of the globule being subsequently dissolved out. One need but recall the surface staining of fat globules with osmic acid and the 'Ringkörper' of Altmann. (b) That the reaction took place in a surface layer of 'formative substance' in which the fat was possibly held in combination (masked form) as in the mitochondria. (c) That the lipoid from the globule in the process of fixation had permeated the surrounding cytoplasm.

For the differential staining of the individual fat granules and globules by the Weigert method, it is apparently necessary to resort to the use of the freezing microtome as Benda and Fischler have done who have stained the fatty acid by means of the Weigert method and the neutral fat with Sudan III. It is possible however that further experimentation will determine a mordantage that followed by celloidin imbedding will permit of the preservation and staining of the individual fat granule. Despite the existing imperfection, however, the method was found to be very useful in the case of the ovary and suprarenal gland.

It might be mentioned that by fixing small heads, such as those of many teleosts, for three or four days in Zenker's fluid and following this by a dichromate mordantage in Muller's fluid of two or three weeks, (shortened by heat if preferred), an excellent Weigert hematoxylin stain of the peripheral nerves may be secured, the acetic acid and dichromate together affording a sufficient decalcification without marked injury of the myelinic sheath. Such material is not as brittle as that secured by long fixation in Flemming's fluid.