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An Improved Method of Staining Lipides: Acetic - Carbol - Sudan.

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INTRODUCTION.

THE literature on the microscopical demonstration of fats and fat-like substances is largely a record of dissatisfaction with previous methods. It is noteworthy how many authors, investigating the lipides of normal or pathological tissues, have felt impelled to turn their attention to improvement of technique. Some of these have gone further and have become absorbed in the problem of fat-staining for its own sake and apart from the difficulties they originally encountered. The technique of Sudan staining is essentially a physico-chemical problem and histologists or pathologists venturing into this realm have sometimes had to run the gauntlet of expert criticism. This danger may act as a deterrent to investigation; but it is comforting to reflect that a successful technique will stand up to a lot of argument about its rationale: a good method will remain, irrespective of the ability of its author to defend or explain the theory underlying it.

Many attempts have been made, either to modify the methods of using existing dyes or to introduce new dyes in the hope of securing improvement in fat staining. Thus we have seen " Sudan III ", " Scharlach R ", and " Sudan IV " successively become the favourite or at least the most highly recommended substances; and there are a number of technical variations depending on the solvents used or on the procedure of making the dilutions from stock solutions.

In spite of all this, it was probably only the Romeis method (to be discussed later) which introduced a radical or qualitative improvement, i.e., actually allowed the staining of lipides which by other Sudan methods remained unstained. For the rest, the modifications have chiefly accomplished improvements in the quantity of fats demonstrated or more especially in the colouration obtained.

Bullard (1916) has justly emphasised that " in the demonstration of fats in tissue sections, methods and technique are of the utmost importance. The wide divergences of opinion among different investigators concerning the occurrence of fat within the cells of normal and pathological tissues is due primarily to differences of technique in preparing the sections for examination." How unsatisfactory the present position is, and how impossible to compare the results of different investigators, becomes apparent when one reflects that the Romeis methods, which beyond doubt give a more complete demonstration of lipides, are scarcely found in histological laboratories.

They have probably been found troublesome and uncertain; and so easier methods, more uniform in their results but unquestionably inferior as micro-chemical tests, continue to be used.

My own interest in Sudan technique arose from the discovery of the existence (at least under pathological conditions) of lipide substances which were unstainable by any known modification of Sudan III, Scharlach R or Sudan IV technique, with the partial exception of the Romeis methods. The latter gave inconstant and usually incomplete results, and as is well known, they are in other ways unsatisfactory. A technique devised to stain these refractory substances proved (as might have been hoped) a highly satisfactory stain for lipides in general. As it arose from pre-occupation with the theory of Sudan staining and from successive attempts to modify the Romeis methods, these aspects will be considered next.

Remarks on the Theory and Practice of Sudan Staining, with Special Reference to the Romeis Methods.

In 1927 Romeis published a technique of staining frozen sections with weakly alcoholic solutions of Sudan III, whereby he claimed that the solvent action of the stronger alcoholic solutions in customary use was avoided and, therefore, a more complete demonstration of fats obtained. Froeboese and Spröhnle (1928) found that the method, on the whole, gave poor results: precipitates were troublesome and in most cases the staining was no more complete than with the ordinary methods. These authors concluded that whatever virtue there was in Romeis's 40 per cent alcoholic solution lay not in its superior preservative power in respect of easily soluble lipides, but in its enhanced staining power: it was not that the reduced alcohol content avoided dissolving the fat, but that the increased water content conferred colloidal properties on the solution. Thus a controversy arose over the theory of Sudan staining which, in my opinion and contrary to the prevailing impression, has never been satisfactorily cleared up.

The following year Kaufmann and Lehmann (1929), by treating tissues with 70 per cent. alcohol and thereafter determining *chemically* their lipide content, showed beyond doubt that alcohol of this strength does dissolve out fatty substances to an apparently alarming extent. Among the more relevant data obtained was that in 70 per cent. alcohol for 20 minutes at room temperature (corresponding to the usual method of staining with Sudan) 38·8 per cent. of phospholipins and 25·5 per cent. of fatty acids may be dissolved out; as compared with the figures 8 per cent. and 1·5 per cent. respectively in the case of 40 per cent. alcohol at room temperature for 24 hours (corresponding to the method recommended by Romeis). These results have been generally accepted as a vindication of Romeis's explanation of the theory underlying his method and as an effective reply to the contentions of Froeboese and Spröhnle. However, while accepting the results of Kaufmann and Lehmann, I doubt whether they have any great significance for the histologist. The substances dissolved by 70 per cent. alcohol may well be the " masked " lipides—highly dispersed fatty substances which are of ultra-microscopic dimensions and so, even if preserved, are not demonstrable by any staining technique. The reasons for this belief will be returned to later. It is not altogether correct to state, as do Kay and Whitehead (1934), that Kaufmann and Lehmann (1929) " demonstrated that the chief merit of the Romeis technique was the fat preserving property of 40 per cent. alcohol ". This is widely believed, but in fact these authors, in the work

referred to, were not using the Romeis stain or indeed any stain at all; their comments on the Romeis stain being entirely inferential from the results of purely chemical studies of the fat-preserving power of 40 per cent. alcohol as such. It is true that in previous work Kaufmann and Lehmann (1926) used a 40 per cent. alcoholic Sudan solution for staining pure lipide substances, not in animal tissues but absorbed by elder pith. The Romeis technique was published later (1927 and 1929) and it is not clear how the 40 per cent. solution which they used was prepared. At all events it was stated by them to be unsuited for use on tissue sections. They did state, however, that this solution would not work on (artificial) material on which a prior attempt had been made to stain with a 70 per cent. solution. Using natural lipides in tissue sections as the test objects, I have not yet found this to be the case.

By the time of Kaufmann and Lehmann's (1929) publication, however, Romeis (1929) had gone much more extensively into the details of the staining technique and published improved methods of making 40 per cent. alcoholic Sudan solutions. Although in this article he criticised Froeboese and Spröhnle for their failure to obtain satisfactory staining with his " 1927 " or " direct " method, the very claim that the new (" 1929 " or " indirect ") methods were so superior to the former carried with it an implied admission that that method was not altogether satisfactory. Indeed, in an addendum to his article, Romeis drew attention to the variability of commercial Sudan III and admitted that this might be responsible for the different results.

In his later article Romeis advocated two alternative methods. In one, by selective precipitation of the fractions of commercial Sudan III, he obtained a solution containing chiefly the ingredient termed by him " Sudan orange ". By the other, this same substance was first prepared in the pure and dried state and then used to make up the staining solution. This last method he was himself unable successfully to repeat, so that it is small wonder that it never came into use. But although the Romeis technique has occasioned some discussion, even the more certain " Sudan orange solution " method has not found acceptance. Of all the handbooks of technique, that of Bensley and Bensley (1938) is the only one to recommend it. The others are either silent or discouraging about it. My experience of the method is that success or failure is intimately dependent on the particular brand or batch of dye that one starts out with, and Romeis did not state what he used. Bensley and Bensley, in publishing the technique, make no mention of this point, whereas it should be made clear that unless one has a suitable sample of Sudan, the method is doomed to failure.

Now it is scarcely conceivable that a method of fat staining which avoided dissolving out large quantities of the very substances which are to be demonstrated[1] would not find immediate and universal acceptance unless it had very serious drawbacks. These drawbacks to the Romeis method certainly exist and they comprise:—

(*a*) The inconsistent behaviour of various samples of Sudan in forming the required colloidal solutions, which has already been mentioned.

(*b*) The capricious results obtained even with the most suitable samples available. Even Romeis himself had to take elaborate precautions, such as a specified relationship between volume of stain container and volume of staining solution used.

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(1) As already stated, I do not consider that this is in fact the rationale of the Romeis method; but it is generally conceded to be such.

(*c*) The occurrence of precipitates, of which the more serious is what has been described as "amorphous" precipitate. Actually it would be more clearly described as globular precipitate. It takes the form of globules or droplets having the colour of the dye itself and therefore exactly resembling fat globules, a source of considerable confusion and annoyance. One has to make sure by careful focussing whether the globules are in or on the section, whether inside the cells or also occurring in tissue or vascular spaces.(2) Crystalline precipitates, although common, are more readily recognised for what they are, but confusion might arise between them and pre-existent fatty acid crystals. In general they are more a disfigurement of the section than an embarrassment in interpretation.

(*d*) The staining times are rather long, which may be an inconvenience in routine work. As for the preparation of the staining solution, this looks more formidable than it really is. But once the stock solution has been prepared, in the Romeis method as in the one to be described, all that is necessary is to anticipate the need for staining solution the day before it is required. This fits in very well with Kay and Whitehead's recommendation to cut sections the day before they are to be stained, so that they become less sticky to handle.

Discussion of the New Method.

The method to be discussed was evolved in an attempt to investigate micro-chemically or at least to achieve satisfactory staining of a "Sudanophobe" lipide which is characteristic of the cells of the contagious venereal tumour of dogs (Jackson, 1943). This substance is soluble in the usual fat solvents and exists as refractile droplets, but is unstained by Sudan III, Sharlach R, or Sudan IV employed according to the prevailing techniques. The Romeis "1927" or direct method gave quite unsatisfactory results with various samples of Sudan III, although occasionally promising results were obtained using Sudan IV (Batch 55572, British Drug Houses). It should be emphasised that this was used, not according to the technique of Kay and Whitehead (1935), who introduced the use of this dye, but to replace Sudan III in the Romeis method. But in general this method of Romeis was capricious and precipitates were troublesome. With the more elaborate "indirect" (1929) method of Romeis, somewhat better results were obtained, but still the staining was uncertain and seldom complete.

It was then found that by diluting Sudan stock solution with solutions of phenol instead of with water, very highly colloidal stains were easily obtained. These colloidal solutions look like blood, as compared with the wine-like molecular Sudan solutions, and they possess high staining powers. This enhanced staining capacity, however, appears to go hand in hand with a tendency to produce precipitates, especially of the globular variety. These precipitates are not prevented (*a*) by preliminary treatment of the sections with alcohol of the same or slightly lower concentration as that of the stain; (*b*) by precautions against evaporation of the stain during the staining period;

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(2) This precipitate is anisotropic under the polarising microscope, a phenomenon that has not been referred to but which is of some use in deciding its nature in cases of doubt and where examination of control unstained preparations has revealed the absence or extent of pre-existent doubly refractile material in the tissue.

or (*c*) by attention to container-volume: stain-volume ratio as suggested by Romeis. Further (*d*) once they had formed they appeared to be tenaciously attached to the section, from which I was unable to remove them by differentiating in alcohols, with or without the addition of acids—at least not without at the same time dissolving out the true staining.

It became necessary to " attenuate " the highly colloidal carbol-Sudan solution. This is achieved by means of adding acetic acid. The more acid one adds the more the solution clears (i.e., becomes more molecular and less colloidal) and the less it tends to precipitate. It is necessary, however, not to overstep the effective end-point of this process, at which the enhanced staining powers of carbol-Sudan are suppressed and at which the high acidity influences the colour reaction obtained in the direction of becoming pale (yellow) instead of deep (red). Effectively prepared solutions may still cause some precipitate, but in this case the precipitate is easily removed by differentiating the sections in a solution of acetic acid in dilute alcohol.

I am inclined to the view that the most perfect Sudan solutions must almost inevitably and by their nature produce precipitate, and that one's only concern should be that this precipitate is subsequently not irremovable. The rationale of Sudan staining, as is well known, depends on the passage of the dye from a solvent in which it is less soluble into the fat, in which it is more soluble. Where lipides are in question which are relatively deficient as solvents for the dye, the ordinary methods of staining break down. The Romeis methods can still succeed, in my opinion because this is compensated for by using a poorer solvent (40 per cent. instead of 70 per cent. alcohol) for the dye; but when the Romeis solutions are made up with the usually available samples of Sudan they may contain so little dye that by extraction into the tissue lipides an equilibrium is reached during the staining process, which may come to an end before it is complete. Carbol-Sudan, on the other hand, appears to contain large quantities of dye, but unlike in molecular solutions, held in an unstable equilibrium from which it still readily passes into the lipides. In this method we achieve an unstable solution without sacrifice of dye concentration and thus fulfil both desiderata (instability and high concentration) of the efficient Sudan solution, in contrast to previous methods which achieve either the one or the other, but never both.

It should be stressed that preliminary treatment of the sections with 70 per cent. alcohol has never reduced the amount of the visible lipides which I have investigated, either when stained thereafter with acetic-carbol-Sudan or according to Romeis. Further, where acetic-carbol-Sudan is used, the optimum alcohol concentration is far above 40 per cent. and good results are obtainable in 65 per cent. alcohol concentration. It is significant that where water is used to dilute the stock solution, the optimum dilution should be so much greater than where carbolic is used. These observations strongly suggest that not fat-preserving but colloidal properties are the crucial ones in both types of technique.

Since certain lipides which are unstainable by standard Sudan or Scharlach techniques stain powerfully with acetic-carbol-Sudan, one should recognise that the terms " Sudanophobe " and " Sudanophil " as applied to lipides become relative and inaccurate. In other words, certain lipides which are "Sudan-moleculophobe " prove to be " Sudan-colloidophil ". As the range of substances stainable by Sudan is extended by this method, these considerations should enhance the reputation already enjoyed by the

Sudan series of dyes as a micro-chemical test: more reliance can be placed on negative results when acetic-carbol-Sudan is used, whereas no conclusions as to the absence of lipides in a given tissue should be drawn from negative results of the previous techniques.

Technique.

As in the case of the Romeis stains, two main alternative methods for preparing colloidal carbol-Sudan solutions present themselves. The " indirect " method given here is to be preferred. A " direct " method will be referred to in a footnote.

1. *Preparation of Acetic-Carbol-Sudan.*—80 per cent. stock solution of Sudan III prepared according to Romeis (1929) [the method is also given by Bensley and Bensley (1933)] is kept on hand in a well-stoppered, preferably sealed bottle.(3) To any desired quantity of stock solution 5 per cent. aqueous carbolic acid is added slowly, drop by drop, from a burette, agitating vigorously, until the alcohol content has been reduced to 60 per cent. (e.g. for small quantities of stain to 6 c.c. stock solution add 2 c.c. carbolic). Time and energy should not be spared in making this dilution. Allow to stand for a few hours, well corked. Glacial acetic acid is then added, drop by drop in the same manner, in the proportion of 2·5 drops per c.c. of carbol-Sudan (i.e., 20 drops in the example mentioned). Allow to stand well corked for 24 hours before filtering and using.(4)

Good results for certain tissues have also been achieved with dilutions other than those suggested above. Thus I have used varying alcohol contents from 70 per cent. to 40 per cent., often with good results. But the amount of acetic acid added must be adjusted for each separate alcohol content. It is very probable that others may for particular tissues wish to modify the dilution to 60 per cent. alcohol content and the amount of acetic acid added.

Acetic-carbol-Sudan has been successfully prepared from a variety of ordinary samples of Sudan III available at this Institute. At present Gurr's Sudan III is being used.

Acetic-carbol-Sudan gradually deteriorates in efficiency, and usually within the course of a week this becomes apparent. It is recommended that it should not be used when more than a few days old. On the other hand, it must on no account be used immediately after preparation.

2. *Staining Technique.*—Frozen sections may be transferred to the staining solution from distilled water or preferably from alcohol of the same or 10 per cent. lower grade. It is probable that the latter step is preferable

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(3) *Preparation of Stock Solution:* 2 gm. of the finely pulverised dye are covered with 450 c.c. of 95 per cent. alcohol and heated on the water bath to simmering, swilling round occasionally. Filter hot. Stopper well and place in the refrigerator overnight. Next day, filter cold. Measure volume of filtrate and dorp in distilled water slowly from a burette, with agitation, to reduce alcohol content to 80 per cent. Allow to stand 24 hours, filter, and keep well corked.

(4) A fairly efficient " direct " carbol-Sudan solution may be more rapidly prepared by simply bringing to simmering excess of the powdered dye in equal parts of 80 per cent. alcohol and 5 per cent. aqueous carbolic acid. Allow to stand 24 hours, filter and use. This gives a 40 per cent. alcoholic solution, which is less highly colloidal, requires longer to stain (say 4 to 24 hours), but has less tendency to produce precipitates, so that acetic acid is not added. What is gained in preparation time is offset by longer staining time. I do not recommend it in preference to the " direct " method, but it may prove to have some use, and is undoubtedly superior to the ordinary techniques.

where only a small quantity of stain is used, in order to avoid carrying over significant quantities of water with the section, which by further dilution is likely to increase the tendency to precipitation. Staining is carried out at room temperature in securely corked small glass containers (tubes) for 1½ hours or longer, according to the type of lipide to be demonstrated. From the stain, transfer direct to a 5 per cent. solution of glacial acetic acid in 50 per cent. alcohol, and differentiate in this for 15 to 60 seconds. This will remove precipitate if this has been formed. If examination has shown that there is no tendency to precipitate, this step may be omitted. In any case the examination of a control section is desirable when new material is being investigated, as a check on possible over-differentiation or modification of the colour reaction due to the acetic alcohol. For some tissues it may be advisable to dilute this acetic alcohol (with 50 per cent. alcohol). Care should be taken to get the section to spread out during differentiation, which it has not much tendency to do of its own accord: precipitate is likely to persist in areas of the section which have remained folded. Wash (1 minute) in distilled water. Counterstain in recently filtered Delafield's haematoxylin diluted with two volumes of distilled water (15 minutes). Differentiate in acid water (0·5 per cent. HCl in aq. dest.) until reddish (10 to 20 seconds). Blue in ammonia water (a couple of drops of Liq. ammon. fort. in a basin of distilled water) about 5 minutes. Wash in distilled water and mount in glycerine-jelly.

RESULTS.

Neutral fats stain a brilliant orange scarlet. The colour given equals in every respect that obtained with the best Scharlach R techniques, so that there is no advantage in this technique of using Sharlach R instead of Sudan III.[5] Normal myelin stains bright orange, the method being a promising one as a substitute for Weigert-Pal. It gives especially good demonstration of the myelin sheaths of peripheral nerves, including the rudimentary sheaths of autonomic nerves in various tissues. It is useful for studies on degeneration in both peripheral and central nervous system and in certain cases has revealed lipides in "granular corpuscles" which did not appear by routine methods.

Two lipides of which the chemical nature is unknown—that of the contagious venereal tumour of dogs and that in the histiocytes in lymph glands of pregnancy disease of sheep (Clark, 1943)—stain like neutral fats.[6] Both these substances are completely "Sudanophobe" by ordinary technique. Fatty acid crystals stain orange-yellow. Where lipide globules are present in leucocytes (e.g., neutrophils) these show up very well.

It appears that acetic-carbol-Sudan will, to say the least, reveal all that Ciaccio's technique will do, but without the artefacts due to liquefaction and fusion of lipide droplets which occur owing to the heat employed in that method.

It is hoped, when material becomes available, to investigate the capability of the method to stain the substances characteristic of the lipoid-histiocytoses—Gaucher's disease and Niemann-Pick disease (kerasin and

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(5) Scharlach R has, however, been used successfully, with the dilutions mentioned. These dilutions do not appear to suit "Sudan IV".

(6) The results obtained will be illustrated in the cited articles of Jackson and of Clark.

sphingo-myelin respectively). The method is to be recommended wherever the completest demonstration of lipides is desired either in normal or abnormal tissues, or where the presence of lipides not stainable by ordinary methods is suspected.

Summary and Conclusions.

1. A method is described of using Sudan III, which gives a more complete demonstration of lipide substances, especially those that are refractory to ordinary Sudan III, Scharlach R, or Sudan IV techniques.

2. This improvement does not appear to depend on superior preservation of lipides, but on enhanced staining powers due to the colloidal nature of the solution.

3. Used in this way, the colouration obtained with Sudan III equals that usually obtained with Scharlach R.

4. The method possesses the advantages of constancy, rapidity, and absence of precipitates as compared with the methods of Romeis, and it succeeds with a variety of samples of the dye.

5. The best variation of the technique is as follows:—

(*a*) Fix in formol or formol-saline.
(*b*) Frozen sections in distilled water.
(*c*) 50 per cent. alcohol 1 minute.
(*d*) Acetic-carbol-Sudan (60 per cent. carbol-Sudan plus 2·5 drops of glacial acetic acid per c.c., prepared as described) 1½ hours (sometimes longer) in a small well-corked container.
(*e*) Differentiate in 5 per cent. acetic acid in 50 per cent. alcohol (10 to 60 seconds).
(*f*) Wash in distilled water (1 minute).
(*g*) Counterstain in filtered Delafield's haematoxylin diluted with two parts of distilled water.
(*h*) Differentiate in acid water (10 to 20 seconds).
(*i*) Blue in ammonia water (5 minutes).
(*j*) Wash in distilled water.
(*k*) Mount in glycerine-jelly.

6. Positive results have been obtained with two lipides which are not stained by ordinary techniques (so-called "Sudanophobe" lipides) and the method is especially to be recommended wherever the existence of such substances is suspected.

7. No conclusions regarding the absence of lipide substances should be drawn from negative results of ordinary Sudan or Scharlach techniques, until acetic-carbol-Sudan has been used.

8. It is anticipated that acetic-carbol-Sudan technique will supersede all previous Sudan methods, wherever critical demonstration of lipides is required.

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[7] Only the pertinent literature, not a complete bibliography of fat staining, is quoted here.