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An Improved Technique for the Demonstration of Tissue Lipids

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AN IMPROVED TECHNIQUE
FOR
THE DEMONSTRATION
OF TISSUE LIPIDS

by
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Graduate School, Marquette University
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April, 1953
Progress in science frequently depends upon proper tools to demonstrate, enhance, integrate, substantiate or refute contentions. Among the means that make possible the advance of biological science is histological technique. Improved microscopic procedures result from a constant refining process and the search for new methods. To merit the adjective "improved" a new or refined technique must display a superiority to similarly employed processes. Such superiority might be shown by its speed, economy, practicality, ease or faithfulness of reproduction, or some other attribute.

This paper portends to present an improved technique for the demonstration of tissue lipids.

By way of acknowledgement I wish to express my appreciation to the Department of Biology of Marquette University and its members for the opportunities afforded me thereby making this work possible. My special gratitude is due to Doctor James C. Perry who gave far and above that which might be expected of the director of a thesis.
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INTRODUCTION

The histological demonstration of lipids has long been a cumbersome task. Such demonstration, nevertheless, is very valuable to the biologist. The endocrinologist, for example, wishes to know the relative quantities of steroid hormone present under various conditions, the site of hormone production, or the periods of most active secretion. The paraffin and celloidin routines are not suitable for such determinations since the lipids are soluble in many of the reagents employed in these techniques. The resulting spaces may indicate the location of fat-like substances but such information is very inconclusive and unsatisfactory.

Occasionally gelatin has been used as a medium for showing lipids (Zwemer '33). This method is time consuming and at best gives sections four to six micra in thickness.

The accepted way of studying lipids microscopically has been by frozen sections. This means has been an invaluable aid in demonstration of tissue lipids. A survey of biological technique texts and periodical literature
readily attests to the wide acceptance and employment of this method. The lipid solubility factor is eliminated. Disadvantages, however, are encountered, chief of which is the difficulty in obtaining thin sections. Average sections run about fifteen micra thereby precluding detailed cytological study. Thinner sections, if obtained, fragment easily during the manipulation necessary in staining and mounting. Ice crystals tear the tissues internally.

There has appeared in the recent literature descriptions of a technique utilizing a group of compounds known as Carbowaxes (Carsten '47; Blank and McCarthy '50; Rinehart and Abul-Haj '51; et. al.). These waxes are polyethylene glycols. They are long straight chain polymers which polymerize ether fashion. The empirical formula is \( \text{HOCH}_2(\text{CH}_2\text{OCH}_2)\text{CH}_2\text{OH} \). The compounds belonging to the group are designated by their average molecular weight with one exception. Thus Carbowax 4000 has an average molecular weight of 4000. The exception is C. 1500 which is a mixture of equal parts of polyethylene glycol 300, a fluid, and the 1540 wax. This mixture has an average molecular weight of 500–600 (Wade '52). The polyethylene glycols employed in histological technique are generally C. 1000, C. 1500, C. 1540, and C. 4000. Solidity, heat stability, and chemical inertness increase with molecular weight.
An outstanding feature of these waxes is their solubility in water. They dissolve in water to form clear, colorless solutions. This property makes it possible to dispense with the dehydration process of the usual slide-making routine. At the same time they may be used as embedding materials in place of paraffin.

Lipids are insoluble in the Carbowaxes at the latter's melting point. The melting point of C. 4000, the wax of the highest molecular weight among this group used for histological purposes, is 52 degrees Centigrade.

The two properties, solubility of the waxes in water and insolubility of lipids in the waxes makes these substances well suited for the dehydration and embedding of tissues in which the demonstration of lipids is desired.

Since in the past the demonstration of lipids in tissue sections has been a troublesome process and since thin sections were not readily produced by such techniques, it was thought that the utilization of the Carbowax method in presenting lipids would offer an improved tool for such histological work.

The adrenal glands were chosen as the subject of this investigation. The known adrenal cortical hormones and their precursors are lipids and appear in these glands in great abundance. In addition, the fact that stress agents can cause a shift, decrease or increase in the lipid
contents (Selye '46; Dosne and Dalton '41; Robinson and Yoffey '50; et al.) makes the adrenal gland an excellent vehicle to test the worth of the Carbowax technique in biological investigations. For the latter reason white male rats were subjected to a stress agent and the adrenal glands of these animals submitted to the Carbowax routine. It was felt that a demonstrated quantitative change or shift of lipid material in the cortex of these glands due to the stress agent would better establish the value of this technique. Such an exposition would provide an example of its quality under experimental rigors.
MATERIALS AND METHODS

Four white male rats, 18 months old, were given estrogen for a period covering 20 days. A subcutaneous administration of 0.05 mg. of this stress agent was given every other day. At the beginning of the experiment the four experimental animals had an average weight of 328 gms.; just prior to killing they weighed 252 gms. The two controls at the outset had an average weight of 312 gms. as against 328 gms. at the time of sacrifice. All animals were killed one day after the last injection by means of an intraperitoneal injection of 1/2 cc. of 60 mg./cc nembutal.

The adrenal glands were removed within three minutes of the nembutal dose. Ten percent formalin served as the fixative. After a period of at least 48 hours fixation the tissues were washed in tap water for four hours. At this point they were introduced into the molten wax. No dehydration process was necessary as in the paraffin and other procedures.

Ordinarily tissues run up via the Carbowax method are placed directly into a mixture of approximately nine parts of C. 4000 to one part of C. 1500 (Blank and McCarthy '50). This would serve as the dehydrating, infiltrating and embedding medium. Blank and McCarthy ('50) prescribe
infiltration time as follows:

The minimum time for impregnation is thirty minutes for a 1mm. section, eighty minutes for a 2mm. section, and proportionately longer times for thicker sections. Some tissues have been immersed in molten Carbowax for periods up to four days with little perceptible distortion. It is recommended that the routine time be three hours.

With previously investigated adrenal glands, however, direct placing into an approximate nine to one ratio wax seemed to cause considerable shrinkage in most of the glands. Such shrinkage was probably due to the sinusoidal nature of the tissues and the loose apposition of the cortex and medulla. To prevent most of the shrinkage this series was worked out:

<table>
<thead>
<tr>
<th>VIAL NO.</th>
<th>PARTS C.1500</th>
<th>PARTS C.4000</th>
<th>DIST. H₂O</th>
<th>MINUTES IN EACH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>20</td>
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<tr>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<td>3</td>
<td>6</td>
<td>6</td>
<td>1</td>
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<tr>
<td>5</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>60</td>
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Evidently the smaller molecules of C. 1500 penetrate more readily and thereby give substance to the embedding mass. Most shrinkage can thus be avoided. The tissues were agitated occasionally because of their tendency to rise to the top of the wax.

Paper boats served as receptacles for embedding. These molds were placed into the refrigerator for fifteen minutes to allow hardening. Care was exercised to prevent
contact with water. Hardening completed, the blocks were trimmed, attached to wooden blocks and sectioned.

Sections were cut at three micra. The ribbons were placed on a floating medium devised by Cobe and Schaeuffle ('46) for paraffin sections and recommended by Blank and McCarthy ('50) for the Carbowax routine. This medium is made up as follows: 0.2 gm. \( K_2Cr_2O_7 \) and 0.2 gm. gelatin are dissolved in 1000 cc. distilled H\(_2\)O by bringing to a boil for five minutes. Cooled and filtered, it is ready for use.

Upon placing the ribbons on this floating medium the wax immediately dissolved out leaving the sections floating. Clean, dry slides were used to pick up the sections. No affixative was necessary, the floating medium serving that purpose. The slides dried rapidly and in about ten minutes time were ready for staining.

Slides to be stained were rinsed in distilled H\(_2\)O to remove the dried floating medium that remained. A rinse in 40% alcohol * followed to rid the slides of water since the water on the slides and carrier would have caused some precipitation of the fat colorant. The sections were then placed into an alcohol solution of Sudan IV for forty-

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*Kaufmann and Lehmann ('29) have shown that 40% alcohol removes a negligible amount of lipid from tissues.
eight hours. The dye was prepared in this manner: Two grams of Sudan IV powder were dissolved in one liter of absolute ethyl alcohol by bringing to a boil. This solution was cooled in the refrigerator overnight and filtered the next day. This served as stock solution. Two hours prior to staining time sixty cc. of distilled H₂O were added dropwise with constant agitation to forty cc. of the stock solution. This latter solution was allowed to stand for at least an hour and no more than two hours and then filtered just prior to usage.

Slides that were not counterstained were taken out of the dye, rinsed in 40% alcohol, washed in tap water, wiped of excess water and coverslipped using white Karo syrup as the mounting medium.

Harris' Hematoxylin served as the nuclear stain for the counterstained slides. The procedure was this:

1) Rinse in 40% alcohol.
2) Rinse in tap water.
3) Harris' Hematoxylin - two minutes.
4) Differentiation in solution of 0.5% HCl in 40% alcohol.
5) Rinse in tap water.
6) Blueing in Scott's Tap Water (Clayden '48).
7) Coverslip using white Karo syrup as mounting medium.
RESULTS

The adrenal cortex is composed of three zones. Immediately below the capsule lies the zona glomerulosa, a narrow zone. The middle layer is the zona fasiculata. The zona reticularis is the innermost zone of the adrenal cortex; it surrounds the adrenal medulla.

The lipid content and/or the width of the zones can vary under conditions of stress. Such variation follows a basic pattern irrespective of the agent used to produce the stress. The response of the organism to stress is known as the General-Adaptation-Syndrome. The basic pattern that is evoked in this Syndrome is described in three stages: Alarm-Reaction, Stage of Resistance, and the Stage of Exhaustion. These stages are reflected in the lipid content of the adrenal cortical zones. During the Alarm-Reaction there is a discharge of lipids from the cortex. In the Stage of Resistance the cortex regains its sudanophilic stores. The lipid content in the Stage of Exhaustion resembles that of the Alarm-Reaction in that there is again a discharge of lipids. (Selye '50).

It will be recalled that estrogen is a stress agent and consequently it might be expected that some stage or
stages of the General-Adaptation-Syndrome would be evident in the results.

The photomicrographs on pages 17 - 21, considering the thinness of the sections, indicates the effectiveness of the Carbowax routine in preserving and allowing the demonstration of tissue lipids. The distribution and concentration of the lipids in the control animals of this investigation match that as described for the frozen section technique used by Cain and Harrison ('47) in investigation the lipid picture in untreated rats of both sexes and varying ages. They state:

With the Sudan Black method, either the outer part of the z. fasciculata was the most heavily loaded or there was even loading throughout the zone. In no case was the inner z. fasciculata more heavily coloured than the outer - .... . The z. reticularis, when present, was as dark as or less dark than the inner z. fasciculata, but never darker except for a small number of scattered and very heavily loaded cells next to the medulla in some individuals of most groups. The z. glomerulosa, on the other hand, was extremely variable; usually there was an inner zone clear of lipoids which appears to be the same as the sudanophobe zone, referred to by many workers .... . In these cases the outer zone was usually heavily loaded, in several cases being often the darkest part of the whole section. Frequently, the z. glomerulosa was not present as a continuous sudanophil rim, but was often interrupted by sudanophobic patches; ....
In the control animals of this investigation (Illus. 1 and 2) the zona fasciculata contained the most lipid material with the greater concentration of sudanophilic material at the outer edges. The zona reticularis is narrower in the control animals than in the experimental animals. Because of a lipid content almost equal to that of the inner zona fasciculata the extent of this zone is a little more difficult to determine. Occasional iuxtamedullary cells show a heavy deposition of lipid material (Illus. 3). Groups of cells in the zona glomerulosa manifest heavy loading while other areas show almost a complete lack of sudanophilic substances.

In three of the experimental animals (Illus. 4, 5, 7, 8) the outer portion of the zona fasciculata again shows a greater concentration of lipid materials than the rest of this zone. The over-all area of this zone, while showing a considerable amount of lipids, does not show as great an amount as evidenced in the same zone of the control animals. The other experimental animal (Illus. 6) displays a definite loss of sudanophilic material in the zona fasciculata. In all four experimental animals the zona reticularis shows apparent enlargement and definite reduction of lipids. Evidence of a change in the zona glomerulosa is not so noticeable. It would appear that there is a reduction in the lipid material when compared with the zona glomerulosa.
of the control animals but the reduction is not very clear-cut.

Along with the loss in weight, the duration of the injection period and the dosage of the stress agent, the near depletion of lipids in the one experimental animal would seem to indicate that this animal is in the Stage of Exhaustion. The definite reduction of lipid materials in the zona reticularis and the apparent reduction in the zona fasciculata and zona glomerulosa coupled with the experimental data would seem to indicate that the remaining three experimental animals were on the verge of the Stage of Exhaustion.
DISCUSSION

The first requirement to be fulfilled by a "new" or "improved" technique is faithfulness of reproduction. Expected results which are obtained by other techniques with the same objective, at least must be matched. The results in this investigation would seem to bear out that the carbowax technique fully carries out this obligation. Expected results are achieved. The lipid picture in the control animals is similar to that described by other authors for normal animals. The lipid picture in the experimental animals reflects that which was obtained under similar experimental procedures by other investigators.

Not only were expected results attained but added advantages were realized. It is these added advantages that label the carbowax technique as an improved procedure. The superiority to other techniques is readily recognizable. Detailed studies can be made of the sections due to the thin sections that can be produced. Sections at two micra can be made routinely. One micron sections are attained with the exercise of a little care. The routine is time saving. Two hours after fixation a completed slide can be ready for microscopic study when the staining time so allows. Such slides apparently are as faithful in cytological details as slides prepared via
the paraffin method. Economy is another feature. The
fact that the carbowaxes are water soluble dispenses with
the entire dehydration process. Tissues are taken direct-
ly from the fixative and placed into the molten wax.
Further economy is achieved in the staining procedure. In
the paraffin technique xylool and the ensuing alcohol series
are necessary to get rid of the paraffin and to prepare
for the staining solution. Carbowax bypasses these steps
since an inexpensive floating medium removes the wax from
the sections so that the dried slide can be placed im-
mediately into the stain. Another advantage lies in the
fact that there is no exposure to any reactive reagent be-
tween the fixative and the wax or between the dried slide
and the stain thus permitting the preservation of cellular
substances.

The technique is not without disadvantages. Since
the polyethylene glycols are hygroscopic, atmospheric
conditions can interfere with the obtainment of good sect-
ions. A warm room with moist air causes the sections to
compress. This can be overcome partially by using more
of the C. 4000 in the embedding mixture. A laboratory
with constant temperature and proper moisture control
would serve to forestall this difficulty completely.
Shrinkage does occur. This likewise may be remedied to
a great extent by starting the infiltration with waxes of lower molecular weight. These molecules of the lower waxes penetrate the tissue easier and evidently give substance to the embedding mass. Serial sections are difficult to obtain since the floating medium sets each section free. Placing the floating medium on the slide would not solve this completely. One other drawback is the lack of standardization of the various waxes. This is the chief disadvantage of the process. Each batch of wax from the supplier must be tested since each lot varies, e.g. C.1500 at one time may contain a preponderance of C.1540 and the next time a greater proportion of C.300. This necessitates adjustments in the proportions of the embedding mixture. This difficulty could be remedied if the manufacturer were to undertake measures to provide a standardized mixture.
CONCLUSIONS

The Carbowax technique augurs well as a histological routine especially for the demonstration of tissue lipids. The reactive agents of other techniques are by-passed. The thick sections obtained in the techniques which do not employ reactive agents are likewise circumvented. These features, the by-passing of reactive agents and the ease with which thin sections are produced plus the speed and economy of the routine definitely establish the Carbowax technique as an improved technique for the demonstration of tissue lipids. The disadvantages encountered may on occasion be annoying, nevertheless they do not detract from the overall merit of the process.

It is fully expected that the Carbowax technique will become increasingly more popular in microscopic studies especially in the investigation of lipids. The technique merits such popularity.
Illustration 1
Control 1 - Heavy lipid concentration at outer edges of zona fasciculata. Considerable amount of lipids in zona reticularis. Border between zona reticularis and zona fasciculata indistinct. 10 x 12.

Illustration 2
Control 2 - Heavy lipid concentration in zona reticularis and zona fasciculata. Border between these two zones indistinct. Occasional laden cells in zona glomerulosa. 10 x 12.
Illustration 3
Control 2 - Enlargement of iuxtamedullary region showing heavily laden individual cells. 10 x 43.

Illustration 4
Experimental 6 - Shows depletion of zona reticularis. Heavily laden cells at border of zona reticularis and zona fasciculata. 10 x 10.
Illustration 5
Experimental 6 - Same slide and same general area as seen in Illustration 4. 10 x 10.

Illustration 6
Experimental 4 - Reduced amount of lipid in all three zones of cortex. 10 x 12.
Illustration 7
Experimental 3 - Shows near depletion in zona reticularis and reduced amount of lipids in zona fasciculata. 10 x 12.

Illustration 8
Experimental 4 - Shows reduced amount of lipids in zona reticularis and zona fasciculata. 10 x 12.
BIBLIOGRAPHY


