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A Study of Some Dehydrating and Infiltrating Agents

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A STUDY OF
SOME DEHYDRATING AND INFILTRATING AGENTS

by

ACKNOWLEDGEMENT

Monica Hoefs

The author wishes to express her appreciation to Doctor William H. Steil for his helpful suggestions and encouragement throughout the entire investigation.

A Thesis presented to the Faculty of the Liberal Arts School, Marquette University, in partial fulfillment of the requirements for the degree of Bachelor of Science.

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Introduction

The ethyl-alcohol-xylol series produces a hardening and shrinking of the tissues. It has long been criticised for this reason.

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Reichardt and Wetzel (1928) used methyl-benzoate alone to overcome the difficulties encountered in infiltration. The hardening of the tissues was overcome, but the grading of the media was made more complicated. Sheridan (1929) proposed the use of normal propyl alcohol. Zirkle (1930) recommended Larbaud's mixture of ethyl and normal butyl alcohol.

Bradbury (1931) proposed the use of iso-propyl alcohol. Margolena (1932) and Stiles (1934) obtained excellent results with animal and insect tissues by using normal butyl alcohol, but Smith (1931) had little success with it. Hewitt (1931) wrote quite favorably in regard to the use of iso-butyl alcohol. Sess (1932) recommended the substitution of ethyl alcohol with acetone.

Graupner and Weisberger (1931) completely omitted the alcohol series by passing the tissues directly from the fixative into dioxan, a reagent which is miscible with water or alcohol

and is a solvent of paraffin. The Turtov News (1934) reported satisfactory results with dioxan. Their experiment was restricted to Zanker-fixed tissues.

Introduction

The ethyl-alcohol-xylol series produces a hardening and shrinking of the tissues. It has long been criticised for this reason. Walls (1932) after his success with the hot celloidin technique, suggested that it was the hot paraffin which shrank and hardened the tissues. Stiles (1934) suggested that it may

Duvel (1879) suggested the ethyl-alcohol-xylol series be used with celloidin to overcome these difficulties. Peterfi (1921) used a methyl-benzoate-celloidin solution and embedded the material in paraffin from xylol. Larbaud (1921) proposed the mixture of ethyl and normal butyl alcohol. Painter (1924) substituted aniline oil for the higher concentrations of ethyl alcohol.

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and is a solvent of paraffin. The Turttox News (1934) reported satisfactory results with dioxan. Their experiment was restricted to Zenker-fixed tissues. Walls (1932) after his success with the hot celloidin technic, suggested that it was the hot paraffin which shrank and hardened the tissues. Stiles (1934) suggested that it may be a matter of dehydration and clearing and not of paraffin impregnation. McClung (1929) omitted the soft paraffin in the alcohol xylene method, and passed directly from the xylene into hard paraffin, thus shortening the time in melted paraffin to a minimum.

Johansen (1935) emphasized the use of dioxan and tertiary-butyl alcohol as dehydrants for plant tissues, but did not elaborate upon the details of his technic. Baird (1936) discussed the application of tertiary butyl alcohol to animal tissues. From nearly two year's experience with dioxan in botanical microtechnic by McWhorter and Weier (1936), they found that dioxan both simplifies and improves paraffin sectioning and that it offers new fields or possibilities in microtechnic.

Lang (1937) suggested several modifications in the use of normal-butyl alcohol. They include a revised series of dehydration solutions for exacting work, and abbreviated schedule of limited usefulness, and a simple method for more rapid paraffin infiltration. Changes in the primary fixation image are significantly less severe by the dehydration with butyl alcohol than with many other reagents.

Such deleterious effects may be further minimized by reducing the time and temperature factors to the practical limit and by substituting acetone for ethyl alcohol in a dehydration series such as that of Zirkle.

According to Stiles (1934) the greatest advantage of the normal butyl alcohol method is the elimination of both hardening agents (the higher percentage ethyl alcohols and xylol or benzol). Another advantage is the great time toleration of the processes of dehydration and infiltration. Tissues have been kept without deleterious effects in normal-butyl alcohol for a year before infiltration. For small insects and vertebrate tissues about five days proved necessary to insure satisfactory infiltration. Normal-butyl alcohol was found to give better results than many other technics in serial sectioning of lightly chitinized insects. Ethyl alcohol is the better dehydrating agent, and for that reason, a small percentage of it is used with normal-butyl alcohol. There is also an added advantage in the flexibility of this method.

Baird (1936) concluded that dioxan causes the least amount of shrinkage, and keeps the tissues from becoming hard and brittle. McClung (1936) substituted dioxan thruout the entire microtechnic process, including staining. He states that dioxan speeds up the entire process and at the same time produces better results.

Guyer (1936) also refers to the use of dioxan as an aid in staining and employs a 0.10% solution of erythrosin as a counter-stain with hematoxylin, because eosin is relatively insoluble in benzene and similar fluids employed for clearing purposes.

Stankovic (1930) found that the higher concentrations of dioxan. Waterman (1937) suggests applying heat to dioxan or adding a few drops of tertiary-butyl alcohol, so that enough eosin will go into solution to stain slowly. He obtained the same result by allowing the dioxan and eosin powder to stand for several weeks, shaking it occasionally. By using eosin in this manner, it stains slowly enough to be observed and can be stopped when the desired intensity has been reached. This eliminated the possibility of overstaining which is so common with the alcoholic method.

Ralph (1938) attributes shrinkage to improper fixation, exposure to air during the process of fixation and dehydration, and the action of reagents. He attributes hardening to the type of fixation used, the influence of hot paraffin, and the action of reagents. He was most successful in his fixations in using Bouin's fluid, and obtained the best results from it, with softer blocks and less shrinkage. He states that the best complete method was found to be Bouin's fixation with dehydration by the slow dioxan method.

Johansen (1935) found that successful dehydration and infiltration is not dependent upon successful fixation but rather that the two are mutually exclusive. Good infiltration occurs when the water is replaced, but the water-absorbing capacity of the tissue is not destroyed. He also states that much of the criticism placed upon certain killing and fixing fluids appears to be wholly gratuitous and should be laid instead against absolute ethyl alcohol, xylol, chloroform, benzene and similar fluids employed for clearing purposes.

Zirkle (1930) found that the higher concentrations of alcohol harden any tissues left in them too long, often before it is completely dehydrated. All the water must be extracted from the specimen before the xylene will penetrate, and this involves the use of absolute alcohol. The xylene itself causes animal cells to shrink and become brittle. In spite of the fact that butyl alcohol diffuses into paraffin more slowly than does xylene, it has several advantages over the latter as a clearing agent. It is slightly lighter than paraffin at the latter's melting point and when the specimen sinks, it remains floating on the paraffin. Xylene is heavier than melted paraffin, and sinks with and surrounds the specimen. An additional advantage in the use of butyl alcohol is that slight traces of it in the paraffin blocks do not render them crumbly as does a like amount of xylene.

Mossman (1937) recommends dioxan in place of alcohols and clearing oils in paraffin embedding, and in the staining of sections. It is unnecessary to dehydrate fresh dioxan before using, and the insertion of other dehydrators and clearers into the series is illogical.

Chamberlain (1924) states that clearing agents are so named because they render objects transparent. When clearing agents are used to precede infiltration with paraffin, the clearing is merely incidental, the real purpose being to replace the dehydrating agent with a solvent of paraffin. Clearing is useful, even in this case, because it indicates when the replacing has become complete. When the clearing agent is used to precede infiltration with paraffin, the material should always be most thoroughly dehydrated

with absolute alcohol before beginning with the clearing agent.

In this study, the author is comparing the reagents used as regards complete dehydration and infiltration, shrinkage, sectioning, and the facility with which the material may be sectioned.

Materials and Methods

The material used in this study was the liver and kidney of the Necturus. The animal was anaesthetized, and the liver and kidney were dissected out, cut into small pieces of equal size and placed into Bouin's fluid for four hours.

Eight pieces of equal size were placed into each of two vials. Eight pieces of equal size of kidney were placed into each of two other vials. The air pump was used on one vial of the liver and on one vial of the kidney. After four hours the fixing fluid was poured off the material, and it was placed into 50% alcohol. The alcohol was changed at regular 24 hour intervals until the alcohol bore no signs of the Bouin's fluid. The material was then placed into 70% alcohol for a period of twenty-four hours. Two pieces of the material from each vial were placed into each of sixteen different vials. The material was divided into four sets. Each set contained two pieces of the liver and the kidney which had not had the air pump used on the material, and each set also contained two pieces of the liver and the kidney which had had the air pump used on the material.

The dehydration and infiltration process was continued with the following fluids: 1) ethyl-alcohol-xylol, 2) tertiary-butyl alcohol, 3) iso-amyl alcohol, 4) dioxan. The methods used with these agents are presented in the following tables.

Table 1 gives the schedule when ethyl-alcohol-xylol was used as a dehydration and infiltration agent on Set 1, showing the time periods and steps involved.

Table 1. Ethyl-alcohol-xylol as a Dehydration and Infiltration

	Agent			
80% alcohol	12 hrs	12hrs	12hrs	12hrs
95% alcohol	21 hrs	21hrs	21hrs	21hrs
Absolute alcohol	1 hr	1 hr	1 hr	1 hr
Absolute alcohol	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr
$\frac{1}{2}$ Abs. and $\frac{1}{2}$ xylol	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr
Xylol and Paraffin	23 hrs	23 hrs	23 hrs	23 hrs
Paraffin	24 hrs	24 hrs	24 hrs	24 hrs
Paraffin	24 hrs	24 hrs	24 hrs	24 hrs
Paraffin	46 hrs	46 hrs	46 hrs	46 hrs

Table 11 gives the schedule for tertiary-butyl alcohol when used as a dehydration and infiltration agent again showing the time periods and steps involved for each set of Necturus in the vials.

Table 11. Tertiary-butyl-alcohol as a Dehydration and infiltration

	Agent			
7 tertiary-butyl	18 hrs	18 hrs	18 hrs	18 hrs
8 tertiary-butyl	25 hrs	25 hrs	25 hrs	25 hrs
9 tertiary-butyl	5 hrs	5 hrs	5 hrs	5 hrs
10 tertiary-butyl	18 hrs	18 hrs	18 hrs	18 hrs
11 tertiary-butyl and paraffin	7 hrs	7 hrs	7 hrs	7 hrs
Paraffin	19 hrs	19 hrs	19 hrs	19 hrs
Paraffin	46 hrs	46 hrs	46 hrs	46 hrs
Paraffin	5 hrs	5 hrs	5 hrs	5 hrs

In this method only the higher grades of tertiary-butyl alcohol were used. The schedule followed for this alcohol was that proposed by Zirkle (1930).

The grades of alcohol used are as follows:

	7	8	9	10	11
Percentage of solution...	85	95	100	100	100
Water.....	15	5	0	0	0
Ethyl alcohol....	50	40	25	0	0
Tertiary-butyl alcohol.....	35	55	75	100	100

Table 111 gives the schedule for iso-amyl alcohol when used as a dehydration and infiltration agent again showing the time periods and steps involved.

Table 111. Iso-amyl-alcohol as a Dehydration and Infiltration

	Agent			
7 iso-amyl alcohol	18 hrs	18 hrs	18 hrs	18 hrs
8 iso-amyl alcohol	25 hrs	25 hrs	25 hrs	25 hrs
9 iso-amyl alcohol	5 hrs	5 hrs	5 hrs	5 hrs
10 iso-amyl alcohol	18 hrs	18 hrs	18 hrs	18 hrs
11 iso-amyl alc. and paraffin	7 hrs	7 hrs	7 hrs	7 hrs
Paraffin	24 hrs	24 hrs	24 hrs	24 hrs
Paraffin	24 hrs	24 hrs	24 hrs	24 hrs
Paraffin	46 hrs	46 hrs	46 hrs	46 hrs

Again in this method as in the tertiary-butyl-alcohol method only the higher grades of the alcohol were used. The grades of alcohol used were the same as those used in the tertiary-butyl series.

Table 1V gives the schedule for dioxan when used as a dehydration and infiltration agent again showing the time periods and steps involved.

Table IV. Dioxan as a Dehydration and Infiltration Agent.

$\frac{1}{2}$ 70% alc. and $\frac{1}{2}$ dioxan	17 hrs	17 hrs	17 hrs	17 hrs
Dioxan I (Used dioxan)	25 hrs	25 hrs	25 hrs	25 hrs
Dioxan II (Pure dioxan) and paraffin	25 hrs	25 hrs	25 hrs	25 hrs
Paraffin	26 hrs	26 hrs	26 hrs	26 hrs
Paraffin	27 hrs	27 hrs	27 hrs	27 hrs
Paraffin	46 hrs	46 hrs	46 hrs	46 hrs

After infiltration with paraffin the material was embedded in the usual manner. The embedded material was then mounted on wooden blocks and sectioned 10 microns in thickness with a rotary microtome. Sections of the material were mounted and stained with Harris's Haemotoxin and Orange G. Thus, with the exception of the continued dehydration and infiltration processes, the sections received similar treatment.

Observations and Discussion

In observing the effect of the dehydrating and infiltrating agents upon the liver and the kidney of the Necturus used in this investigation the following points were considered:

1. Facility with which the material may be sectioned,
2. Shrinkage, 3. Effects on the cytoplasmic and nuclear structure of the cell, and 4. Staining properties, especially the facility with which the material may be stained.

Difficulty in sectioning was experienced with the material prepared in ethyl-alcohol-xylol. The use of alcohol and xylol seemed to harden the material. After placing the mounts in ice-water, they sectioned with more ease. This may have been due to the softening of the paraffin. The mounts prepared with

tertiary-butyl-alcohol, iso-amyl-alcohol and dioxan were sectioned with ease. These dehydrating and infiltrating agents seemed in no way to harden the material. The kind of dehydrating and infiltrating agent used produced no effect on the finer cytological structure. A microscopic examination of the finished slides showed that these agents caused no shrinkage, or any other injury to the cytoplasmic and nuclear structure of the cell. The results were equally good. However, the sections which had the air pump used on them sectioned with greater ease than did those without the use of the air pump. It seems that the air pump helps to make the dehydration process more complete.

The good results obtained no doubt were due to the fact that in the primary dehydration only the lower grades of ethyl alcohol were used.

Johansen (1937) claimed that, "Extensive experimentation has demonstrated that the most satisfactory dehydration is predicated upon the use of ethyl alcohol in conjunction with tertiary-butyl alcohol, as with practically all other dehydration fluids." In his work for preliminary dehydration, the tissues, after being washed, were passed through a series of 5%, 11%, 18%, and 30% or 35% ethyl alcohol allowing one hour each percentage. The writer used a more simple series of ethyl alcohol than did Johansen. She passed the material from the Bouin's fixing fluid directly into 50% ethyl alcohol. The material was retained in the 50% alcohol, changing at 24 hour intervals, until the alcohol retained it's clear color. From the 50% it was passed into the 70% alcohol. Since good dehydration was obtained, it can be assured that the closely graded series of alcohol used by Johansen are not absolutely necessary.

Another advantage of the series used in this study is that the material may be left in the 70% ethyl alcohol for a considerable length of time without any detrimental effect upon the tissue. It has been found that the material can be left in the 70% ethyl alcohol for only 2 hours and 40 minutes.

From the 30% ethyl alcohol Johansen transferred the material to the 50% alcohol mixture of water, 95% ethyl alcohol, and tertiary-butyl alcohol and continued through the various grades as indicated in the following table. He allowed two hours for each grade.

Percentage of alcohol.....	50	70	85	100	100
Distilled water.....	30	30	15	0	0
95% ethyl alcohol.....	40	50	50	45	0
Tertiary-butyl alcohol....	10	20	35	55	75
100% ethyl alcohol.....	0	0	0	0	25

Comparing these grades of alcohol with those used by the author we find that Johansen's schedule is more complicated and involved. In using the schedule in this study, there is a complete dehydration assured because another change of 100% tertiary-butyl-alcohol is made. The use of absolute ethyl alcohol was eliminated. For the time periods Johansen claimed that, "For not too large pieces of animal tissue an hour in each solution is sufficient; for larger animal tissues or organs and for the majority of plant tissues, two hours in each fluid is required." Johansen further claimed, "Tissues may, if necessary, be left overnight in any of the various percentages but may become slightly hardened as a result."

In this respect the author found that no hardening resulted even though the material was left in some grades of the alcohol as long as 18 hours.

Using a graded series of tertiary-butyl alcohol and water, Ralph (1938) tried to eliminate differences due to variations of the density of the agents but discovered considerable shrinkage by this method.

The General Biological Supply House (1934) published a study of dioxan in conjunction with ethyl alcohol. Using Zenker's as a fixing agent, human tissues were used. After washing with 70% alcohol, the tissues were passed through, 1. 1/3 dioxan and 2/3 alcohol, 2. 2/3 dioxan and 1/3 alcohol, 3. pure dioxan with one change of dioxan at intervals of 1½ hours for each step. The results showed no shrinkage or distortion of the tissue. Using dioxan in conjunction with 70% alcohol the author found that two steps, after preliminary dehydration with ethyl alcohol and before infiltration, were sufficient for good results. This may be attributed to the fact that in each step the material was kept in dioxan for longer periods.

Other workers used either the pure dioxan, "fast dioxan," or dioxan mixed with water, "slow dioxan." Baird (1936) prefers the slow dioxan method to the fast method and claimed that "grading dioxan as one would alcohol proved to be of no advantage." They did encounter some hardening but this was remedied by placing the paraffin blocks in water before sectioning. Mossman (1937) in summarizing

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the available information in regard to the dioxan method claims that "...One is inclined to believe that many of the faults attributed to the use of dioxan may actually have their origin in some other part of the technic or in the careless use of the dioxan itself." Ralph (1938) also found that the slow dioxan method is the better method of dehydration. The author agrees with Mossman, for in using dehydration. The author agrees with Mossman, for in using dioxan in conjunction with ethyl alcohol, preceded with a preliminary dehydration in ethyl alcohol, excellent results were obtained.

All of the material used in this study was embedded in Tex-Wax which has a melting point of 51°C. All of the blocks containing the material sectioned into nice long ribbons without any tearing or cracking. The only exceptions were those dehydrated according to the ethyl-alcohol-xylool series. The long periods of infiltration with paraffin did not have any shrinking or hardening effect upon the material.

The staining process was not affected by the dehydrating and infiltrating agents, for all of the material stained well and readily regardless of the dehydrating and infiltrating agents used.

Summary and Conclusions

1. The ethyl-alcohol-xylool series produces a hardening effect upon the tissue.
2. Successful dehydration and infiltration is obtained when ethyl alcohol is used in conjunction with tertiary-butyl

alcohol, iso-amyl alcohol or dioxan.

3. Keeping the material in the higher grades of tertiary-butyl alcohol, iso-amyl alcohol or dioxan for long periods of time is not detrimental to the finer details of the cell structure.
4. The material sectioned with ease when the mounts were placed into ice water just before sectioning.
5. After 70% ethyl alcohol, one grade of ethyl alcohol and dioxan is sufficient, before passing to pure dioxan, for complete dehydration.
6. Long periods of infiltration with paraffin did not have any shrinking or hardening effects upon the material.
7. The type of dehydrating and infiltrating agent used did not effect the facility with which the material was

stained.

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