A COMPARATIVE STUDY OF SOME DEHYDRATION AND CLEARING AGENTS

PAUL RALPH, *Oklahoma Agricultural and Mechanical College, Stillwater, Oklahoma*

Reprinted from STAIN TECHNOLOGY, Vol. 13, No. 1, January, 1938

A COMPARATIVE STUDY OF SOME DEHYDRATION AND CLEARING AGENTS

BY

PAUL RALPH

BACHELOR OF ARTS WESTMINSTER COLLEGE FULTON, MO. 1936

SUBMITTED TO THE DEPARTMENT OF ZOOLOGY

OKLAHOMA AGRICULTURAL AND MECHANICAL COLLEGE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

1937

APPROVED:

Geo. A. Moore — In Charge of Thesis

R. O. Whitenton — Head of the Department of Zoology

D. C. McIntosh — Dean of the Graduate School

A COMPARATIVE STUDY OF SOME DEHYDRATION AND CLEARING AGENTS[1]

PAUL RALPH, *Oklahoma Agricultural and Mechanical College, Stillwater, Oklahoma*

ABSTRACT.—**An experiment to determine the advantages of dioxan, iso-butyl alcohol, tertiary butyl alcohol, and ethyl alcohol as dehydrants and chloroform, toluol, xylene, benzol, methyl benzoate, methyl salicylate, and acetone as clearers is described. Materials fixed in Bouin's fluid, Zenker formol, and 10% neutral formalin were dehydrated, embedded, sectioned, and stained. Bouin's fluid produces less hardening, shrinkage and distortion than the other fixatives employed. Slow dioxan is the best method of dehydration. All the picric acid need not be removed from tissues to be embedded in paraffin. Tissue blocks not more than 4 mm. thick may be dehydrated and impregnated with paraffin by slow dioxan in 13 hours, fast dioxan in 10 hours, iso-butyl alcohol and tertiary butyl alcohol in 14 hours, and ethyl alcohol-chloroform in 17 hours without incurring any distortion due to rapidity of dehydration and infiltration.**

Recently many workers have been concerned with the problem of finding reagents which would produce a minimum of distortion, shrinkage, and hardening in the paraffin method of embedding animal tissues. Their investigations have followed three lines: that of finding reagents to take the place of ethyl alcohol as a dehydrant, the search for an improvement upon xylene as a clearer following ethyl alcohol, and the use of different types of fixatives.

The purpose of this study has been to coördinate these three lines of investigation and if possible to arrive at some conclusion as to which reagent or reagents produce the least amount of shrinkage, hardening, and distortion. Dioxan, iso-butyl alcohol, tertiary butyl alcohol, and ethyl alcohol were used as dehydrants. Chloroform, toluol, xylene, methyl benzoate, benzol, methyl salicylate, and acetone were the clearers used to follow ethyl alcohol. Fixatives used were 10% neutral formalin, Zenker formol, and Bouin's fluid, chosen because they represent the three most widely used reagents.

Shrinkage has been attributed to improper fixation, exposure to air during the process of fixation and dehydration, and the action of

[1]Contribution from the Zoology Laboratory, Oklahoma Agricultural and Mechanical College; prepared under G. A. Moore. For helpful suggestions the writer is indebted to Dr. J. E. Lynch, Department of Fisheries, University of Washington.

reagents. Hardening has been attributed to the type of fixative used, the influence of hot paraffin, and the action of reagents. Distortion has been largely attributed to changing tissues from liquids of varying densities and temperatures, and to pronounced shrinkage of particular organ elements such as connective tissue. In this study an effort was made to remove any possibility of differences in results due to factors other than the characteristic effects of the dehydrant, clearer, and fixative.

Four tissues were used—thyroid gland, intestine, liver, and skeletal muscle. These were taken from the same animal and fixed at the same time. From each tissue 5 blocks were fixed in Zenker formol, 5 in formalin, and 11 in Bouin's fluid. Small adjacent pieces, 4 mm. × 4 mm. × 1 cm. were fixed (24 hours) to insure best results. Blocks fixed in Bouin's fluid were transferred directly from the fixative to the first step of dehydration. Those in formalin were washed in running tap water for 10 hours, those in Zenker formol for 15 hours, before starting dehydration.

It will be noted that except in the case of "fast dioxan," dehydration was effected gradually in order to eliminate differences due to variations in the density of the reagents. This has not been taken into account in some previous studies. "Slow dioxan", fast dioxan, iso-butyl alcohol, tertiary butyl alcohol, and ethyl alcohol, followed by the clearers already mentioned, were used. Because of the relative immiscibility of iso-butyl alcohol with water, it was found advisable to extract most of the water with ethyl alcohol. The remainder of the water and the ethyl alcohol were removed with iso-butyl alcohol, with which ethyl alcohol is quite miscible.

The paraffin mixtures were kept at 54–56 degrees C. during the process of embedding. The embedding medium was a 3.5% mixture of yellow beeswax in soft paraffin.

The detailed procedures for dehydration and clearing are as follows:

Iso-butyl alcohol: 30% ethyl alcohol, 2 hr.; 50% ethyl alcohol, 2 hr.; 70% ethyl alcohol, 2 hr.; 95% iso-butyl alcohol, 3 hr.; iso-butyl alcohol, 1 hr.; 50% iso-butyl alcohol, 50% paraffin, 1 hr.; hard paraffin, 2 hr.; hard paraffin, 1 hr.; embed.

Tertiary butyl alcohol: 30% tertiary butyl alcohol, 2 hr.; 50% tertiary butyl alcohol, 2 hr.; 70% tertiary butyl alcohol, 2 hr.; 95% tertiary butyl alcohol, 3 hr.; tertiary butyl alcohol, 1 hr.; 50% tertiary butyl alcohol, 50% paraffin, 1 hr.; hard paraffin, 2 hr.; hard paraffin, 1 hr.; embed.

Slow dioxan: 33% dioxan, 2 hr.; 67% dioxan, 2 hr.; dioxan, 4 hr.;

33% paraffin, 67% dioxan, 1 hr.; 67% paraffin, 33% dioxan, 1 hr.; hard paraffin, 2 hr.; hard paraffin, 1 hr.; embed.

Fast dioxan: Dioxan, 2 hr.; dioxan, 4 hr.; 50% paraffin, 50% dioxan, 1 hr.; hard paraffin, 2 hr.; hard paraffin, 1 hr.; embed.

Ethyl alcohol-chloroform: 30% ethyl alcohol, 2 hr.; 50% ethyl alcohol, 2 hr.; 70% ethyl alcohol, 2 hr.; 95% ethyl alcohol, 3 hr.; ethyl alcohol, 1 hr.; 50% ethyl alcohol, 50% chloroform, 1 hr.; chloroform, 1 hr.; 67% chloroform, 33% paraffin, 1 hr.; 33% chloroform, 67% paraffin, 1 hr.; hard paraffin, 2 hr.; hard paraffin, 1 hr.; embed.

Ethyl alcohol-toluol,-xylene,-benzol,-methyl benzoate,-methyl salicylate, and -acetone: the procedures for these clearers are the same as that of ethyl alcohol-chloroform except that a mixture of 50% clearer and 50% paraffin is used for 1 hr. instead of the two chloroform-paraffin baths for 2 hr.

The blocks were cut at 10 μ, the room temperature ranging from 22.5 to 31° C. The sections were flattened by placing the ribbon on water at 40° C. This permitted perfect flattening of the sections without stretching or distortion. The sections were floated on slides lightly coated with Mayer's albumen. The slides were dried, decerated in toluol, passed down to absolute alcohol after which they were immersed in 0.5% pyroxylin, and dried in air until a slight film appeared on the pyroxylin. The slides were plunged into 95% alcohol and downgraded to Harris's hematoxylin in which they were stained. After upgrading they were counterstained with triosin in 95% alcohol, after which they were soaked in absolute alcohol until the pyroxylin was removed. The slides were then immersed in 1% pyroxylin[2] which was hardened in chloroform, after which they were cleared in toluol. A comparison was made of two sets of slides which differed only with regard to the second coat of pyroxylin. The slides without the second coating showed much more shrinkage, particularly of the connective tissue layers. This was due, no doubt, to the shrinking effect of the toluol in clearing.

Tables 1 and 2 give the results of the procedures mentioned above. Due to the fact that there was no objective means of measuring the qualities of the blocks and slides, they were examined independently by two investigators and their joint findings compiled. In regard to impregnation it was found that for tissue blocks of this size, three hours in paraffin were sufficient to bring about complete infiltration (for results see tables—column 2 under impregnation). In order to investigate the reagents in regard to impregnation the experiment was repeated with the same procedure except that the tissues were left in

[2]Equivalent of celloidin as suggested by Galigher (1934).

TABLE 1. TABLE SHOWING THE EFFECT OF DEHYDRATING AND CLEARING REAGENTS ON THYROID GLAND AND SKELETAL MUSCLE. SD, SLOW DIOXAN; FD, FAST DIOXAN; IA, ISO-BUTYL ALCOHOL; TA, TERTIARY BUTYL ALCOHOL; EA, ETHYL ALCOHOL; CHL, CHLOROFORM; TOL, TOLUOL; BEN, BENZOL; M BEN, METHYL BENZOATE; M SAL, METHYL SALICYLATE; ACE, ACETONE; SL, SLIGHTLY

TISSUE	FIXATIVE	DEHYDRATION	CLEARER	IMPREGNATION				SECTIONS				MICRO-EXAM					
				1		2						Shrinkage			Cracking		
				Com	Inc	Com	Inc	Soft	Medium	Hard	Shatters	None	Medium	Bad	None	Medium	Bad
THYROID	ZENKER	SD		+		+		+				+				+	
		FD		+		+		+					+			+	
		IA		+		+		+				+			+		
		TA				+			+	.			+			+	
		EA	CHL	+		+		+					+		+		
	FORMALIN	SD			+	+			+				+		+		
		FD		+		+		+					+		+		
		IA		+		+		+					+			+	
		TA				+		+					+		+		
		EA	CHL	+		+		+						+	+		
	BOUIN	SD		+		+			+			+			+		
		FD			+	+		+				+			+		
		IA		+		+		+					+			+	
		TA				+		+					+		+		
		EA	CHL	+		+		+					+			+	
		EA	TOL		+	+		+					+				+
		EA	XYLOL	+		+		+					+			+	
		EA	BEN	+		+		+					+			+	
		EA	M BEN		+	+		+						+		+	
		EA	M SAL		+	+		+					+			+	
		EA	ACE		+	+		+					+			+	
SKELETAL-MUSCLE	ZENKER	SD			+	+			+		+			+			+
		FD		+		+				+	+			+		+	
		IA		+		+				+	+			+			+
		TA				+				+				+			+
		EA	CHL	+		+				+				+		+	
	FORMALIN	SD		+		+		+					+			+	
		FD		+		+		+					+			+	
		IA			+	+				+	+			+			+
		TA				+			.	+	+			+		+	
		EA	CHL	+		+				+	+			+		+	
	BOUIN	SD		+		+		+				+			+		
		FD		+		+				+		+			+		
		IA		+		+			+		SL		+		+		
		TA				+			+		SL		+		+		
		EA	CHL	+		+		+					+		+		
		EA	TOL	+		+				+			+		+		
		EA	XYLOL	+		+			+		+		+		+		
		EA	BEN	+		+			+		SL	+			+		
		EA	M BEN	+		+				+	+		+		+		
		EA	M SAL	+		+			+		+		+		+		
		EA	ACE	+		+			+		SL		+			+	

TABLE 2. TABLE SHOWING THE EFFECT OF DEHYDRATING AND CLEARING REAGENTS ON LIVER AND INTESTINE. SD, SLOW DIOXAN; FD, FAST DIOXAN; IA, ISO-BUTYL ALCOHOL; TA, TERTIARY BUTYL ALCOHOL; EA, ETHYL ALCOHOL; CHL, CHLOROFORM; TOL, TOLUOL; BEN, BENZOL; M BEN, METHYL BENZOATE; M SAL, METHYL SALICYLATE; ACE, ACETONE; SL, SLIGHTLY

Tissue	Fixative	Dehydration	Clearer	Impregnation 1 Com	Impregnation 1 Inc	Impregnation 2 Com	Impregnation 2 Inc	Sections Soft	Sections Medium	Sections Hard	Sections Shatters	Micro-Exam Shrinkage None	Shrinkage Medium	Shrinkage Bad	Cracking None	Cracking Medium	Cracking Bad
LIVER	ZENKER	SD		+		+		+									
		FD		+		+		+									
		IA			+	+		+									
		TA				+		+									
		EA	CHL	+		+			+								
	FORMALIN	SD		+		+		+									
		FD		+		+		+									
		IA		+		+		+									
		TA				+		+									
		EA	CHL	+		+		+									
	BOUIN	SD		+		+		+									
		FD		+		+		+									
		IA			+	+			+								
		TA				+		+									
		EA	CHL	+		+		+									
		EA	TOL	+		+		+									
		EA	XYLOL	+		+		+									
		EA	BEN	+		+		+									
		EA	M BEN	+		+		+									
		EA	M SAL	+		+		+									
		EA	ACE	+		+		+									
INTESTINE	ZENKER	SD		+		+		+				+			+		
		FD		+		+		+					+		+		
		IA			+	+		+					+			SL	
		TA				+			+					+		+	
		EA	CHL		+	+		+					+		+		
	FORMALIN	SD		+		+			+					+	+		
		FD		+		+				+			+		+		
		IA		+		+				+				+		SL	
		TA				+			+				+			SL	
		EA	CHL	+		+			+				+			+	
	BOUIN	SD		+		+		+				+				+	
		FD		+		+			+			+				+	
		IA		+		+		+					+				+
		TA				+			+				+			+	
		EA	CHL	+		+		+					+				+
		EA	TOL	+		+		+					+				+
		EA	XYLOL	+		+		+					+			+	
		EA	BEN	+		+		+				+					+
		EA	M BEN	+		+		+				+					+
		EA	M SAL	+		+		+				+				+	
		EA	ACE	+		+		+					+				+

hard paraffin only two hours. The results of this experiment are tabulated in column 1 under impregnation. Iso-butyl alcohol shows the greatest difficulty in impregnation, probably due to its limited miscibility with paraffin and water. Dioxan showed a tendency to require more time in infiltration than all other dehydrants except iso-butyl alcohol. Slow dioxan after fixation in Zenker formol and in formalin produced much softer blocks than all other methods. After fixation with Bouin's fluid, ethyl alcohol-chloroform, ethyl alcohol-xylene, ethyl alcohol-benzol, ethyl alcohol-methyl salicylate, and ethyl alcohol-acetone produced blocks equally soft, but with some shattering. Of all the clearers tried chloroform produced the softest blocks and toluol and methyl benzoate the hardest blocks. Xylene, benzol, methyl salicylate, and acetone were of equal value. Slow dioxan and fast dioxan seemed to be equally effective in eliminating shrinkage and excelled all other methods. The most shrinkage was produced by iso-butyl alcohol and tertiary butyl alcohol. Ethyl alcohol-chloroform, ethyl alcohol-toluol, ethyl alcohol-methyl benzoate, ethyl alcohol-acetone, ethyl alcohol-benzol, and ethyl alcohol-methyl salicylate were of equal value. There was less cracking with slow dioxan, fast dioxan, and tertiary butyl alcohol than with other methods.

In regard to fixation, Bouin's fluid produced by far the best results, with softer blocks and less shrinkage. There was little shattering with even the most difficult tissues. To determine the effects of the presence of picric acid in tissues an experiment was conducted with duplicate tissue blocks and with the same procedure, except that in one all of the picric acid was washed out with lithium carbonate in 70% alcohol. There were no differences in cutting consistency between blocks prepared in this manner and those in which much of the picric acid was left.

Zenker formol produced the hardest blocks of the three fixatives used. The hardening took place mostly during fixation. In regard to shattering Zenker formal and 10% formalin were of approximately the same value, quite inferior to Bouin's fluid. Formalin produced initial softness but much hardening took place during dehydration and embedding. Formalin fixation was attended in all cases by a disintegration, and an alternation of the staining reaction, of thyroid colloid, and produced considerable shrinkage. It was the least satisfactory of the fixatives used.

The best complete method was found to be Bouin's fixation with dehydration by the slow dioxan method. For student use, however, this has the objection of expense coupled with some danger from inhalation of the fumes. Perhaps the most satisfactory method for student work is Bouin's fixation followed by ethyl alcohol-chloro-

form. This method produces soft blocks with little shrinkage or cracking.

Conclusions

(1) Bouin's fixative produces less hardening, shrinkage, and distortion than the other fixatives employed.

(2) Slow dioxan is the best method of dehydration.

(3) Picric acid need not all be removed from tissues to be embedded in paraffin.

(4) Tissue blocks 4 mm. thick may be dehydrated and impregnated with paraffin by slow dioxan in 13 hr., fast dioxan in 10 hr., and ethyl alcohol-chloroform in 17 hr., without incurring distortion due to rapidity of dehydration and infiltration.

BIBLIOGRAPHY

Baird, T. T. 1936. Comparative study of dehydration. Stain Techn., **11**, 13–22.

Bradbury, O. C. 1931. A new dehydrating agent for histological technique. Science, **74**, 255.

Farmer, W. F. A. 1929. A paraffin method without ethyl alcohol. Arch. Pathol., **7**, 1040–1.

Feldman, W. H. 1927. The carbon disulphide-paraffin method of embedding tissues, and their subsequent handling and staining by haematoxylin and eosin. Arch. Pathol. & Lab. Med., **4**, 979–83.

Galigher, A. E. 1934. The Essentials of Microtechnique. Albert E. Galigher, Inc., Berkeley, California.

Graupner, H. and Weissberger, A. 1931. Über die Verwendung des Dioxans beim Einbetten Mikroskopischer Objekte. Mitteilungen zur mikroskopischen Technik I. Zool. Anz., **96**, 204–7.

1933. Die Verwendung von Lösungen in Dioxan als Fixierungsmittel für Gefrierschnitte. Zool. Anz., **102**, 39–44.

Johansen, D. A. 1935. Dehydration and infiltration. Science, **82**, 253–4.

1937. Tertiary butyl alcohol methods. El Palo Alto News, **1**, 1–3 and 5–6.

Kisser, J. 1933. Neue Wege und Erfahrungen auf dem Gebiete der Paraffinmethode. Cytologia, **4**, 288–304.

McWhorter, F. P. and Weier, E. 1936. Possible uses of dioxan in botanical microtechnic. Stain Techn., **11**, 107–17.

Sass, J. E. 1932. Acetone as a substitute for alcohol in microtechnic. Stain Techn., **7**, 65–6.

Tarkhan, A. A. 1931. The effect of fixatives and other reagents on cell size and tissue bulk. J. Roy. Micr. Soc., **51**, 387–400.

Underhill, B. M. L. 1932. The rate of penetration of fixatives. J. Roy. Micr. Soc. **52**, 113–20.

Weissberger, A. J., Young, J. Z., and Carleton, H. M. 1934. On the use of dioxan for transference of tissues direct from water to paraffin wax. Lancet, **227**, 1279–80