

STUDIES ON CELL DIVISION IN THE ALBINO RAT (MUS NORVEGICUS, VAR. ALBA)

II. EXPERIMENTS ON TECHNIQUE, WITH DESCRIPTION OF A METHOD FOR DEMONSTRATING THE CYTOLOGICAL DETAILS OF DIVIDING CELLS IN BRAIN AND TESTIS

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ONE TEXT FIGURE AND TWO PLATES

CONTENTS

Introduction: the purpose of the experiments.....	565
The scope of the experiments.....	566
Experiments on fixatives.....	567
The fixative which proved best.....	569
Results of dehydration experiments.....	570
The efficacy of the drop method.....	570
An automatic apparatus for dehydrating and washing.....	571
The value of anilin oil as a substitute for the higher alcohols.....	574
Results of clearing experiments.....	575
Range of oils tried.....	575
The best clearing oils.....	575
Detailed account of the technique as finally developed.....	576
Discussion of the results of the technique.....	577
Cytological.....	577
Volumetric.....	580
Conclusions.....	583
Bibliography.....	585

INTRODUCTION

This paper deals with a series of experiments on technique by which a method has been developed for demonstrating the details of mitosis in the central nervous system and testis of the albino rat without producing distortion of the other parts of the tissues. The work was begun because of difficulty experienced

in positively identifying dividing cells while conducting experiments on influencing the rate of cell division in the cerebellum of the albino rat. In the external granule layer of a sick individual seven days old, I found a large number of peculiar cells which oftentimes resembled certain phases of dividing cells. The tissue had been fixed in Carnoy's fluid and treated according to the method previously used in my study determining the duration of cell division in the central nervous system of the albino rat (Allen '12). At Professor McClung's suggestion I attempted to secure a technique which would insure the cytological details desired.

King ('10), after a thorough and exhaustive set of experiments on various standard fixatives for brain tissue, showed that for general histological purposes Ohlmacher's fixing fluid followed by double imbedding with celloidin and paraffin gave the best results. This method, however, fails to prevent the chromosomes from fusing, and consequently was inadequate for the purpose in hand.

My experiments grew to a series of tests dealing with each step in the preparation of sections, finally resulting in a method which not only clearly differentiates dividing cells in all stages, but also prevents fusing, or 'clumping,' of the chromosomes, a distinct gain in the technique for mammalian tissue. Other workers in the Zoological Department of the University of Pennsylvania are experimenting on other tissues. The results of their work will also be published, so that this paper forms one of a series on the problems of technique.

THE SCOPE OF THE EXPERIMENTS

It is needless to record all the details of the work done or of the results obtained. The general plan and scope of the work are as follows: While a few experiments were made on a pure trial and error basis, with the hope that something desirable would result, most of them were carefully planned along the line of modifying approved methods. The success of each experiment was measured by two criteria: (1) the isolation and definition of the

chromosomes and (2) the 'general fixation.' The term 'general fixation' covers the points of shrinkage or swelling of the whole mass, and the degree of distortion of one part or of one tissue. A good general fixation describes the condition of a preparation which has suffered only slight changes in total volume, either by shrinkage or swelling, and in which the individual parts maintain their normal relationship of volume and structure; that is, the connective tissues are not torn, the limiting membranes retain their size and smooth outlines, while the cells show no vacuoles about them nor cytolysis of any kind.

For a long time it looked as if excellence of general fixation would have to be sacrificed to distinctness of the chromosomes, so that I was particularly pleased when the best chromosome differentiation obtained was accompanied by the best general fixation. A comparison of figures 2, 9, 10, 13 and 15 with figures 4, 7, 12, 14 and 17 respectively will show the contrast between poor and good preparations according to the standards just described.

EXPERIMENTS ON FIXATIVES

The first line of investigation followed was that of modifying the proportions of constituent parts of Bouin's fluid, since this fixative had proved so excellent for general purposes. The proportions are given in table 1. The first set of experiments was with the first six modifications shown in table 1. One promising fluid appeared in modification no. 2. It failed, however, upon further trial to yield constant or fully satisfactory results, though it seems to be a better fixative for albino rat tissue than Bouin's combination.

After thorough trial of the picro-formol-acetic combinations as far as no. 14 in the table, the following fluids were used: Flemming's, Worcester's, Lavdowsky's, Carnoy's (often called van Gehuchten's), Ohlmacher's, King's potassium-bichromate-acetic-sublimate, and Tellyesniczky's. Urea was added to each in varying proportions for studying its effects; fixatives were made up in normal salt solution; different temperatures—0°C., room and 30 to 40°C.—and different fixation periods were tried with any

TABLE 1

Showing the various combinations of picric acid, formol, acetic acid, corrosive sublimate, chromic acid and urea employed. Fluids are measured in cubic centimeters; solids, in grams; the percentages are thus indicated

SOLUTION	PICRIC ACID; SATU- RATED AQUEOUS SOLUTION	FORMOL	GLACIAL ACETIC ACID	CORRO- SIVE SUB- LIMATE SATU- RATED; AQUEOUS SOLUTION	SALT; CRYSTALS	UREA; CRYSTALS	CHROMIC ACID; CRYSTALS	WATER
Bouin ¹	75	25	5					
No. 1	75	15	5					
2	75	10	10					
3	75	15	10					
4	75	10	5					
5	75	5	5					
6	75	10	10	5				
7	50	10	10					25
8	90	5	5		0.9	0.5		
9	75	10	10		0.9	1.5		
10	75	15	10		0.9	1.5		
11	75	10	10			0.1		100
12	75	10	10			1.5		300
13	75	25	5			4.0		300
14	75	25	5		0.9	0.5		
15	75	25	5			2.0	1.5	
16	75	20	3			1.0	1.0	
17	75	5	1			1.0	0.5	
18	75	20	7			0.3	0.5	

¹ Lee ('13).

fluid which seemed hopeful on first use. Various strengths of formalin, with and without sugar, and different combinations of acetone and formalin were also tried. See table 2.

A second promising fluid resulted from the addition of a little dilute chrome-acetic mixture (one of the parts of Flemming's fluid) to the Carnoy fluid. The general fixation was excellent and the dividing cells in the brain showed better cytological detail than with anything else tried up to that time. This combination was therefore tested. The constituents were mixed in different proportions; and different temperatures, periods of fixation and quantities of urea tried out. Chromic acid yielded better and better results upon the chromosomes as its percentage was increased, but the mitotic figures were still not satisfac-

TABLE 2

Showing some of the various combinations of fluids used based upon either formol, acetone or Carnoy. The measurements are the same as in table 1

SOLUTION	FORMOL (NEU- TRAL)	ACE- TONE	ALCO- HOL (100 PER CENT)	CHLO- ROFORM	GLACIAL ACETIC ACID	CHRO- MIC ACID; CRYSTALS	CORRO- SIVE SUBLI- MATE; CRYSTALS	UREA; CRYSTALS	SUGAR; CRYSTALS	WATER
No. 1 } McClendon }	10								2.0	88
2	10							1	1.0	90
3	10								0.5	90
4	1							1	1.0	98
5	2							1	1.0	98
6	5							1	1.0	94
7	2		48					1		50
8	1	90						1		10
9	10	50						1	1.0	40
10	5	50						1	1.0	40
11	10		50					1		45
12	5		50					1	1.0	45
Carnoy			60	30	10					
13			60	30	5	1.0				
14			98			5.0				2
15			60	30	10	1.5		2		
16	5		60	30		1.5		2		2
17			60	30		1.5		2		2
Ohlmacher			80	15	5		Sat- ura- tion			

tory for accurate work when the concentration had reached a point in which the rest of the tissues were torn and distorted worse than the results of Flemming's fluid shown in figure 13. Some of the combinations used are shown in table 2, nos. 13 to 17. Formol and acetic acid both swell tissues. For this reason formol was substituted for the acetic acid in several trials, only one of which is shown in the table (no. 16, table 2).

Finally one half of a seven day brain was put into the combination of chromic acid and Carnoy's fluid which had given the best results, and the other half in some Bouin's fluid to which I added at the same time some chromic acid and urea. The half placed in the latter fluid showed all the good points of general

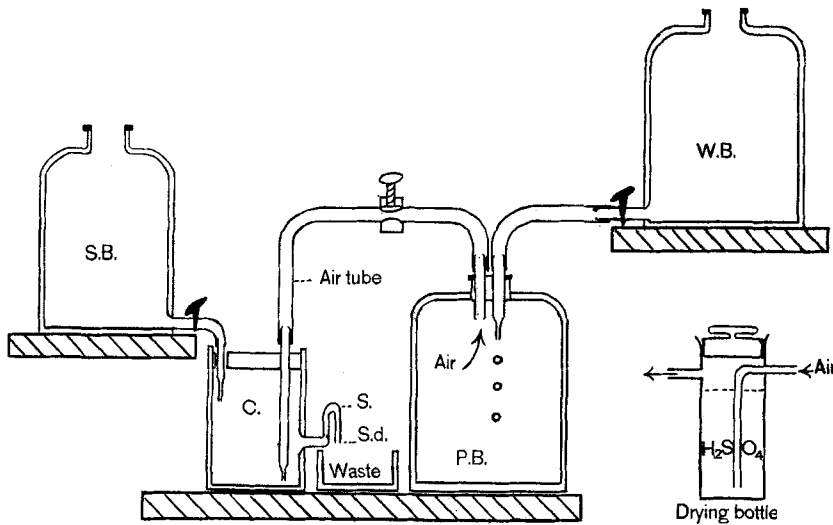
fixation gotten by Bouin's fluid with the addition of well differentiated chromosomes, while the other half was useless in comparison. Further trials of the new fixative (no. 15, in table 1) yielded practically constant results and later when applied to the testis gave the material from which figures 12, 14 and 17 were made. The exact proportions used appear in table 1, no. 15. This fixative will henceforth in this paper be spoken of as the picro-formol chrome-acetic in urea fixative, or for convenience 'B-15.'

DEHYDRATION

The well recognized necessity of a very slow change from a fluid of one density and osmotic pressure to another which differs in these respects, in order that cytolysis may be avoided, led to a consideration of how this change might be easily and effectively accomplished. Professor McClung while working on grasshopper testis found that if the higher grades of alcohol were added drop by drop better results were obtained than by transferring the tissue from water to 35 per cent, 50 per cent, 60 per cent alcohol, etc. To accomplish this purpose he used the principle of capillarity in a cotton cord or a fine glass siphon. This method, however, when applied to more than two or three cubic centimeters of fluid did not insure mixing, the lighter liquid remaining on top of the heavier. Even when the lighter liquid is introduced through a tube to the bottom of the heavier, it rises immediately and accumulates on top of the heavier in an independent layer and remains there for days, as is readily shown by introducing colored alcohol in the manner described. I have found that a flow of air gently and rapidly mixes the fluids of different density. The current may be obtained from a pressure bottle when compressed air is not available in the laboratory. The apparatus devised for this purpose is shown in text-figure 1. It consists of two bottles of equal volume, one for water and the other for air pressures, marked *W.B.* and *P.B.* One of these is of the aspirator type. The remainder of the apparatus is a supply bottle for storing the alcohol or other replacing fluid, *S.B.*, also fitted with bottom stopcock; a container

for the tissue, *C*, fitted with a siphon opening from the side, *S*; a jar for receiving waste; glass and rubber tubing for connections as shown; corks and screw clamps or stopcocks of small bore, as indicated in the figure.

The size of the water and pressure bottles should be two or more liters, in order that little attention need be given the supply of air. A variety of sizes of the container is desirable, since a small amount of tissue needs only a small amount of fluid. For two or three pieces of 0.5 cc. volume a vessel of 30 cc. total



capacity is ample. Shell vials or shallow stender dishes are very suitable. One should avoid narrow, tall containers as handling the tissue in them is difficult. The supply bottle for alcohol may be large or small, depending upon the quantity of fluid needed. Where large quantities of tissue are being dehydrated or changed, several supply bottles will be convenient, one for each kind of fluid that is in use.

If aspirator bottles are not available, ordinary bottles fitted with siphons through their corks may be substituted.

The connections should be made as shown in the diagram. The cork for *P.B.* fits tightly; that for *C* is loose-fitting, or else the

hole for one of the tubes is slightly larger than the tube, to allow for escaping air. The cork for *S.B.* must also fit loosely.

To operate: after seeing that all connections are adjusted, fill the water bottle *W.B.* and open its stopcock slightly until the water begins to drop into the pressure bottle. As soon as the air pressure in this bottle overcomes the pressure of the fluid in *C*, bubbles will emerge from the mouth of the air tube and pass to the top of the fluid. A steady stream of air instead of bubbles is better, and may be produced by drawing out the end of the air tube into almost a capillary and clamping its rubber tube almost shut, thus forcing the air to accumulate under pressure in *P.B.*

Next start the alcohol dropping at the desired rate. The air and alcohol will continue to flow until their supply is exhausted in each case or until stopped by the operator. The siphon will remove the excess of fluid, so that the concentration of the alcohol is steadily rising in *C*. The waste jar must be large enough to accommodate the excess fluid.

A two-liter bottle for *W.B.* if filled will run all night at the rate of a drop a second. When the water has run over into the pressure bottle it is poured back into the water bottle and thus used repeatedly.

When the apparatus is used for higher alcohols or for oils, the air should be dried by passing it through a tube containing calcium chloride or through sulphuric acid. The latter method is easily accomplished by a gas washing bottle, shown at one side in the diagram.

As a concrete illustration, suppose that the tissue is to be raised from water to 75 per cent alcohol. It is placed in the container *C* and covered with the fixing fluid or with whatever fluid it may be in at the time. The air and alcohol are set flowing as previously described. Sometimes a difficulty appears in connection with the stopcocks or pressure clamps. Stoppage will occur in the rubber tubing so clamped after the clamp has been on for some days. Consequently the clamp must be moved occasionally. The stopcocks need turning wide open occasionally and then readjusted to the desired rate of flow.

The rate of dropping is affected by the pressure of the water above the stopcock in the water bottle, this pressure varying directly with the quantity of water. A little experimenting will usually suffice to regulate the flow satisfactorily.

If the tissue is very delicate and is seen to be moving about in the container, the rate of air admission may be slowed or a glass slide interposed between the air and the tissue. This should fit loosely enough to allow of free interchange of liquids about its edges but to prevent rapid currents at the same time. Another excellent scheme is to put the tissue into a smaller bottle, the sides of which have been perforated with numerous holes. This receptacle prevents the air currents from forcibly disturbing the tissue while at the same time it permits very free circulation of the fluids.

The siphon may be adjusted to discharge constantly, once it has started, or intermittently. If its discharge is at the same level as the point where it leaves the container (as shown in the cut), the flow will be constant. If the descending arm is longer than the ascending arm, thus lowering the discharge point, the flow will be intermittent.

By measuring the amount of water in the container at the beginning, one can readily calculate how much alcohol of a given strength will be required to bring the fluid in the container up to 70 per cent, or any other percentage desired. This quantity can be put into the supply bottle *S.B.*

The percentage of alcohol to be used at first varies somewhat with conditions. If the tissue is small compared with the volume of fluid in the container, 95 per cent may be used. On the other hand, if the quantity of fluid is small compared with the tissue it is better to start with 50 per cent, or 70 per cent and continue this until the tissue is in 50 per cent when 95 per cent may be run in. The new fluid may be introduced with constantly increasing speed as its concentration rises in the container.

The same apparatus is very convenient for washing out picric acid or corrosive sublimate, as the current of air hastens the process considerably, since the agitation constantly removes the layer of fluid which exudes from the tissue, and the interchange

is thus not dependent solely upon diffusion of fluids. If desired, alcohol of the same strength may be kept running into the container from the supply bottle; or the fluid in the container may be changed at intervals.

For passing to the higher alcohols and clearing oils, the same apparatus is employed in the same way as for bringing the tissue up to 70 per cent.

THE USE OF ANILIN IN PLACE OF THE HIGHER ALCOHOLS

While carrying on these experiments at Woods Hole, Dr. E. E. Just called my attention to Suchannek's ('90) use of distilled anilin oil as a substitute for the higher alcohols, and spoke of his own success in its use on heteronereis eggs. I tried it after 75 per cent alcohol. It produced no appreciable change in the volume of the tissue even if left in it for twenty-four hours, and I have since employed it entirely except after a Flemming fixation. Tissue so fixed is turned very black by it, even though previously very thoroughly washed.

Anilin does not mix with paraffin but is readily followed by any clearing oil. I have had no difficulty with its use. On the other hand, it seems to improve the tissue for sectioning and staining. By one distillation (the distillation point is 184°C.) anilin becomes a clear, pale-yellowish fluid. This keeps well if stored in the dark. The Kahlbaum anilin comes a pale red and can be used without distillation, but may darken the tissue somewhat.

King ('10) found that double imbedding prevented shrinkage of the nerve cell body. She says:

When a brain that has been fixed in Ohlmacher's solution is imbedded in paraffin after being cleared with chloroform or with any other substance commonly used for this purpose, there is invariably a shrinkage of the cell body, . . . and a condensation or vacuolization of the cytoplasm. If, however, the brain is imbedded in celloidin-paraffin, . . . there is no shrinkage evident anywhere in the cell.

referring to large cells in the cerebral cortex. I am confident that the use of anilin obviates this difficulty, and consequently avoids the shrinkage due to the higher alcohols essential to the

use of celloidin, and furthermore gives perfectly flat sections. The photographs in figures 3 and 4 are typical of the appearances produced by my method of treatment. Neither large nor small cells show vacuolization of cytoplasm nor shrinkage of the nucleus.

CLEARING

Xylol, as is well known (Lee '13) shrinks tissues considerably. I experimented with chloroform, oils of cedar, cassia, wintergreen and bergamot. Each produced less shrinkage than xylol. The effects in each case were measured on the whole mass of tissue, on the smoothness of outline of the individual tubules of the testis, and on nerve cells. The least shrinkage occurred with bergamot; wintergreen is a close second. Cassia (cinnamic aldehyde) seemed excellent for the testis, but since this does not mix with paraffin it must be followed by a paraffin solvent. It has the advantage of mixing with 85 per cent alcohol freely, and it is therefore useful after Flemming to avoid the higher alcohols. The pure synthetic preparations of all the oils are better than the natural products. The following illustrations serve to show the extent of shrinkage during the clearing process.

	cc.
Volume of brain when put into xylol.....	0.65
Volume of brain 2½ hours later.....	0.60
Volume of brain 23 hours later.....	0.50

The loss in this case was 23 per cent. In some cases it was as high as 25 per cent.

The effects of bergamot oil were compared with those of a special cedar oil which has the merit of mixing with alcohol in any proportion. I passed the halves of the same cerebellum through these oils respectively, and then measured ten undeveloped Purkinje cells from each preparation, carefully selecting those cut in the same way. Drawings were made with the aid of the camera lucida. The mean diameter of the ten fixed in bergamot oil was 9 units; that of the ten fixed in the special cedar oil was 8.1 units, a loss of 10 per cent. The diameters of the latter were consistently smaller throughout.

Testis fixed in Flemming seems less susceptible to change in volume at this stage in the technique than when fixed in one of the picro-formol-acetic combinations, an indication that this fixative (Flemming) has brought the tissue more nearly to its limit of shrinkage.

DETAILED STATEMENT OF THE METHOD

Formula for the fixative. (B-15; table 1, no. 15)

	cc.
Picric acid, saturated aqueous solution.....	75
Formol.....	25
Glacial acetic acid.....	5

In this fluid dissolve at time of fixing 1.5 gram of chromic acid in crystals, and then 2 grams of urea in crystals.

Fixation. Heat the fixative to 37° or 38°C., place the tissue in it and hold at that temperature. Pieces of brain 0.5 cc. in volume fix in one hour; pieces of young testis of the same or less volume require a little longer. The adult testis should be given two to three hours, depending on the age of the animal.¹ The external membranes of the tubules and the connective tissues become, with age, progressively less easy to penetrate. Plenty of fixing fluid is used. It is agitated several times during the fixation. To secure quick penetration of the testis it is necessary to cut the outer covering (the tunica albuginea), and in addition snip the tubules apart with scissors.

A low temperature paraffin bath is convenient for keeping the fluid at the desired temperature.

Dehydration. The fluid and contained tissue are cooled to room temperature and slow, gradual dehydration begun with alcohol as described previously (p. 578). The rate of dropping should be about one drop per second unless the quantity of fluid is very small, when it should be less rapid.

After this fixative, it is desirable to bring the tissue up to 75 per cent within an hour (for pieces 0.5 cc. in volume) if the tissue is soft; harder tissues need a little more time.

¹ The young mature testis is better for the study of spermatogenesis than the old.

Since the picric acid must be washed out, fresh 75 per cent alcohol to which a few drops of a saturated aqueous solution of lithium carbonate has been added is placed on the tissue in the supply bottle, and the flow of air started very slowly. By agitation and constant drainage, the time for washing out picric is lessened to one-third that required when the tissue simply soaks in the same solution, which is renewed from time to time. Very small pieces will wash in a few hours. Large pieces must be left until no yellow appears in the fluid.

It is desirable to keep the tissue in alcohol as short a time as possible. As noted in table 3, the shrinkage that occurs in getting the tissue into this grade of alcohol is considerably more than occurred in the fixative.

The anilin oil is started dropping slowly. It is heavier than alcohol so that a stronger current of air may be needed to insure quick mixing. The eye will readily detect the flow of air necessary. When nearly pure anilin is reached, the tissue is left in pure anilin until it is clear like amber.

Clearing. The oil follows by the same method. The tissue should be changed once after being placed in the pure oil, as it is quite essential that all of the anilin be removed, or poor infiltration by paraffin results.

Infiltration with paraffin. This is accomplished by warming the oil and tissue slightly and adding to it every ten minutes a few drops of melted paraffin, which is then thoroughly mixed with the oil by a pipette. This is continued until the mixture is 85 per cent or 90 per cent paraffin. It is then transferred to the melted paraffin. I use one melting point only—52° or 55°C.

If bergamot oil has been used, at least four changes of paraffin should be made, the tissue remaining in each a half hour, and in the fifth paraffin at least one hour. Testis requires longer. I cannot see that twelve to twenty hours in paraffin of 55°C. injures brain or testis.

Imbedding. This is done in the usual way.

RESULTS OF THE METHOD DEVELOPED

Cytological. As stated in the introduction, the object of the experiments was to obtain a method which would positively differentiate dividing cells in the brain of the albino rat. This object was attained. In addition, the same method has given the most satisfactory preparation of the testis yet obtained for the study of spermatogenesis, as simultaneously with the work on the brain tissue the testis was also tested. On this latter material cold Flemming's fluid (0°C.) to which was added 2 or 3 per cent urea gave some very admirable results, but of many testes fixed only two came out well, and these were good only in a few places, while the tubules that showed chromosomes in good condition for study were invariably badly torn, as is illustrated in figures 13 and 15. Figure 10 was made in order to show that this torn condition is general and not confined to one or two places. All stages of spermatogenesis are shown in the tubules of figure 10. It is evident that any conclusions relative to chromosomes drawn from tissues so badly distorted should be fully controlled by a preparation in which no such distortion is to be seen. It is believed that figure 14 illustrates such a preparation.

In the Flemming-fixed material, the early prophases seem more sharply defined, as if the chromatin were compressed into more of a dense thread than in the picro-formol-acetic preparations. It is likely that the latter fixation preserves them in a more nearly normal condition. When chromic acid and urea are added to the picro-formol-acetic mixture the metaphase chromosomes of both spermatocytes are held in some way from clumping, just as they are in the best Flemming preparations, while the prophase threads are not hardened down appreciably. The chromatin maintains its diffuse character between the chromomeres. It may be that on this account the study of these prophase chromosomes will be easier in Flemming-fixed material if one desires to differentiate a single chromosome in the large number possessed by rat and some other mammals, but for the study of the structure of the individual chromosomes, it would seem that the

B-15 preparation is decidedly preferable. I shall have more to say upon this subject in the paper bearing upon the spermatogenesis of the albino rat.

In this paper, however, I wish merely to show the possibilities of isolating chromosomes in the metaphases of spermatocytes by the addition of urea to Flemming's fluid and fixing at 0°C. Figure 15 is to be contrasted with figure 16, where the chromosomes are so clumped as to be hopelessly unresolvable with the best microscopic combinations available. Figure 17, prepared by the B-15 fixative, does not show its full worth in this figure. I had no slide of that preparation which showed several metaphase conditions in polar view, and was compelled consequently to use lateral views at this magnification, trusting that the higher magnification shown in figures 14 and 14 a would bear witness to the real excellence of the preparation. Furthermore only a few cells in figure 17 focussed at the same level, while many did in figure 15. The excellence of general fixation comes out clearly in both figures 14 and 17.

In this connection, attention is called to the apparent excellence of fixation in figure 11. This contrasts favorably with figure 10. Examination of figure 16, however, which is made from the same slide as figure 11, shows the clumped condition of the chromosomes and some spaces about the cells. Neither of these conditions is manifest in figure 17. The spaces near the center of this last figure are the normal lumen of the tubule.

Consider now the cytological details of the brain tissue as illustrated in the figures. The superiority of the general fixation shown in figures 3 and 4 over that shown in figure 2 is manifest. The spaces about the cells in figure 2 do not appear in tissue fixed by B-15 and subsequently treated by the method described in this paper. Tissue fixed in Carnoy's fluid, in formalin, in alcohol and in Ohlmacher's fluid show these spaces throughout. Figures 3 and 4 are typical of the preparations by the method which I have employed in connection with the picroformol-chrome-acetic-urea fixative. No such spaces appear about cells of any size. Large cells have been purposely chosen for the figures, as they would show such spaces most strikingly if present.

In these cells the characteristic fibrillation of the larger protoplasmic process appears distinctly. Fibers are also distinct between the cell bodies. The chromatin masses in the nucleus are more delicately treated than in the other fixations.

The equal action of B-15 throughout the tissue is illustrated in figures 6 and 7. The latter figure shows the outer part of the external granule layer, or where this layer lies on the outside of the cerebellum, while figure 6 shows the same layer deep within a sulcus, as at *I*, figure 1. The cells in each region seem alike. Those in figure 9, on the contrary, are markedly different from those in figure 8. Figure 9 shows the outer cells and figure 8 the deep-lying ones. The appearance in figure 9 is characteristic of Carnoy, alcohol and Ohlmacher preparations, which therefore prove very unsatisfactory fixatives if one desires to study this layer of cells throughout.

The demonstration of dividing cells in photographs was found very difficult for several reasons. One is that the chromosomes are very small and numerous, and that they do not form a flat equatorial plate. A photograph of any equatorial plate would therefore appear to show some fusing. In fact, if one wishes to be absolutely sure of the number of chromosomes, the cell must be slightly inclined to the plane of section, since in a strict polar view one or more of the chromosomes may be entirely hidden by another chromosome. Figure 5 shows a dividing cell in early anaphase, in which some of the chromosomes may be seen separately at this focus. In the preparation they are perfectly distinct from each other. In figure 6, one of the daughter nuclei of a dividing cell in late anaphase is seen (*D. c.* in lower part). This stage is the most critical one in the fusing of the chromosomes. In all other fixatives they mass into a solid body at this stage. In this figure, however, they will be seen as distinct, although too small to be isolated in the photograph. The dividing cell in the upper part of this figure is out of focus, but it can be seen that its edge is not as hard and smooth as the dividing cell in figure 8, which is typical of all other fixatives except B-15.

SHRINKAGE DUE TO FIXATION

Several brains were weighed to show the change in volume due to fixation. The data appear in table 3. The method of weighing is as follows: as soon as the brain is removed it is dipped for a few seconds into the fixative, then removed and drained on filter paper. It is then placed in a weighing bottle and the weight of brain and bottle determined. The brain is then removed and

TABLE 3

Showing loss in weight of different-aged brains in the fixing fluid which gave the best results, viz., picro-formol chrome-acetic urea. See table 1, no. 15

NUMBER OF RAT	SEX	AGE IN DAYS AFTER BIRTH	NUMBER OF HOURS IN FIXING FLUID	WEIGHT IN GRAMS BEFORE FIXATION	WEIGHT IN GRAMS AFTER FIXATION	LOSS OF WEIGHT IN FIXING FLUID	PERCENT-AGE LOSS OF WEIGHT IN FIXING FLUID
133	♂	2	1	0.2860	0.2640	0.0220	7.6
119	♀	3	1	0.2992	0.2770	0.0221	7.4
117	♂	7	1	0.6605	0.6024	0.0581	8.8
118	♀	7	1	0.6799	0.6206	0.0593	8.7
135	♂	8	1	0.9433	0.8969	0.0464	4.9
136	♀	8	1	0.9271	0.8871	0.0400	4.3
137	♂	25	1 $\frac{3}{4}$	1.3590	1.2759	0.0831	6.1
138	♀	25	1 $\frac{1}{2}$	1.2848	1.2031	0.0817	6.4
139	♂	90	2	1.7927	1.6598	0.1329	7.4
139			48	1.7927	1.4398	0.3529	19.6
140	♂	63	2	1.6050	1.4852	0.1198	7.4
140			48	1.6050	1.3611	0.2449	15.2

the bottle weighed before cleaning. The difference between the first and second weights is taken as the brain weight. If the brain is weighed as it comes from the animal, its condition is not the same after being in the fixing fluid, as some of the fluid will remain on its surface. This may be sufficient, even after draining, to counterbalance the loss of weight due to the action of the fixative.

The specific gravity of B-15 is about that of the brain. Any loss of weight while the tissue is in the fluid is therefore due to loss of water. King ('10) found that the loss of weight during fixation in Ohlmacher's fluid varied from 8 per cent to 15 per cent, when left in the fixing fluid from 1 to 6 hours. The 8 per cent

loss was on the brain left in 6 hours. She concludes that length of time in the Ohlmacher fixing fluid does not make much difference in the shrinkage. Her method of calculation, however, was to weigh the brain after fixation and compare this weight with the computed weight for a rat of this size and body weight.

The percentage losses of weight shown in table 3 run from 4.3 to 8.8 per cent when the tissue is left in the fixing fluid one to two hours, but jump to as high as 19.6 per cent when left in 48 hours. Age seems to make little difference, if any, when fixation time remains the same.

SHRINKAGE DUE TO ALCOHOL

It is well known that alcohol shrinks tissue. Table 4 shows that this is considerable if the tissue remains in the alcohol long, running as high as 17.6 per cent when left in 48 hours. These figures show the desirability of passing the tissue through alcohol as quickly as possible.

Table 4 shows also the total loss in weight due to fixing fluid and alcohol up to 60 per cent. These figures run on the whole a little less than those given by King ('10) for Ohlmacher fixation followed by 48 hours in 70 per cent alcohol, these running from

TABLE 4

Showing typical losses in weight of brains in alcohol after fixation as described in table 3, and the total loss of weight due to the effects of the fixing fluid and the alcohol, this loss increasing with the length of time the tissue is left in each fluid

NUMBER OF RAT	SEX	AGE IN DAYS AFTER BIRTH	NUMBER OF HOURS IN ALCOHOL	WEIGHT IN GRAMS WHEN THE ALCOHOL WAS STARTED DROPPING	WEIGHT AT END OF THE TIME INDICATED IN COLUMN 4	LOSS OF WEIGHT	PERCENT-AGE LOSS OF WEIGHT	TOTAL LOSS OF WEIGHT IN FIXING FLUID AND ALCOHOL (60 PER CENT)	TOTAL PERCENTAGE LOSS OF WEIGHT
133	♂	2	2	0.2640	0.2360	0.0280	10.6	0.0500	17.4
133			48	0.2640	0.2294	0.0346	13.0	0.0666	23.6
135	♂	8	2	0.8969	0.8780	0.0189	2.1	0.0650	6.9
135			48	0.8969	0.7458	0.1511	16.8	0.0975	20.7
136	♀	8	2	0.8871	0.8664	0.0207	2.3	0.0607	6.5
136			48	0.8871	0.7309	0.1562	17.6	0.1962	21.1
137	♂	25	48	1.2759	1.1046	0.1713	13.6	0.2544	18.7
138	♀	25	48	1.2031	1.0145	0.1886	15.6	0.2703	21.1

16 per cent to 30 per cent, but most of them being in the neighborhood of 21 per cent. There is probably, then, very little difference, if any, between the shrinkage effects of these two fixatives and methods of preparation up to 70 per cent alcohol.

Some rough areal measurements of mesial views of brains indicate that the chief shrinkage is due to alcohol in the method described in this paper, taking the whole technique from fixation through paraffin to the slide. Some substance for replacing alcohol is therefore a decided need in technique.

CONCLUDING REMARKS

I have searched the literature for some mention of the use of chromic acid with a picro-formol-acetic combination, but find none. It would seem that my special contribution to microscopic technique as a result of these experiments is the fixative and the method of agitating fluids automatically as described in this paper.

I am greatly indebted to Professor McClung for constant criticism and advice; also for as constant encouragement when attainment of the desired result seemed hopeless. From him came the suggestion to add to the fixatives some urea, which had proved efficacious in some experiments carried on by Miss Eleanor Carothers at his direction. As an account of these experiments will be printed later by Professor McClung, no further reference need be made in this paper.

The Zoological Department of the University of Pennsylvania has provided me with laboratory facilities at Woods Hole and has placed its exceptional photographic facilities in the University at my disposal.

Professor Donaldson has shown a cordial interest in this work and his encouragement and advice have been very helpful. Wistar Institute has supplied the rats, the number of which passed the hundred mark, and has also provided equipment for carrying on these experiments.

GENERAL CONCLUSIONS ON TECHNIQUE

For cytological work, the slightest gain at any point in the technique is worth working for.

Very gradual change of fluids, agitation of the fluids during changing, and slow infiltration appear essential in order to get the best results from any fixative.

The addition of a low percentage of urea to fixing fluids results in sharpening the chromosomes and preserving the structure of the achromatic nuclear material. It may help penetration of the fluid.

Picro-formol-acetic mixtures are more effective when used at about 38°C. Cold is detrimental.

Flemming's fluid is more effective if used at 0°C. or a few degrees lower.

Flemming's fluid is of no value as a brain fixative at any temperature. It at times (if urea is added) isolates metaphase and anaphase chromosomes in spermatocytes somewhat better than any other fixative tried (except B-15), but is hard on the rest of the tissue and shrinks heavily.

Anilin oil is an excellent substitute for the higher alcohols.

Xylol shrinks tissue more than the vegetable oils.

Special conclusions applicable to the albino rat

B-15 is the only fixative tried which clearly differentiates brain chromosomes. It is superior to all others as a general fixative for developing and adult testis and central nerve tissue. It insures cytological detail in the testis better than any other.

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EXPLANATION OF PLATES

The figures are photographed from material fixed in five different ways. They show the essential differences between poor and good preparations.

Plate 1 illustrates the effects of fixatives upon the brain; Plate 2, upon the testis.

Modes of preparation. Figures 1, 3 to 7, 12, 14 and 17 were prepared by the method described in this paper. Figure 2 was fixed in Carnoy's fluid plus 1 per cent chromic acid and 2 per cent urea (table 2, no. 15), and carried through by the method described herewith. Figures 8 and 9 are from tissue fixed in Carnoy's fluid, dehydrated and double-imbedded as described by King ('10). Figures 10, 13 and 15 are from tissue fixed in Flemming's fluid and carried up by the method described in this paper. Figures 11 and 16 are from tissue fixed in Bouin's fluid and carried through by the method described in this paper. Sections were cut at 6 to 10 micra and stained with Iron Haem. and Or. G.

Photographing. Figures 1, 10, 11 and 12 were made with low-power projection lenses; figures 15, 16 and 17, with Zeiss 4 mm. apoc. obj. and Zeiss No. 4 projection ocular; figures 2 to 9, 13 and 14 were made with Zeiss 2 mm. oil immersion apoc. obj. and Zeiss No. 4 projection ocular. All were reduced for publication to the magnifications indicated below.

PLATE 1

EXPLANATION OF FIGURES

1 Sagittal section through cerebellum and medulla of 7-day rat. *Ext. gr. layer* is the region of actively dividing cells, magnified portions of which appear in figures 5 to 9. Only a small portion of the medulla is shown. $\times 15$.

2, 3 and 4 show typical effects of different fixation upon cells in the medulla. Tissue is from 7-day rats. $\times 600$.

2 Fixation: Carnoy plus 1 per cent chromic acid and 2 per cent urea.

3 Fixation: Picro-formol chrome-acetic in urea ('B-15').

4 Fixation: same as figure 3.

5 Dividing cell at inner edge of external granule layer of 7-day cerebellum shown in figure 1. Fixation by B-15. $\times 1200$.

6 to 9 illustrate the external granule layer of the cerebellum of 7-day rat under different fixatives. The contrast is between the surface and the deep-lying portions. $\times 600$.

6 Deep-lying tissue. Fixation: B-15.

7 Surface layer. Fixation: B-15. From same section as figure 6.

8 Deep-lying tissue. Fixation: Carnoy.

9 Surface layer. Fixation: Carnoy. From same section as figure 8.

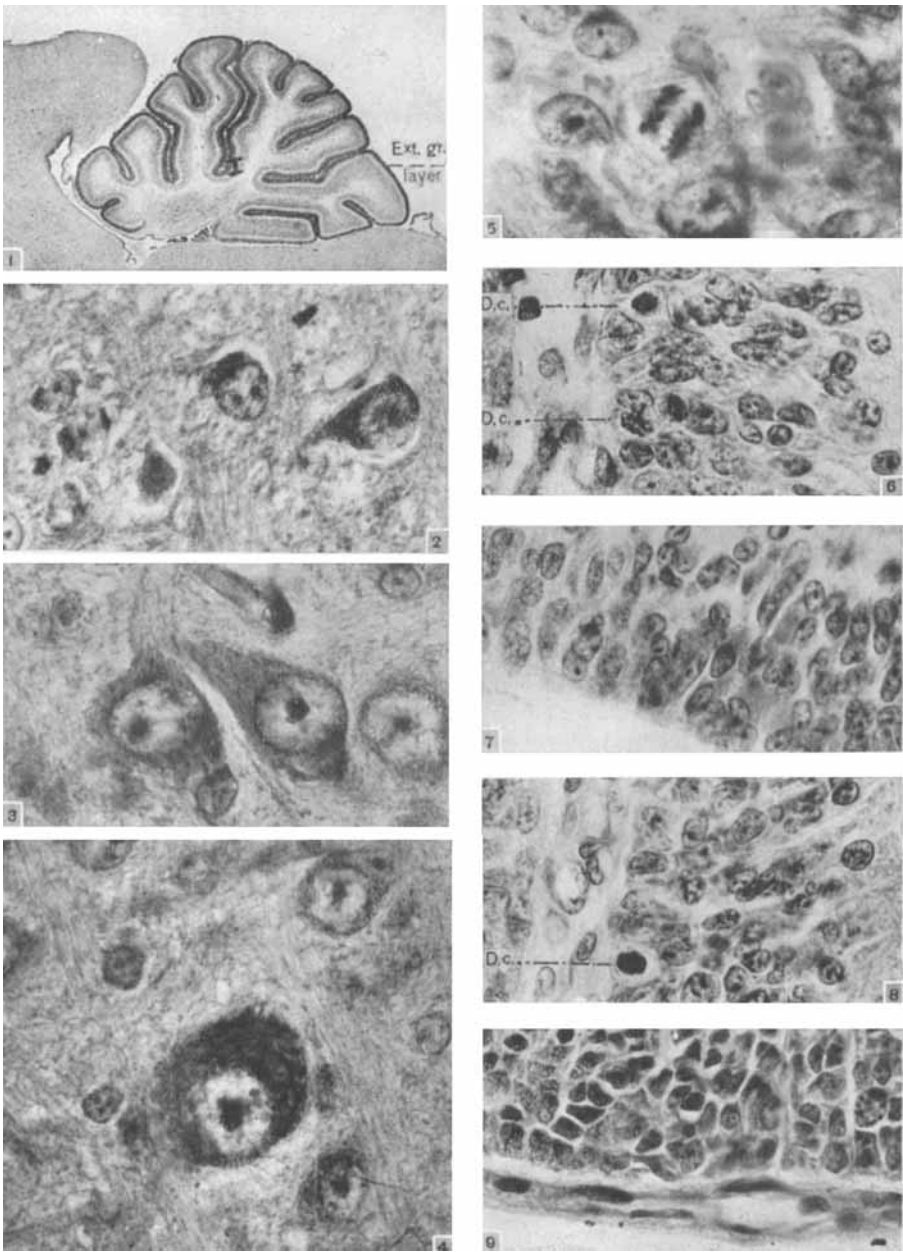


PLATE 2

EXPLANATION OF FIGURES

- 10 Testis fixed in Flemming's fluid. $\times 24$.
- 11 Testis fixed in Bouin's fluid. $\times 24$.
- 12 Testis fixed in B-15. $\times 24$. This tissue is from a younger animal than those shown in figures 10 and 11.
- 13 Same preparation as shown in figure 10. From adjoining slide. $\times 600$.
- 14 From same slide as shown in figure 12. $\times 1200$.
- 14a This figure shows the cell directly above it in figure 14 at a different focus. $\times 1200$.
- 15 From same slide as shown in figure 10. $\times 300$.
- 16 From same slide as shown in figure 11. $\times 300$.
- 17 From same slide as shown in figure 12. $\times 300$. The chromosomes appear at some disadvantage in this figure compared with those in figure 15. The reasons for this are two: (1) they are lateral views in figure 17 as opposed to polar views in figure 15; (2) the cytoplasm is much better preserved in figure 17. A third reason may be suggested: they are less shrunken than in figure 15.

