Department of Research, Wills Hospital, Philadelphia, and Princeton Hospital, Princeton

# TISSUE FIXATION CHANGES*

H. SHAPIRO and T. S. HARVEY

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## Introduction

A considerable literature, dating more than 3/4 century, exists on the technic of fixing and staining tissues in preparation for microscopic examination. Histologists and cytologists are aware of the ever-present problem of artifact arising from the very nature of the multifarious chemical and physical processes to which the preparations are subjected. Systematic researches are consequently bound to be tedious and perhaps unrewarding in relation to the effort expended, and these difficulties and uncertainties have acted as a deterrent in this field.

The present study originated from problems of interpretation of eye sections. Since the eye is a composite of several well-defined tissues, we thought it appropriate to study also the effects of fixation upon other discrete tissues of the body.

### Technic

In order to determine the absolute volume changes undergone by tissues, two widely used fixatives, Bouin's fluid, and formaldehyde, and dilutions thereof, have been employed. Formaldehyde has great penetrating power, affords good preservation of tissue elements, and good staining of cells. It has been extensively used for the preservation of the central and peripheral nervous system. The composition of the Bouin's fluid was

| | |
|---|---|
| Picric acid, c. p. ..... | 10 g |
| Formaldehyde, 37% (w/v) ..... | 333 ml |
| Glacial acetic acid ..... | 67 ml |
| Distilled water ..... | 1000 ml |

At room temperature the picric acid crystals required several days for complete solution, Formaldehyde solutions were made by diluting the 37% solution (preservative, 12% $CH_3OH$), without taking into account volume changes occurring on dilution. Formaldehyde (alone), 0,92% was calculated to be isosmotic with 0,9% NaCl. Four, 10, and 37% formaldehyde (alone) were calculated respectively to be 4,34, 10,83, and 40,1 times as concentrated osmotically as the 0,92% formaldehyde.

Muscle, liver, brain and nerve from adult and baby rabbits were used. Observations on eye tissues will be reported elsewhere. With the exception of nerve, pieces ranging in size from approximately 200 to 400 mg were cut out, weighed, and dropped into about 40 ml of

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the fixative. No attempt was made to keep the tissues at constant temperature, and throughout the prolonged series of observations the temperature in fact fluctuated widely. Temperature proved, however, not to be a critical factor in the interpretation of the results. The tissues were weighed on a torsion balance of nominal range 0—500 mg, and sensitivity 0,1 mg. By the use of a supplementary counterweight, the range could be extended to 1 g. In weighing, tissues were blotted lightly with filter paper to remove surface moisture, and could be weighed usually in less than $^1/_4$ minute. The tissues were kept in tightly stoppered 40 ml bottles, to minimize loss of fluid by evaporation. The weighings on tissue samples were reproducible to better than 1%.

## Results

### *Adult tissues in Bouin's fluid*

The changes undergone by L. sciatic nerve, abdominal muscle, liver, and parietal cortex in Bouin's fluid are represented in Fig. 1. In this, as in all the

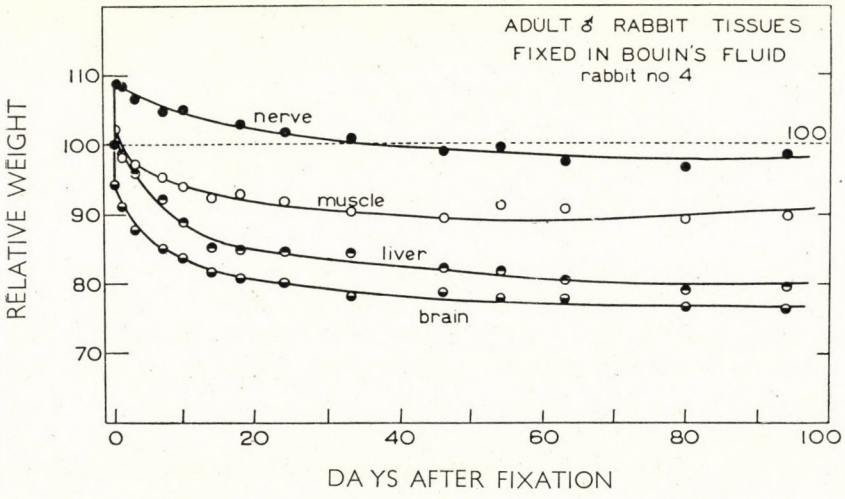


*Fig. 1.* Changes in moist weight of adult rabbit tissues fixed in Bouin's fluid

other curves, the initial weight of the freshly dissected tissue, prior to immersion in the fixative, is taken as 100. The course of the changes was followed over a period of weeks or months. Despite the fact that Bouin's fluid is considerably hypertonic to living mammalian tissues, nerve showed an initial swelling of 8,9% in the first 3 hours, from which it slowly declined. Over a period of 33 days it fell to its initial weight, and in the ensuing 61 days, lost 2,3% of the original weight. Measurements carried out to 116 days (not shown on the curve) showed a stabilization at about 97% of the fresh weight. Muscle underwent only a slight initial increase (2,1%), followed by a weight loss of some 10%. Liver and brain declined steadily to final weight losses of 21% and 23% respectively. The observed weight losses are not due to loss of tissue flaking away or removed by contact with the filter paper. They are real changes occurring within the tissue, as will be demonstrated in the sequel. It is evident that the changes

occurring with a given fixative will be determined by the nature of the tissue. There may be an initial weight increase followed by a slow decline, or the decline may occur at the outset and steadily progress. Most noteworthy is the long period of time during which the changes occur, the bulk of the weight loss occurring within a month, but continuing slowly thereafter, tending to approach asymptotically a final value. The directions given in texts for the duration of fixation may vary considerably. In view of the fact that changes occur slowly and over a protracted period of time, it is apparent that the point at which passage from fixative to the subsequent steps in the technique takes place should be undertaken with a recognition of the weight change factor as a function of time.

SCHMIDT (1944, p. 149) states that in stirring a saturated solution of picric acid with a protein solution, the protein will usually be precipitated quantitatively from the solution as a picrate. Adjustment of pH may, however, be necessary. In the compounds formed, apparently the picric acid and the protein form definite reproducible compounds, which can be used as a quantitative measure of the protein.

One of the first questions arising on examining curves such as those of Fig. 1 is whether the changes are due to slowness of penetration of the fixative through diffusion barriers. Were the tissue alive, one would expect the permeability to formaldehyde and acetic acid to be considerably greater than that to picric acid. Part of the fixative, at least, should penetrate to the center of the tissue in a relatively short time. However, the tissue is quickly killed by the full strength fixative, and presumably the diffusion barriers set up by the living cell membranes are removed, facilitating an even more rapid penetration, governed by physical and geometric parameters. In the case of nerve, where the tissue dimensions are very small, weight changes continued nevertheless to be measurable for many days.

### *Effect of concentration of the fixative. Muscle*

In addition to fixative composition, the other obvious variable is that of fixative concentration. That a striking effect exists is apparent from Fig. 2, where adult rabbit abdominal muscle was fixed in Bouin's fluid, full strength 1, and 1/2, 1/10, and 1/50 the concentration of the original formula. The abdominal muscle samples came from a single animal, with the exception of that used in full strength Bouin. Apart from initial changes, the concentration resulting in least weight changes was 1/2 Bouin. Full strength resulted in shrinkage of 14%, whereas muscle fixed in 1/10 and 1/50 Bouin swelled to final values of 121% and 170%, respectively.

Histological observations at this point appeared desirable, hence 2 sets of samples of the tissues of Fig. 2 were processed for sectioning. The 3 muscle

samples in Fig. 3A were tied together, and processed simultaneously in dehydration, embedding and sectioning maneuvers of the paraffin technique. They were embedded together in one paraffin block and sectioned. The same was done with the 3 samples of Fig. 3B, carried through celloidin. All the muscle in Fig. 3 was taken from the same animal, and the samples labelled 1/2 (Bouin) were derived from the same original piece of muscle fixed in 1/2 Bouin, and then

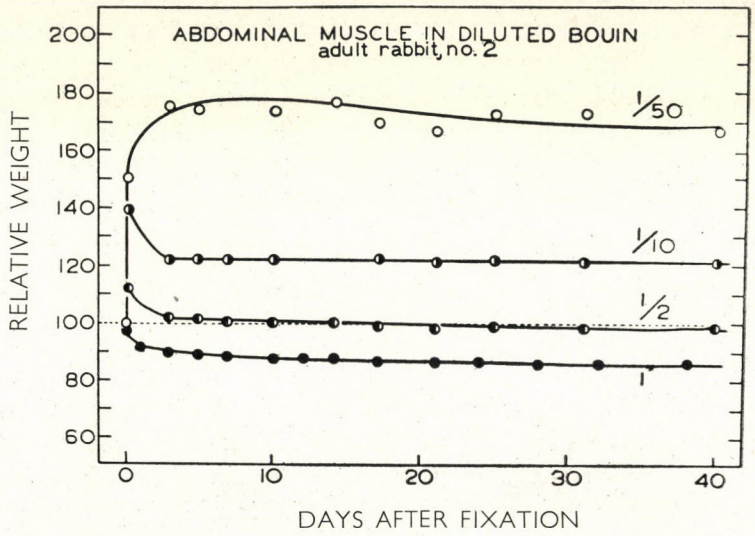


*Fig. 2.* Effect of fixative concentration (Bouin) on adult rabbit abdominal muscle

subdivided after 42 days fixation for processing in paraffin and celloidin (Figs. 3A and 3B, respectively). A correlation between the histological picture and the curves of Fig. 2 can be seen. Taking Fig. 3A, which is a low power photograph of the 3 sections in paraffin, dilution of the fixative leads to the accumulation of fluid between the muscle bundles. Under higher power, (125×) Fig. 4A, B, C, further details of the fixation can be seen. All the sections illustrated in this paper were stained with hematoxylin and eosin. Muscle in 1/50 Bouin, Fig. 4C, shows the large quantity of fluid not only between the muscle bundles, but between some of the individual muscle fibers. As to the adequacy of the fixation in the diluted Bouin's fluid, high power views (1200×) are contained in Fig. 5. The muscle spindle (1/10 Bouin) apparent above the letter B, in Fig. 4B (125×), is shown in Fig. 5A (1200×). In Fig. 5B, 1/10 Bouin, details of nuclei and chromatin granules, as well as some cross striations, are apparent in fibres cut parallel to their length. In Fig. 5C, cross sections of fibre again show details of nuclear chromatin.

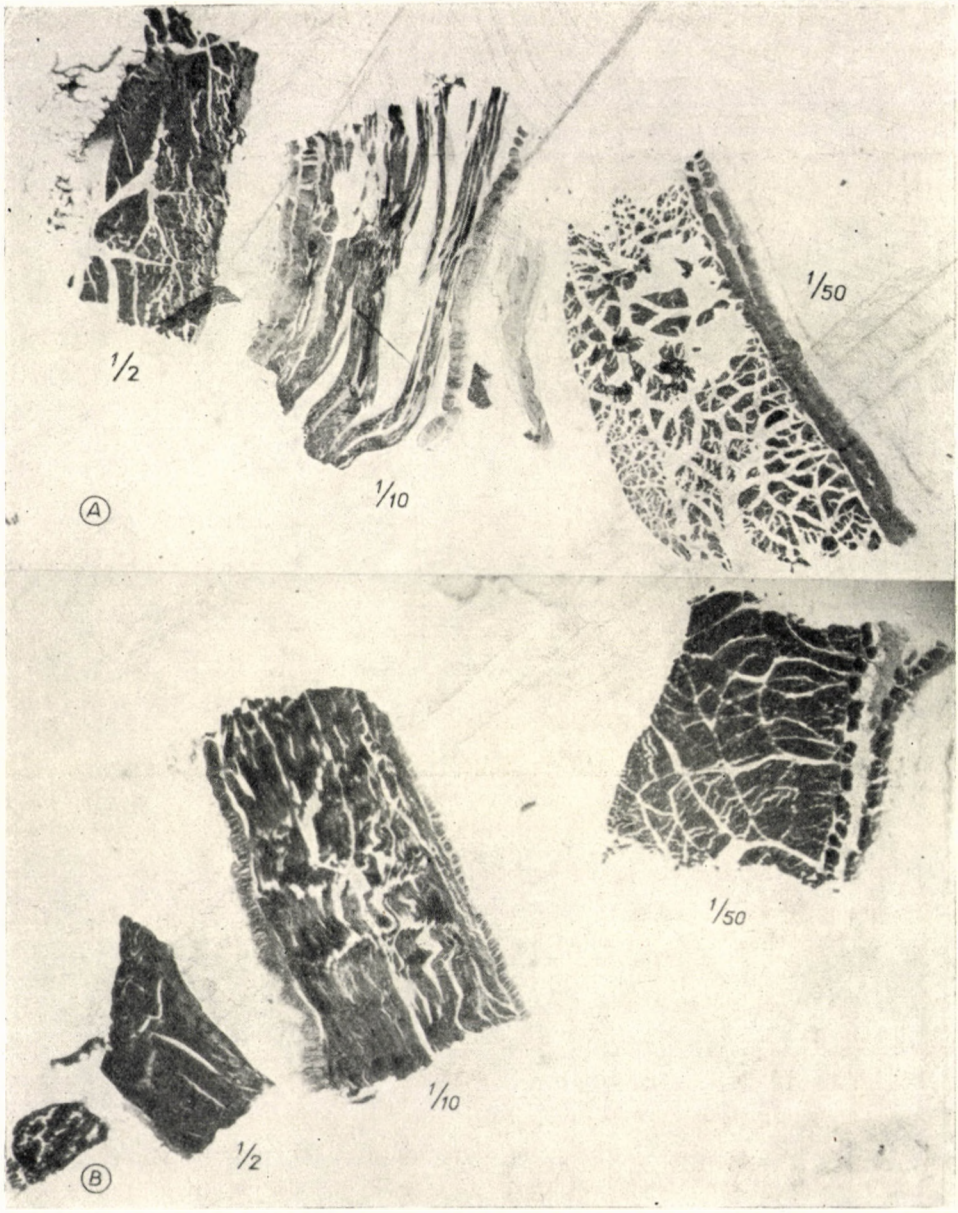


*Fig. 3.* Low power views of adult rabbit abdominal muscle after 42 days' fixation in $\frac{1}{2}$, $\frac{1}{1}$ and $\frac{1}{50}$ Bouin. Simultaneous processing in paraffin (Fig. 3A); in celloidin (Fig. 3B)

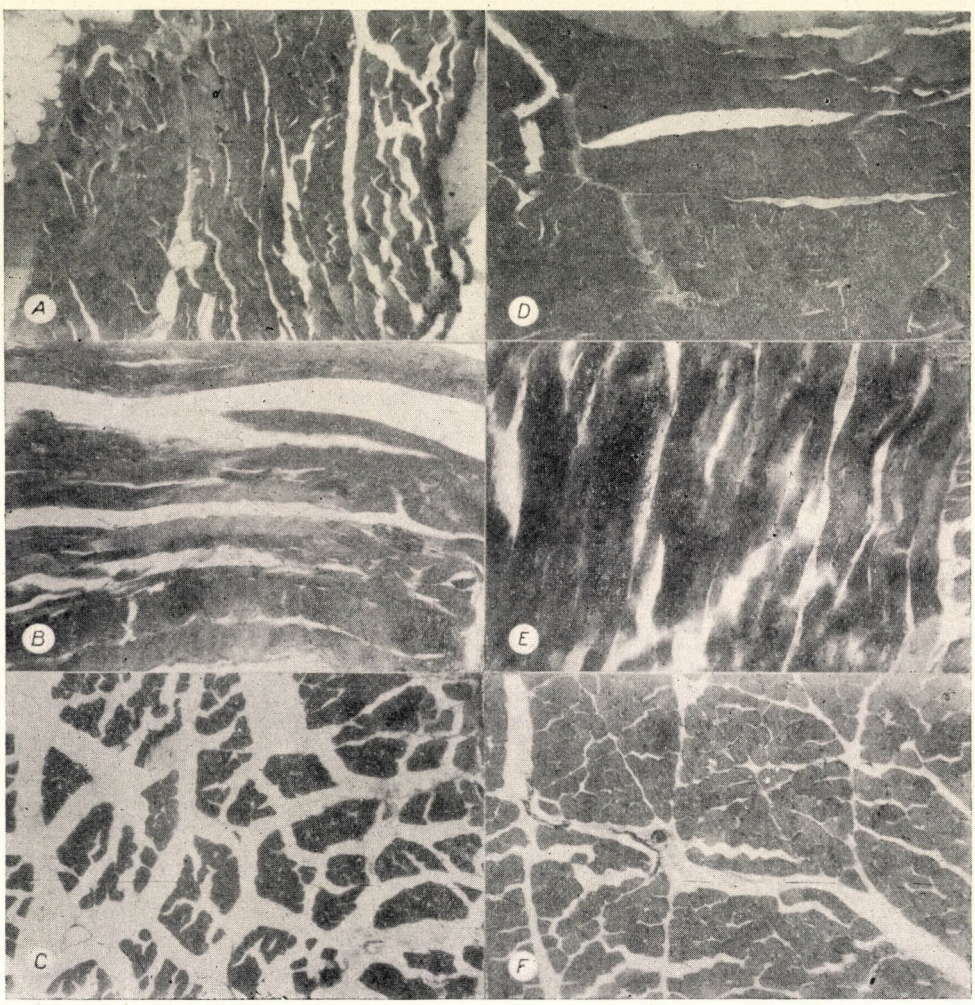


*Fig. 4.* 125×. Same tissues as in Fig. 3A, B, and C in $\frac{1}{2}$, $\frac{1}{10}$, $\frac{1}{50}$ Bouin, paraffin. D, E, and F in $\frac{1}{2}$, $\frac{1}{10}$, $\frac{1}{50}$ Bouin, celloidin. Note muscle spindle above letter B, shown also in Fig. 5A

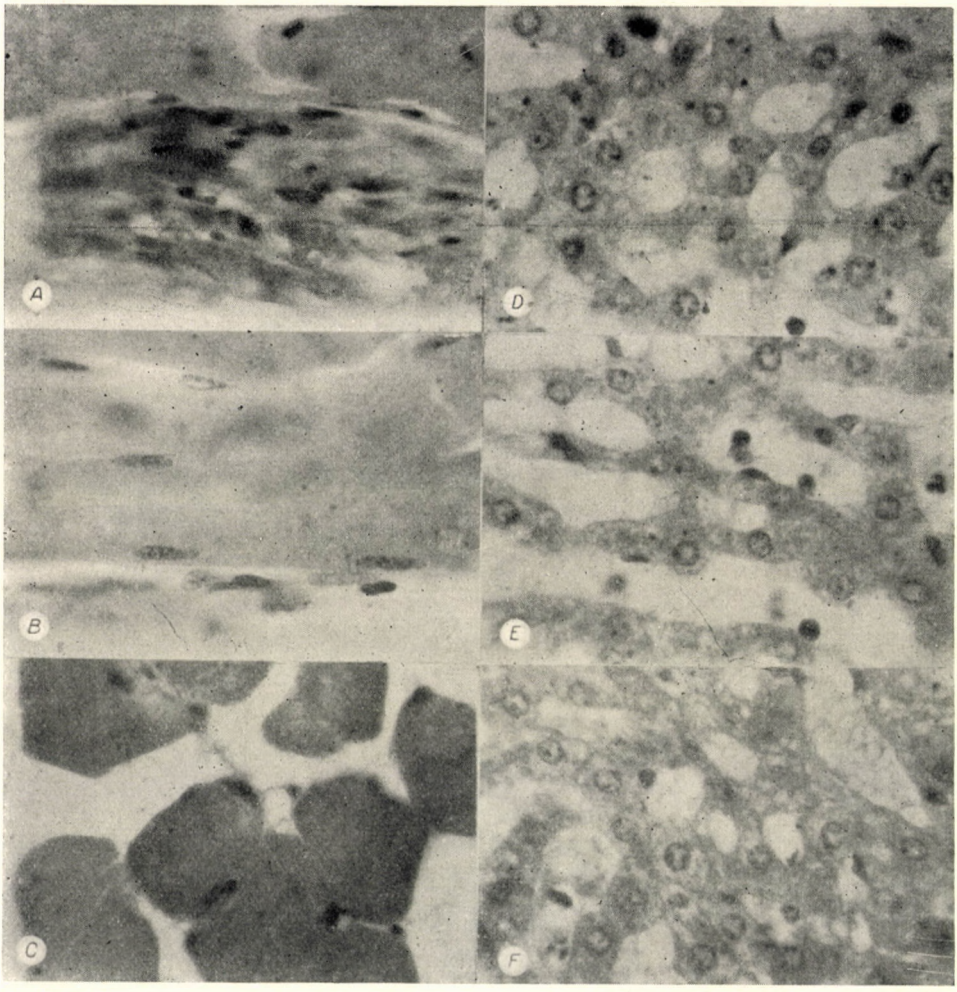


*Fig. 5.* 1200×. A, muscle spindle of Fig. 4B ($^1/_{10}$ Bouin). B, muscle fibres, $^1/_{10}$ Bouin, longitudinal section. C, muscle fibres, $^1/_{50}$ Bouin, transverse section. D, E, and F, liver in ½, $^1/_{10}$, and $^1/_{50}$ Bouin, processed simultaneously in paraffin technique

### *Comparison of celloidin and paraffin*

Inspection of Fig. 3 demonstrates that the swelling effect observable in the paraffin tissues, Fig. 3A, is preserved in celloidin, Fig. 3B, though it is not as striking. The celloidin technique shrank all the tissues, thus counteracting the swelling effect of the fixative. This is of interest in the light of the fact that the heat of molten paraffin tends to contract the tissue, if such heat is allowed to become excessive. The heat apparently acts upon tissue protein already coagulated by fixative. Higher power details of the celloidin sections (125 ×)

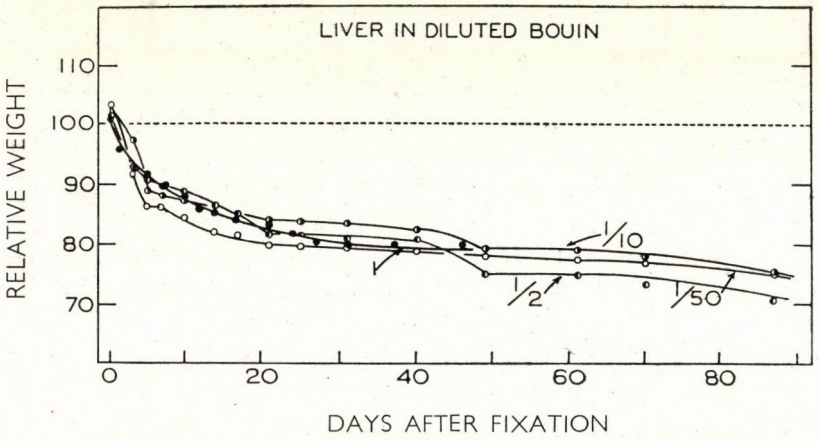


*Fig. 6.* Adult rabbit liver in 1, ½, $^1/_{10}$, $^1/_{50}$ Bouin

are seen in Fig. 4D, E, F. (1/2, 1/10, 1/50 Bouin). All sections in Figs. 5, 7 and 15 were processed in paraffin.

### *Effect of concentration of the fixative. Liver*

Fig. 6 illustrates the weight changes in pieces of liver fixed in 1, 1/2, 1/10, 1/50 Bouin. In all concentrations, a far more uniform effect is found than in muscle. All the tissues shrank in a comparable course to a limiting value lying between 72% and 75% at 123 days. Liver in 1 Bouin was observed 46 days, whereas liver in 1/2, 1/10, and 1/50 Bouin, was measured for 123 days. Fig. 6 shows the measurements up to the 87th day. In consonance with this result, the high power (1200 ×) views of Fig. 5D, E, F in 1/2, 1/10, 1/50 Bouin, show no marked differences, yet histological and cytological details are preserved in the diluted Bouin fixation. Neither do low power views 62× and 125× reveal any significant difference when viewed on a grosser scale.

*Effect of concentration of the fixative. Brain*

The curves in Fig. 7 are derived from brain slices from the same animal, with the exception of the slice fixed in 1 Bouin. All the tissues ultimately underwent distinct shrinkage, with brain in 1/10 and 1/50 Bouin passing through

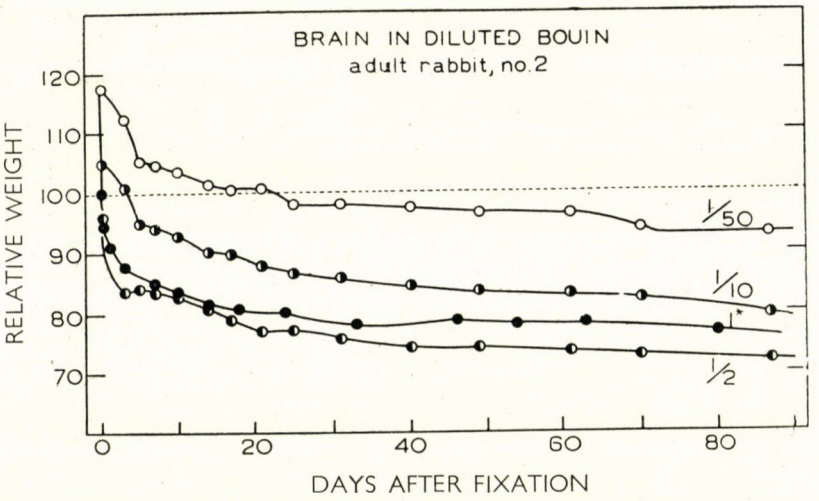


*Fig. 7.* Brain cortex of adult rabbit, in 1, ½, 1/10, 1/50 Bouin

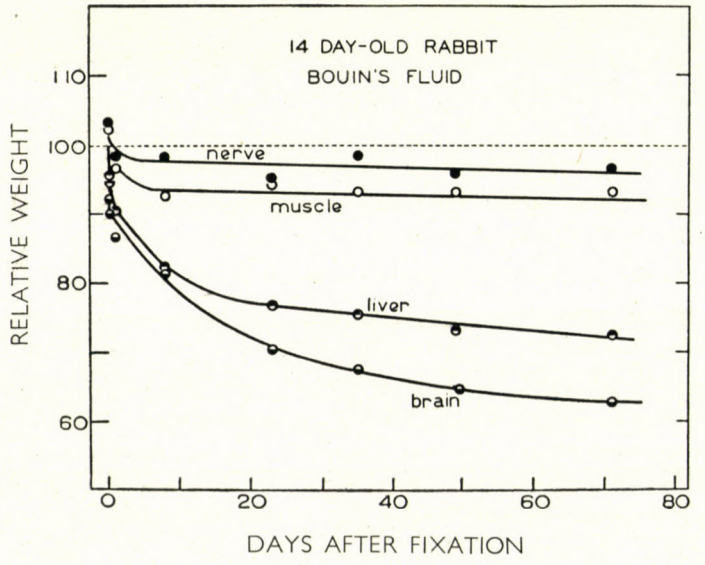


*Fig. 8.* Nerve, muscle, liver and brain of 14 day rabbit in 1 Bouin

an initial swelling phase. Brain preserved in 1/50 Bouin showed the least weight distortion.

### *Influence of animal's age. Bouin's fluid*

Nerve, muscle, liver and brain were excised from a 14 day old rabbit and fixed in 1 Bouin. The results, summarized in Fig. 8, are to be compared with

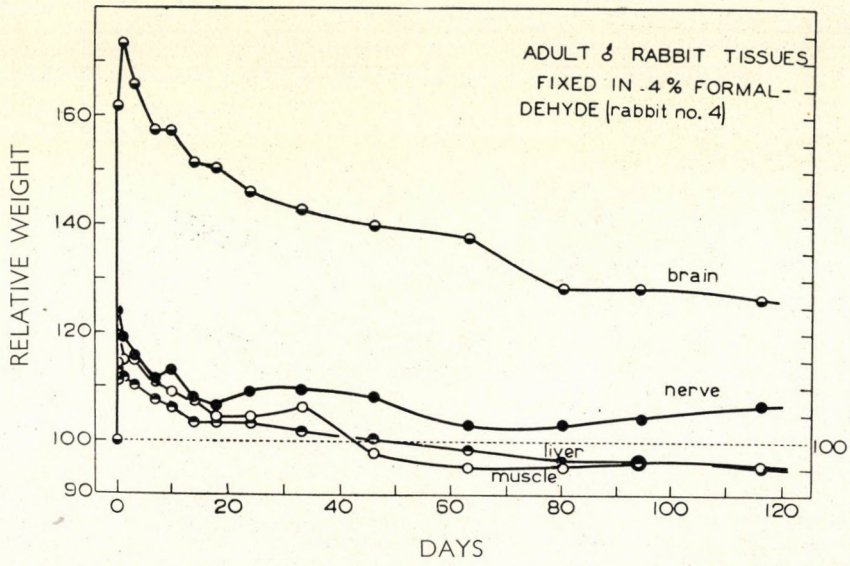


*Fig. 9.* Adult male rabbit tissues, in 4% formaldehyde. Tissue from same animal as in Fig. 1

those obtained on an adult rabbit, Fig. 1. Qualitatively, the results are similar with the curves falling in the same order. Adult liver and brain underwent less absolute change than the infant tissue.

### *Formaldehyde fixation. Adult rabbit*

Using the same animal (Rabbit No. 4) whose tissues were measured in Bouin's fluid, sample tissues were fixed also in 4% formaldehyde. The results, Fig. 9, are to be compared with those for Bouin's fluid Fig. 1. Both qualitatively and quantitatively the curves are entirely different. Brain, showing a marked shrinkage in Bouin, undergoes to the contrary, a considerable swelling in formaldehyde. All the tissues swell initially. Liver and muscle in subsequent shrinkage, stabilize at about 95% of original weight. Nerve stabilizes at about 105% of the fresh weight.

### *Formaldehyde fixation. Effect of age*

In a 1 day old rabbit (Fig. 10), the tissues in 4% formaldehyde all showed an initial swelling, as in the adult. The curves are qualitatively similar to those for the adult (Fig. 9).

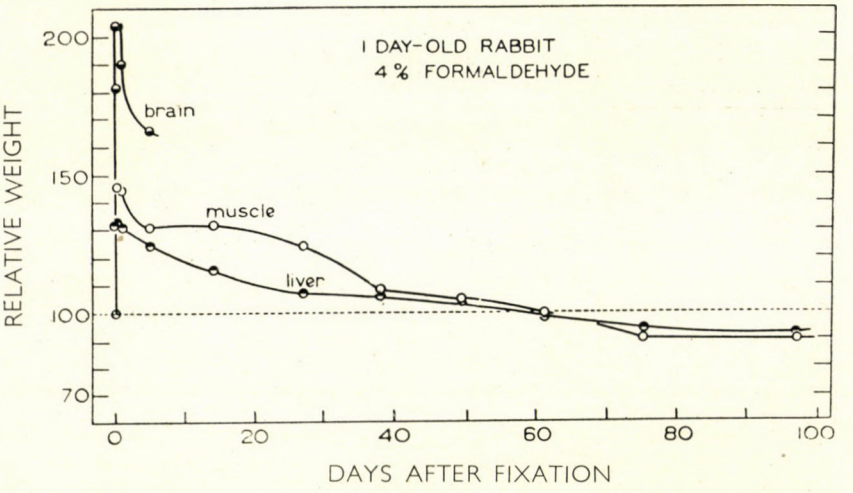


*Fig. 10.* Tissues from 1 day old rabbit, fixed in 4% formaldehyde

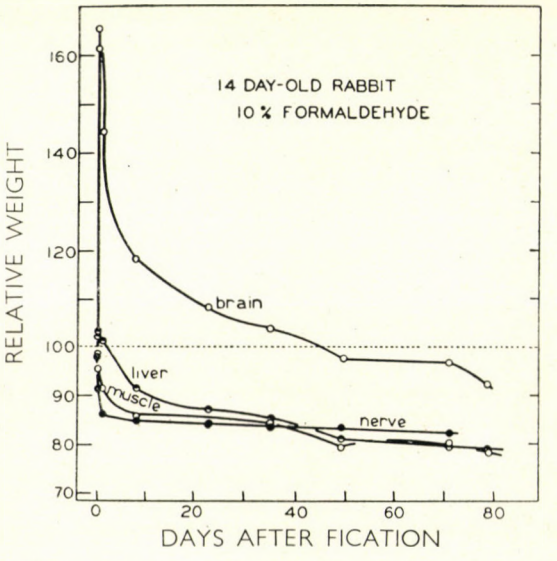


*Fig. 11.* Tissues from 14 day rabbit, fixed in 10% formaldehyde

*Formaldehyde fixation. Effect of age and concentration*

With a higher concentration of formaldehyde (10%), and tissues from a 14 day old rabbit (Fig. 11) the family of curves is lower as a whole, in comparison with adult tissues in 4% formaldehyde (Fig. 9). With the exception of brain, the initial swelling is considerably reduced, and the stabilized values after 79 days appreciably lower. This graph is directly comparable with Fig. 8, which sets forth the results for tissues from the same 14 day rabbit, fixed in Bouin. Note

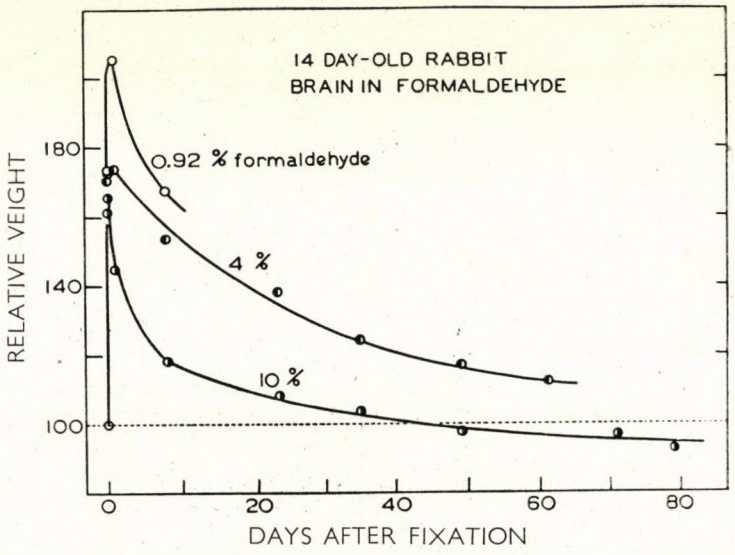


*Fig. 12.* Tissues from 14 day rabbit, in 0,92%, 4%, and 10% formaldehyde

the entirely different response of the same tissues, in Fig. 11, as compared with Fig. 8.

Taking samples of brain from the same 14 day old rabbit a concentration effect is readily apparent (Fig. 12). In all the concentrations of formaldehyde used, a large initial swelling is recorded, which subsides gradually. The measurements of brain in 0,92% formaldehyde (isosmotic with blood) were discontinued because of adherence of the tissue to the filter paper, and fragmentation. The 10% formaldehyde, after about 45 days, brought the tissue back closer to its original weight than did 4%. Brain fixed in 0,92% formaldehyde remained very soft, whereas 4% and 10% left the tissue harder, the hardness increasing with concentration.

In Fig. 13, full strength formaldehyde, 37% is compared with 10% in its effect upon cortex of a 2 day old rabbit. The direction of change falls in line with that manifest in Fig. 13, 37% producing the greatest shrinkage.

It is clear from the data presented in Figs. 9, 12 and 13 that any comparative study of vertebrate or human brains on the basis of weight, should preferably be made on freshly dissected unfixed tissues, since large weight changes occur in preserved brains, which are a function of fixative concentration and duration of fixation.

*Histological observations on formaldehyde fixations of brain*

Sections of brain cortex of the 14 day old rabbit are shown in Fig. 14. Figs. 14A and 14C are cortical sections with cell populations sparser than Figs.

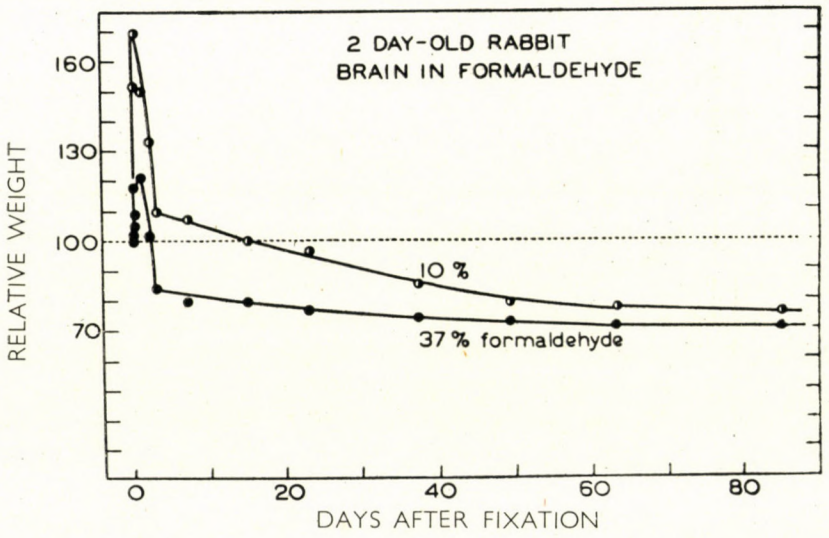


*Fig. 13.* Brain of 2 day rabbit, in 10% and 37% formaldehyde

14B and 14D. Fig. 14A, 10% formaldehyde, is to be compared with Fig. 14C, 4% formaldehyde. The approximately 20% difference in the end portions of the curves in Fig. 12 are not apparent through gross inspection of Figs. 14A and 14C. A similar comment appears to apply to a comparison of Figs. 14B and 14D, which are sections of cortex with more closely packed cells fixed in 4% and 0,92% formaldehyde, respectively. Figs. 14E and 14F are 1200 × magnifications of dense and sparse areas of cortex fixed in 4% formaldehyde.

Even on the assumption that the weight changes shown in the curves were reflected in cell volume changes alone (which does not hold, as is evident from inspection of Fig. 3) it might still be difficult to detect and identify such differences histologically. The following consideration makes this clear. What one

sees in a histological preparation is a cross section of a population of cells, and in the case of the cortical sections of Fig. 14 these are not spherical cells, nor are the sections in general through the middle of the cell. Since the volume V of a spherical cell is $4/3\ \pi\ r^3$, the ratio of the diameters of 2 cells whose volumes $V_1$ and $V_2$ differ by 20% is calculated as follows :

$$V_1 = \frac{4}{3}\pi\ r_1^3$$

$$V_2 = \frac{4}{3}\pi\ r_2^3$$

$$\frac{V_1}{V_2} = \frac{1}{1{,}2} = \frac{{}^4/_3\pi\ r_1^3}{{}^4/_3\pi\ r_2^3}$$

$$1{,}2\ r_1^3 = r_2^3$$

$$\frac{r_2^3}{r_1^3} = \left(\frac{r_2}{r_1}\right)^3 = 1{,}2$$

$$\frac{r^2}{r_1} = \sqrt[3]{1{,}2} = 1{,}063$$

i. e. the radii (and diameters) would differ in absolute values by only 6,3%,

An analogous calculation for abdominal muscle, fixed in Bouin, Fig. 2. comparing 1 Bouin with 1/50 Bouin, and taking a difference of 84% in the final stabilized weight differences, yields a ratio of $r_1/r_2 = 1{,}225$.

## Discussion

Part of the differences manifested in the curves must in any case arise from the specific chemical constitution generally characteristic of a given tissue in a healthy, normally functioning state. The chemical composition of the normal organism varies not only from youth to old age, but is in a constant state of flux from day to day and from moment to moment. The grosser changes in ratios of chemical components are more striking in the course of embryological and fetal development. In relation to the changes under examination in this study, it is relevant to list some of the major chemical differences in the tissues selected.

Citing determinations of ABDERHALDEN and WEIL (1913), PAGE (p. 205) quotes the following relative values for total amino acid content of brain, cord, and peripheral nerve :

| | |
|---|---|
| Gray substance | 2,48 |
| White substance | 2,53 |
| Spinal cord | 1,68 |
| Peripheral nerve | 2,21 |

In rats' brains, DONALDSON and HATAI (PAGE, p. 306) observed that the percentage of water reached a maximum at 4 to 8 days of age, falling rapidly until the 50th day, just before puberty, and then slowly declining further. The olfactory bulbs had the greatest water content, forebrain and cerebellum were intermediate, and the stem contained the least.

KOCH and KOCH (PAGE, p. 307) found a rapid increase in all solid constituents, especially phosphatide and protein, in the young albino rat in the first 10 postnatal days. This period is one of marked growth and is characterized morphologically by development of fibres from the cells and increase in their size.

DONALDSON showed that about 88% of the volume of adult brain is composed of axons and their sheaths ; cell bodies with their dendrites and supporting tissue together make up the remaining 12% (PAGE, p. 308).

The protein content of brain (ALBRITTON, 1954) is approximately half that of liver, which in turn is less than that of muscle (if correction be made for fat tissue content). In both Bouin and formaldehyde, the curve for brain (with significantly lower protein content) is at the periphery of the family of curves for different tissues (Figs. 1, 8, 9, 10, 11). But the behaviour in formaldehyde (Figs. 9, 10, 11) is both qualitatively and quantitatively different from that in Bouin (Figs. 1, 8). On the assumption that protein swelling and shrinkage play a major role in the tissue weight changes, there are evidently specific effects awaiting further detailed analysis. These protein changes must be considered in connection with water exchanges of the cells, and in the tissue spaces, as is apparent from Fig. 3.

Some of the earlier literature (summarized in MANN, 1902; e. g., pp. 89—91) describes experiments on the chemical effect of formaldehyde on serum albumin and edestin in which a prolonged action (2 months as compared to "a short time") of aldehyde leads to much larger quantities of aldehyde being fixed chemically by the cell. Egg- and serum-albumin, after having been acted upon by formaldehyde, did not coagulate on heating, but were precipitated by the addition of acids, alcohol and acetone ; on addition of water they were rendered soluble again.

**Summary**

1. To study the effects of fixatives in altering tissues, measurements were made of weight changes as a function of time after fixation.

2. Tissues studied were mammalian (rabbit) liver, abdominal muscle, brain cortex, peripheral nerve of young (Figs. 8 and 11) and adult animals (Figs. 1 and 9).

3. Fixatives were Bouin's fluid and dilutions of $\frac{1}{2}$, $\frac{1}{10}$, and $\frac{1}{50}$; (Figs. 2, 6, 7) and formaldehyde, 0,92, 4, 10, and 37% (Figs. 12 and 13).

4. For a given tissue and fixative, characteristic changes were observed which began soon after immersion of the tissue. Apart from initial changes, slow, long-term weight changes of considerable magnitude occurred, which lasted for weeks or months before becoming stabilized or approaching asymptotic values (Figs. 1 and 9).

5. The type of curve found depended upon the nature of the tissue, type of fixative and fixative concentration.

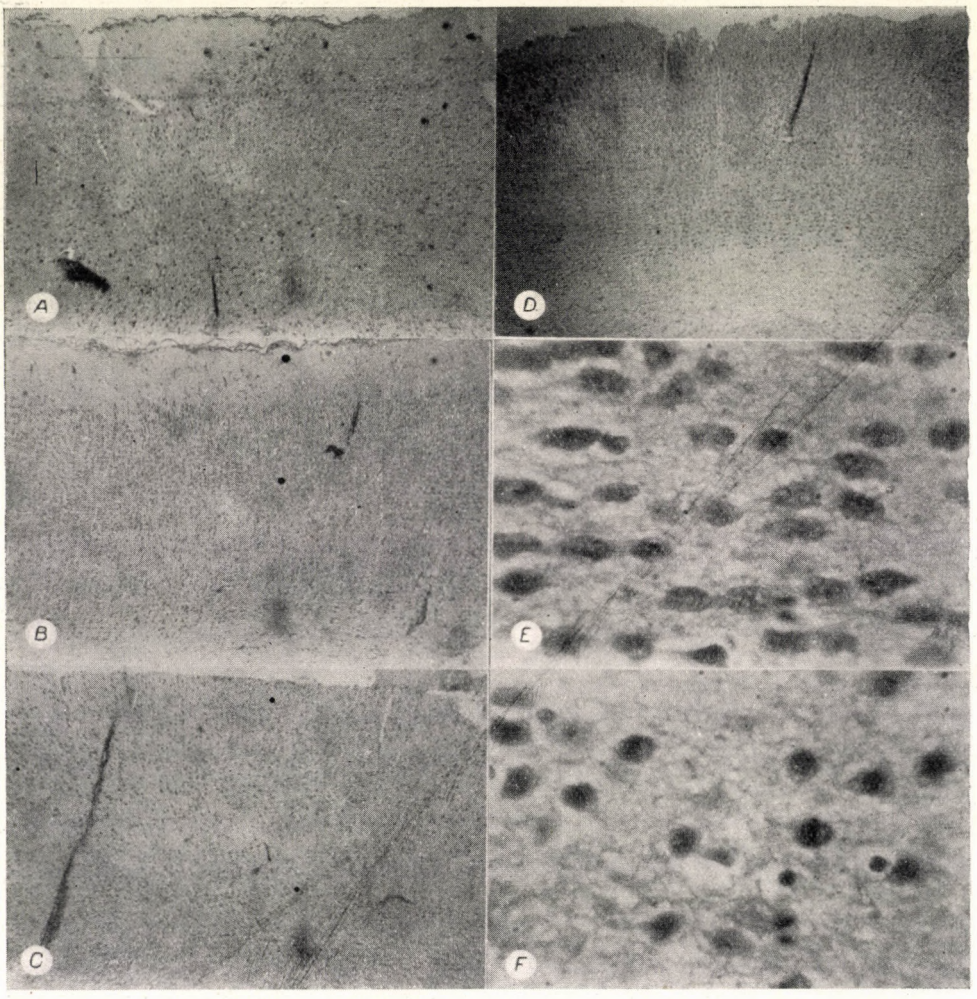


*Fig. 14.* 14 day rabbit brain cortex. A, B, C and D, 62×; E and F, 1200×. All in paraffin. B and D, dense cellular areas, compared to A and C, which are sparser. A, 10% formaldehyde. D, 0,92% formaldehyde. E and F, 4% formaldehyde. E, dense cellulation; F, sparser

6. An attempt was made to correlate these curves with the histological appearance of the tissue by suturing tissue samples together so that they would be subjected to the same processes in embedding, sectioning and staining (Figs. 3, 4, 5, 14).

7. Data such as those presented in this paper are of value in selecting a fixative which will least distort the tissue.

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## ДЕЙСТВИЕ ФИКСАТОРОВ НА ИЗМЕНЕНИЕ ТКАНЕЙ

Х. ШАПИРО и Т. С. ХЭРВЕЙ

1. В целях исследования действия различных фиксаторов на ткани, авторы установили изменения веса в зависимости от истекшего после фиксации времени.

2. Исследовались ткани молодых и взрослых животных. Исследованию подлежали следующие ткани: печень, мускулатура живота, кора головного мозга и периферические нервы млекопитающих (кроликов).

3. Исследовались следующие фиксаторы: смесь Буэна и разведение последней в соотношении 1 : 2, 1 : 10 и 1 : 50; формальдегид в концентрациях 0,92, 4, 10 и 37%.

4. При исследовании данных тканей с упомянутыми фиксаторами авторы наблюдали характерные изменения, проявляющиеся немедленно после погружения тканей в фиксирующие растворы.

Кроме изменений в начальной фазе, произошли также и значительные медленные изменения в весе в течение длительного времени, которые продолжались несколько недель или даже месяцев, после чего они стали постоянными или достигали асимптомических величин.

5. Тип полученной кривой был различным в зависимости от природы ткани, от качества и концентрации фиксаторов.

6. Авторы пытались согласовать эти кривые с гистологической картиной тканей, путем сшивания тканевых кусков, причем последние подвергались одним и тем же процессам разреза и окрашивания.

7. Сообщенные в настоящей статье данные могут быть полезными при выборе такого фиксатора, который меньше всего оказывает вредное действие на ткани.

## GEWEBSVERÄNDERUNGEN AUF WIRKUNG VON FIXIERMITTELN

H. SHAPIRO und T. S. HARVEY

1. Zwecks Untersuchung der Wirkung von verschiedenen Fixiermitteln auf die Gewebe wurden die Gewichtsveränderungen in Abhängigkeit von der seit der Fixation vergangenen Zeit gemessen.

2. Es wurden die Gewebe von jungen und erwachsenen Tieren untersucht. Die untersuchten Gewebe waren: Leber, Bauchmuskulatur, Gehirnrinde und periphere Nerven von Säugetieren (Kaninchen).

3. Als Fixiermittel wurden verwendet: Bouinsche Lösung und ihre Verdünnungen im Verhältnis von 1 : 2, 1 : 10 und 1 : 50; Formaldehyd in 0.92, 4, 10 und 37prozentiger Konzentration.

4. Im Falle eines bestimmten Gewebes und Fixiermittels wurden charakteristische Veränderungen beobachtet, die kurz nach dem Eintauchen der Gewebe in das Fixiermittel in Erscheinung traten.

Außer den anfänglichen Veränderungen wurden langsam vor sich gehende, langanhaltende Gewichtsveränderungen von bedeutendem Ausmaß beobachtet, die wochen- oder

monatelang dauerten, um dann konstant zu werden oder asymptomatische Werte zu erreichen.

5. Der Typ der gefundenen Kurve war in Abhängigkeit von der Natur des Gewebes, von Art und Konzentration des Fixiermittels verschieden.

6. Es wurde der Versuch unternommen die erhaltenen Kurven mit dem histologischen Bild der Gewebe in Einklang zu bringen, indem man Gewebestücke zusammennähte, und sie den gleichen Schnitt- und Färbungsverfahren unterwarf.

7. Die im vorliegenden Aufsatz mitgeteilten Angaben können sich bei der Auswahl von Fixiermitteln mit am wenigsten schädigender Wirkung auf die Gewebe nützlich erweisen.

Dr. Herbert Shapiro, 5800 N. Camac St. Philadelphia 41, Pa. U. S. A.