The Value of 'Spreading Factors' in the Demonstration of Tissue Neural Elements

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With three plates (figs. 1, 2, and 3)

SUMMARY

1. A description is given of the value of 'spreading factor' (hyaluronidase) used in conjunction with various neurohistological methods for the display of tissue neural elements.

2. It has been shown that after its use nerve fibres and their terminals in a variety of tissues, including the cornea, closely resemble those seen in fresh specimens of cornea under phase-contrast conditions.

3. The specimens prepared after treatment with hyaluronidase are almost free from artifacts. The staining or impregnation process can be controlled with certainty and precision to produce evenly stained specimens in the tissues we have used.

4. It is suggested that some tissues may need special treatment to obtain optimal results.

5. The use of 'spreading factor' makes it possible to determine the cause of the artifacts commonly observed after employing standard neurohistological techniques.

6. The rationale of using 'spreading factor' in conjunction with neurohistological techniques is discussed.

INTRODUCTION

METHODS for displaying tissue neural elements suffer from defects of which two are outstanding from the point of view of functional interpretation. (1) The selective staining or impregnation of nerve fibres and their terminals is never completely uniform. Thus, contrast is lost and it becomes impossible to distinguish fine detail, a circumstance which has led some observers to conclude erroneously that no nerve fibres are present in particular positions in certain tissues. (2) The majority of neurohistological methods distort and some even destroy a proportion of the tissue elements. This makes it difficult either to determine the exact relationship of the different tissue elements to one another or to distinguish artifacts introduced by the method from structures present in the living state.

Since some distortion is inevitable in fixed tissues, the most reliable method of assessing the detailed anatomy of the finest tissue elements is to examine them in the living state. In transparent tissue such as the cornea, the anatomy of the neural elements can be assessed by examining fresh slices under phase-contrast conditions (Weddell and Zander, 1951). The nearest approach to this in less transparent tissues is to stain them with methylene blue in the living animal and to examine fresh preparations before the dye 'fades' (Ohmori, 1924). This method is notoriously capricious and, despite recent improvements in technique (Weddell, 1941; Feindel, Allison, and Weddell, 1948), it

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 389-397, September 1954.]

is still not possible to control the concentration of the dye reaching a given tissue within the fine limits necessary for selective staining which will not distort the neural elements or give rise to artifact-formation. Attempts to 'fix' the dye and make the tissue available for microscopical examination as a relatively permanent preparation result in further distortion of the neural elements and the introduction of still more artifacts (Weddell and Zander, 1950).

Other methods of staining tissue neural elements (such as silver, gold, osmium tetroxide, iron haematoxylin, &c.) involve the submission of the tissue to an even longer and more complicated series of processes than vital methylene blue staining, all of which are liable to produce artifacts.

In an effort to overcome the defects of patchy staining, distortion of nerve fibres, and the introduction of artifacts during the process of preparing tissues for microscopical examination, we have exposed them to 'spreading factor' before subjecting them to specific neurohistological techniques. It is the purpose of this paper to give a general account of the results which we have obtained and to discuss the value of such enzymes and the rationale of their use for neurohistological purposes.

Material

Our observations were made chiefly on skin obtained from living animals and human subjects at operation, but we were also successful in obtaining good preparations from the cornea, mesentery, stomach, intestine, uterus, kidney, and suprarenal of the rabbit.

Method

A 1 c.c. ampoule of 'Hyalase' (Benger) equivalent to 1 mg. of hyaluronidase was diluted with 5 c.c. of normal saline and injected into the tissue (in the case of skin, both intra- and subcutaneously), the usual precautions with regard to strict cleanliness being observed. Not less than 20 minutes later, the tissue was carefully removed and fixed in 15 per cent. formalin in preparation for impregnating with silver according to the techniques of Weddell and Zander (1950), or of Bielschowsky-Gros, or for staining for myelin by Weil's method. In the case of methylene blue staining a solution of 0·02 per cent. or 0·04 per cent. methylene blue (Merk) in normal saline was infiltrated into the area treated with hyaluronidase until it was stained a uniform blue colour. The treated tissue was then massaged at about 5-minute intervals over a period of 20 minutes, after which it was removed and treated in the manner described by Weddell (1941).

Observations

Methylene blue staining by local injection

It is difficult to compare histological techniques of the kind in question objectively, for they are all capable of yielding an almost perfect result in a

limited region and, on rare occasions, over a wider area. It is, however, possible to state that on 40 successive occasions we have obtained almost uniformly successful methylene blue staining of the cutaneous nerve fibres and their terminals in skin from the dorsum of the rabbit ear after the use of hyaluronidase; whereas our notes show that in only 20 instances out of 40 were the results uniform over even small areas of skin in preparations stained with methylene blue, when hyaluronidase was not used. Moreover, in none of these untreated cases were all the nerve fibres and their terminals perfectly displayed throughout the uniformly stained areas.

We do not claim that the use of hyaluronidase in the manner described by us will make it possible for the axis cylinders of the nerve fibres to be stained uniformly, specifically, and without distortion in all tissues and on all occasions. We have, however, been able to obtain consistently good results in all the tissues which we have examined and our standard for comparison has been the appearance of the axis cylinders of nerve fibres teased apart from a nerve trunk in normal saline and the appearance of fine axons in the fresh cornea under phase-contrast conditions (Weddell and Zander, 1951).

One striking advantage of the method is the minimal pressure required to inject methylene blue into the tissue. Thus, with practice, it is possible to stain axons throughout the skin of the dorsum of the rabbit ear through a single puncture by subcutaneous infiltration of the dye solution. To stain nerve terminals in the epidermis satisfactorily, however, it is necessary to inject the dye intradermally also. Various other modifications have to be made, depending upon the tissue which is to be stained. In the case of the rabbit uterus, for instance, the dye had to be injected intravascularly after the uterus had been infiltrated with hyaluronidase, to obtain the best results (Pallie, Corner, Weddell, 1953).

The effect of injecting hyaluronidase before the dye is twofold. Not only are the nerve fibres stained more uniformly over a wider area but they hardly appear to be distorted at all. The finest nerve fibres are beaded, but the size of the beads is less than in preparations made without hyaluronidase and they are always connected to one another by threads of axoplasm. If the concentration of methylene blue is increased, the size of the beads increases, and conversely. Low concentrations of dye may sometimes display fine nerve fibres which are virtually smooth in outline, but they are then difficult to see.

Fine nerve fibres ending in relation to hair follicles and those found within cellular capsules are particularly well seen, but we have found that such terminals, in whatever region of the body they are found, are best displayed if the strength of the dye is increased to 0·04 per cent., although this inevitably leads to some distortion (i.e. swelling and irregularity) of the axons serving them and to some generalized tissue staining.

These observations, together with more detailed findings, are best particularized by reference to a series of photomicrographs. Fig. 1, F is from a preparation of the skin of the rabbit's ear, stained with methylene blue. A single optical plane through a small nerve-bundle is shown. The axis cylinders are specifically

stained. They are smooth in outline and of uniform diameter. Nodes of Ranvier can be seen in relation to each myelinated axon. Fig. 1, A is from a similar preparation but at lower magnification and shows the manner in which a nerve-bundle similar to that seen in the figure gives rise to a 'cutaneous plexus' of axons. In this preparation the concentration of methylene blue was 0·04 per cent., which has resulted in slight generalized tissue staining, together with staining of the nuclei of a number of cells in the connective tissue, as well as those of the sebaceous glands. In addition, some of the finer, more superficial, axis cylinders are not specifically stained but are seen to be enveloped by Schwann cells. Fig. 1, G is from a similar preparation and is included to illustrate the distortionless, specific, and uniform staining of an isolated myelinated axis cylinder. Preparations of this kind provide an opportunity for measuring the lengths of axon between adjacent nodes of Ranvier.

FIG. 1, C is from a similar preparation and again demonstrates the value of hyaluronidase in obtaining undistorted preparations. The bundles of axis cylinders are displayed specifically and without distortion. The axons remain quite independent of one another and do not break up into neurofibrillary strands nor do they enter into a reticular formation. Fig. 2, A shows how clearly it is possible to display fine axoplasmic filaments ending in relation to the wall of a small artery. These filaments are derived from stem axons by a process of arborization and each terminates freely and extracellularly. The filaments do not fuse with one another to form protoplasmically continuous nets. This latter point is more convincingly demonstrated in Fig. 2, F, in which axoplasmic filaments can be seen terminating freely in relation to a small arteriole. Fig. 2, D shows fine axoplasmic filaments arising from stem fibres, and ending in relation to a hair follicle in skin from the rabbit ear stained by methylene blue. It is very difficult to display the fine nerve terminals in this situation without prior treatment with hyaluronidase unless an excessively high concentration of dye (0·05 per cent.) is used, and this leads to gross distortion of the stem axons. High concentrations (0·04 per cent.) of dye can be used after treatment with hyaluronidase, for the fluid spreads so widely and evenly that the relative concentration of the dye is never excessive and thus distortion is minimal. Details of the innervation of hairs will be given in a subsequent paper but our illustration shows the essential characteristic of nerve termination in the skin—stem axons giving rise to arborizations of separate axoplasmic filaments.

FIG. 1 (plate). A is from a specimen of skin from the rabbit's ear, stained with methylene blue (0·04 per cent.).

B is from a silver-impregnated specimen of skin from the rabbit's ear.

C is from a specimen of skin from the rabbit's ear, stained with methylene blue.

D is from a specimen of skin from the rabbit's ear stained by Weil's method.

E is from a silver-impregnated specimen of skin from the rabbit's ear.

F is from a specimen of skin from the rabbit's ear, stained with methylene blue.

G is from a specimen of skin from the rabbit's ear, stained with methylene blue.

H is from a silver-impregnated specimen (Romane's method) of nerve trunk from the rabbit's ear.

Fig. 2, G shows an encapsulated ending stained with methylene blue, from the pad of a human toe. It shows a myelinated axon giving rise to a series of fine axoplasmic filaments which terminate freely and extracellularly, often in small swellings. These lie among the cells of the capsule which can only be seen under phase-contrast conditions, for they are not stained with methylene blue in preparations of this kind. Fine terminals ending freely in between the cells of the stratum germinativum in the neighbouring epidermis can also be seen; they are indicated by an arrow.

Fig. 2, H shows stem fibres giving rise to fine-beaded freely-ending naked axoplasmic filaments in the epidermis of a rabbit tongue stained with methylene blue. The separateness and extracellular position of the terminals is clearly shown by the use of hyaluronidase, for the tissue can be fixed without distortion and the terminals do not collapse upon one another to simulate a protoplasmically continuous peripheral nerve net.

Fig. 2, B shows bundles of fine nerve fibres in the adventitia of the suprarenal gland of the rabbit. A number of fine filaments can be seen passing to the cortex where they ramify among the gland cells.

Fig 3, A–C are from rabbit stomach stained by local injection of methylene blue after treatment with hyaluronidase. The absence of shrinkage and distortion of the fine nerve fibres and ganglion cells is striking. It is possible to follow individual axons in nerve bundles, where they always remain separate, towards their termination in the muscle coat or mucous membrane. The termination always takes the form of an arborization into fine axoplasmic filaments which end freely either in relation to a nerve ganglion cell or to one of the tissues of the stomach wall. We did not see any nerve fibres giving rise to a series of anastomosing fibrillae. Fig. 3, F is from a preparation of rabbit uterus stained with methylene blue. It shows stem axons giving rise to fine naked axoplasmic filaments ending freely in relation to the uterine myometrium. The nerve supply of the rabbit uterus has been the subject of a separate communication (Pallie, Corner, and Weddell, 1953).

Silver impregnation.

We have found that it is possible to obtain pictures comparable with those seen after methylene blue staining if certain precautions are observed. The tissue should remain in fixative for at least 24 hours but not longer than 3–4

FIG. 2 (plate). A is from a specimen of skin from the rabbit's ear, stained with methylene blue.

B is from a specimen of suprarenal gland from the rabbit, stained with methylene blue.

C is from a silver-impregnated specimen of skin from the lip of the monkey.

D is from a specimen of skin from the rabbit's ear, stained with methylene blue.

E is from a silver-impregnated specimen of skin from the finger pad of the monkey.

F is from a specimen of the skin of the rabbit's ear, stained with methylene blue.

G is from a methylene blue stain specimen of skin from the pad of a human toe, stained with methylene blue.

H is from a specimen of mucous membrane from the rabbit's tongue, stained with methylene blue.

I, J, and K are from silver-impregnated specimens of skin from the finger pad of the monkey.

days, and to obtain uniform impregnation it is essential that the sections should be of uniform thickness. To impregnate the axon specifically it is necessary to control the period during which the section remains in the ammoniated silver bath: with practice, this is not difficult. Careful control during this stage also makes it possible to demonstrate the relationship of axis cylinders to the Schwann elements surrounding them. In this section, too, we shall attempt to particularize our observations by reference to a series of photomicrographs; but the results are less dramatic than in the case of methylene blue staining, for sections as opposed to whole preparations give flat, strictly two-dimensional, pictures. It is also necessary to impregnate the surrounding tissue to some extent, for otherwise the pictures are merely of fine black lines and this means that the finest axoplasmic filaments often appear as insignificant threads and do not stand out from the background.

Fig. 1, B is from a silver preparation of the skin of the rabbit's ear. It shows axis cylinders of the cutaneous nerve plexus specifically impregnated. Fig. 1, E is from a similar preparation. In this case, however, the specimen was allowed to remain slightly longer in the ammoniated silver bath and this has resulted in the Schwann cell sheath being clearly outlined. Fig. 2, C is from a silver preparation of skin from the lip of a Rhesus monkey. It shows stem fibres giving rise to fine axoplasmic filaments ending in relation to a large hair follicle. The filaments are extremely fine and hence they do not appear so dark on the print as we would have liked. Moreover, it is difficult to display long lengths of nerve in the single plane offered by a thin section. It will be noticed that in this specimen the stem nerve fibres are slightly distorted. This is due to somewhat prolonged immersion in the ammoniated silver bath. As in the case of methylene blue staining, the display of fine axoplasmic filaments in this position requires treatment which is rigorous enough to damage stem axons to a small degree.

Fig. 2, E shows a stem axon giving rise to a leash of fine axoplasmic filaments coursing in close relationship to a capillary in the dermis of monkey skin. The filaments are fine and smooth and remain separate throughout their course. Fig, 2, I–K show encapsulated nerve endings from the finger pad of a monkey. In both pictures stem axons can be seen as well as the fine axoplasmic filaments to which they give rise. The filaments lie in between the cells of the capsular wall and end freely.

Fig, 3, D shows stem fibres giving rise to fine axoplasmic filaments ending on the cell bodies of nerve ganglion cells in the wall of the rabbit stomach. The number of filaments is large but they are all separate and end freely in an extracellular position. Fig. 3, E shows fine axoplasmic filaments which terminate among the cells of the stomach mucous membrane of the rabbit. The filaments are derived from stem axons and end freely and extracellularly.

FIG. 3 (plate). A, B, and C are from specimens of rabbit's stomach, stained with methylene blue.

D and E are from silver-impregnated specimens of rabbit's stomach.

F is from a specimen of rabbit's uterus, stained with methylene blue.

The value of 'spreading factor' in relation to a more routine neurohistological procedure is illustrated in Fig. 1, H. This is part of a nerve trunk from the dorsum of the rabbit ear which had been treated with hyaluronidase before removal and impregnation according to the method of Romanes (1950). The absence of gross distortion is particularly well illustrated in the appearance of the capillaries.

Myelin impregnation

Fig. 1, D shows myelinated axons in close relation to hair follicles in the rabbit ear. The specimen was prepared by Weil's method and shows how the myelin sheath tapers towards its termination. There is comparatively little distortion, although impregnation was somewhat prolonged as can be seen by the faint but general nuclear staining.

Discussion

Duran-Reynals first demonstrated that the introduction of testicular extract into rabbit skin facilitated the spread of vaccinia virus in 1929 and it was soon discovered that the clinical use of this substance made it possible to introduce subcutaneously large quantities of fluids which diffuse readily without causing pain or damage.

We first employed hyaluronidase to promote the spread of methylene blue in the skin of the rabbit's ear, as did McGregor (1953). We hoped that it would enable us to obtain more uniform staining of axis cylinders. Further acquaintance with hyaluronidase, however, showed that not only does it assist the spreading of methylene blue and the penetration of nerve-impregnating substances, but it also alters the properties of the skin and other tissues to such an extent that when they are fixed in either alcohol or formalin the axis cylinders are much less distorted by the fixative than usual. We also found that the results of Weil's method were improved by preparatory treatment with 'spreading factor'.

The mechanism underlying the phenomenon of spread is complex but some of the factors are now known. The matrix of connective tissue is highly viscous and consists of a complex of proteins and mucopolysaccharides. Its viscosity is due in particular to hyaluronic acids, and the hyaluronidase enzymes, which are active principles in the testicular extract mentioned above, cause a chemical reaction which involves the depolymerization and hydrolysis of the hyaluronic acids, thereby greatly reducing the viscosity.

Since mucopolysaccharides are demonstrable histochemically in blood-vessels in the dermis (more particularly in the region of the papillae) and around hair follicles, the introduction of hyaluronidase into the skin might have been expected to lower the viscosity in these regions and to facilitate the permeation of fluid throughout the skin generally. That this is the case we have demonstrated by the results obtained after the injection of methylene blue. Since the spaces between connective tissue fibrils and other structures are

rendered fluid, nerve fibres will be more freely and evenly exposed to staining or impregnating agents than is usually the case and thus distortion and artifacts due to the physical process of the injection of methylene blue will be virtually eliminated and, in addition, the concentration of the dye can be more accurately adjusted to obtain specificity of action without distortion, and the staining process itself is more even.

Further, the use of 'spreading factors' assists the process of rapid and even tissue fixation and thus eliminates artifacts which are known to result from the slow penetration of fixatives. Similarly, the various agents used to stain or impregnate nerve fibres after fixation penetrate more rapidly and evenly and the results obtained are thus far more uniform.

Finally, it has been shown in attempts made to wash out mucin from tissues that a viscous connective tissue matrix can, when subjected to tension, cause distortion and displacement of collagen fibres and presumably any nerve fibres that may be passing among them. Thus artifacts resulting from mechanical handling of tissues are also eliminated if the connective tissue matrix is rendered less viscous.

We do not claim to have exhausted the possibilities of this technique for our observations have so far been limited to certain specified regions of the skin. Also, the effect of 'spreading agents' is known to be modified by age and by the presence or absence of certain hormones, oestrogens tending to increase it and corpus luteum hormones to decrease it. There are, in addition to hyaluronidase, lytic agents such as collagenase which might prove of great value in specific instances. It is, however, clear from our observations that the use of hyaluronidase as a 'spreading factor', when used in conjunction with each of the three most widely used neurohistological methods (i.e. methylene blue staining, myelin and silver impregnation), gives sections in which the tissue elements are more consistently and uniformly stained and in which the nerve fibres stand out so clearly that they can be traced to their ultimate point of termination; further, they are relatively free from artifacts. This result is due to its property of allowing rapid and even penetration of fluids throughout tissues and its ability to render them relatively more homogeneous in their behaviour towards various reagents. The observations of Irving and Tomlin (1954), which have appeared since this paper went to press, suggest that in the case of silver impregnation, hyaluronidase also renders reticular and collagen fibres non-argyrophil.

There is one other point worth mentioning, which concerns skin in particular. Khanolkar (1951) has recently clarified some essential features of leprosy and has shown that this disease is neural at its inception, inasmuch as the spread of micro-organisms is either in or along the finest cutaneous nerve fibres in the initial stages, probably gaining entrance through hair follicles. The bacilli are then attracted towards the degenerating or regenerating nerve fibres in the cutaneous plexus. Wyburn and Bacsich (1950) have shown the rapidity and ease with which hyaluronidase penetrates into nerve trunks and they also found that the effect of hyaluronidase used in this manner lasted 24

hours and showed no ill after-effects. Any therapeutic measure designed to control the course of the disease would have to reach the axonal substance so as to be able to exert its influence on the bacilli. Our own observations on the use of hyaluronidase in rendering cutaneous nerve fibres and terminals accessible to staining or impregnating agents would seem to be of particular importance in this connexion.

This work was made possible by a grant from the Rockefeller Foundation which is gratefully acknowledged. We would also like to thank Miss Jean Gurden and Mr. Frank Blackwell for their skilled technical assistance.

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