Experiments on Vital Staining with Methylene Blue

By JOHN E. HARRIS AND A. PETERS

(From the Department of Zoology, University of Bristol)

SUMMARY

A new method is described for vital staining of nervous tissue with methylene blue; it involves impregnation of whole embryos or tissue-fragments with the rongalit-reduced dye under anaerobic conditions at pH 5, followed by development of the stain in an oxygenated neutral saline solution. Determinations of the solubility of leuco-methylene blue and experiments on vital staining with it and with the oxidized dye at different pH values are consistent with the hypothesis that the dye penetrates cell membranes and tissues mainly as the leucobase, but may pass along nerve (and muscle) fibres in the oxidized form. Penetration of the leucomethylene blue below pH 5 is hindered by its ionization; above pH 5 the penetration falls off because of reduced permeability of the cell membranes.

SOME four years ago, with the help of Miss L. Morris, one of the present authors developed a new method of vital staining of the nervous system of the embryo dogfish, *Scyliorhinus canicula*, using a modification of the old-established rongalit methylene blue technique. The success of this method led to an experimental study of some of the factors involved, and the present account deals mainly with these studies. The work is concerned almost entirely with the penetration of the stain into the tissues; the part, if any, played by the specific chemical combination of nerve-cell constituents with the dye-stuff in its final site is not considered.

Since the method used originally on the dogfish has not previously been published, and has been very little modified as a result of the later work, it will be convenient to outline it first.

A TWO-STAGE METHOD FOR VITAL STAINING WITH RONGALIT METHYLENE BLUE

The stock solution of rongalit-reduced methylene blue was prepared according to the method of J. E. Smith (1947).

A dogfish egg-case was cut open and the yolk together with the embryo was carefully transferred to a dish of dogfish saline (Bialaszewicz, 1933). The embryo was dissected off with as little damage as possible, any surplus yolk being removed by a jet of water from a fine pipette; it was then transferred to a thick-walled glass test-tube containing 10 ml. of the saline. A well-fitting rubber stopper, pierced with a single hole, sealed the tube mouth and served to connect the tube to a gas line including a simple mercury manometer, a rotary vacuum pump, and a gas-cylinder of nitrogen (oxygen-free).

[Quarterly Journal of Microscopical Science, Vol. 94, part 2, pp. 113-124, June 1953].

The air in the tube was evacuated; tapping the walls of the tube helped to liberate dissolved air, and the pressure was reduced until the solution just began to boil at room temperature (15–20° C.) As soon as this occurred the vacuum line was shut off and nitrogen admitted until atmospheric pressure was re-established. The stopper was removed, and 0·1–0·5 ml. of rongalit methylene blue stock solution was added, the tube being closed again as soon as possible. The evacuation and admission of nitrogen was repeated to remove the last trace of oxygen and the system was then left for about an hour for impregnation of the embryo with the dye (or rather with its leuco-derivative) to be completed.

Within a minute of the deoxygenation the embryo ceased to show any of its typical rhythmical movements. At intervals during the hour the contents of the tube could be agitated by rotating it between the palms of the hands; the actual time used for this stage, and the amount of rongalit methylene blue stock added, varied with the age of the embryo.

After this phase was completed the tube was unsealed and the contents poured out into a Petri dish. The embryo was taken up in a pipette, and transferred to a dish of fresh well-aerated saline and kept under examination with the high power of a binocular dissecting microscope; it was illuminated (by reflected light) only at intervals. Within a few seconds it became uniformly blue; after a minute or two normal body movements started again. At first there was no visible differentiation of the nerves, but in a time which varied from 10 minutes to half an hour or more, the muscles and other non-nervous elements became paler and more transparent while the nerve cells and fibres took on the typical intense dark blue stain.

The dye used was obtained from Grübler, Gurr, British Drug Houses or Imperial Chemical Industries, and was in each case the zinc-free chloride for vital staining. All brands were successfully used, the only difference being in the extent to which the nervous system was differentiated from other tissue elements, which were sometimes stained to a greater or lesser extent. A rongalit methylene blue (RMB) stock which was pale pink in colour invariably gave better differentiation than one which was yellow, though the nerves were always well stained. We believe that staining of non-nervous elements in these specimens is brought about by other thionine derivatives, probably present as impurities. Heller, Starr, and Davenport (1949) have indicated this and the present writers can confirm that in the case of the dye 'new methylene blue', the use of a rongalit-reduced solution leads to a very well developed staining of other tissue elements without any staining of nerve fibres. It appears that tetramethyl thionine (methylene blue in the strict sense) is highly specific in its nerve staining; the trimethyl, dimethyl and monomethyl derivatives and thionine itself (Lauth's violet) show a decreasing ability to stain nerve cells and fibres together with an increasing affinity for other tissues. Since the lower methyl homologues are present to some extent in all commercial methylene blues (from which in fact they are more or less impossible to remove), it is reasonable to suppose that our Grübler dye sample, which produced a typical

pink RMB stock, and gave the best *differentiation* of the nervous system, contained the smallest amount of these impurities. The difference between the four samples was, however, small. While rongalit white supplied by Gurr was used in all the later studies, other batches of different origin and of unknown age were used with equal success in the early work.

Not only was the source of dye and rongalit relatively unimportant, but the ageing of the RMB stock was also immaterial. Kept in a well-stoppered or wax-sealed tube it has been used at all times after preparation, from immediately after it has been filtered up to 2 months later. It is generally stated that the solution should be used only after 24–36 hours and up to 8–10 days (Pantin, 1946). The objection to its immediate use, we believe, is the presence of an excess of hydrogen sulphide in the freshly prepared stock-solution; this penetrates organisms very readily (Osterhout, 1925) and is highly toxic. The evacuation of the staining solution removes the hydrogen sulphide as effectively as it does the oxygen; embryos will remain alive and healthy in it for long periods, whereas they are rapidly killed by an RMB solution which has been used in the normal manner, too soon after preparation of the stock.

The poor results obtained from a stock solution which has been aged too long are partly due to reoxidation of the leucomethylene blue, the solution taking up atmospheric oxygen and turning blue in the process. This does not occur if the stock bottle has a very small free air space and is tightly sealed.

One of the most striking features of the two-stage method of vital staining described above was the almost simultaneous development of the stain in all the nerve cells and their processes, both central and peripheral. It is clear that during the impregnation stage a large and fairly uniform concentration of dye in the reduced condition accumulated throughout the organism. As oxidation proceeded, the dye appeared to migrate into its final site; the muscles and other non-nervous tissues lost their early blue colour while the nerve cells and fibres became more and more intensely stained. So far as we can see, nerve staining was complete in all the functioning neurons and their processes. At what stage in its life a neuroblast becomes capable of concentrating this enormous amount of dye we do not exactly know, but some yet-unpublished work suggests that it is less than 48 hours before it begins to conduct impulses.

An Experimental Study of the Impregnation Process

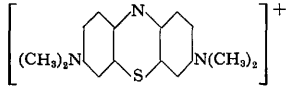
1. *The equilibrium of reduced methylene blue in the staining solution.*

It was fortunate in many ways that the method had been tried first on dogfish embryos, for, as will become apparent later, the conditions provided by the experiment were accidentally almost ideal for its success. Similar tests with embryos of the lamprey and trout were less successful until it was realized that the lamprey and trout Ringer solutions used were buffered by the addition of phosphate. Owing to the hydrochloric acid added in its preparation, RMB stock solution is acid and the staining solution made with dogfish

saline was at about pH 5; that made with the other buffered Ringer solutions was much nearer to pH 7. Clearly an investigation of the effect of pH on the impregnation was required, and also a study of the dissociation of the leucomethylene blue under the conditions of impregnation.

According to the work of Clark, Cohen, and Gibbs (1925) the essential features of the oxidation-reduction equilibrium of methylene blue may be briefly summarized as follows: methylene blue chloride (the form in which it is used for vital staining) exists in solution in the oxidized form as a completely dissociated salt at all pH values of biological significance.

The structural formula of the cation may be written:



Reduction of this radicle gives leucomethylene blue by producing a negative charge on the bridging N-atom, which fixes a hydrogen ion in consequence. This gives an uncharged molecule which we may write as HB, where B is the radicle shown in the structural formula above (neglecting changes in electronic configuration brought about by the reduction). The unionized leucomethylene blue HB is colourless and forms the principal component present in neutral or alkaline reducing solutions. In acid solutions, however, the leucobase adds on first one and then a second hydrogen ion to the dimethylamino-groups, giving radicles which can be schematically represented by the formulae H_2B^+ and H_3B^{++}.

The unionized leucobase HB is poorly soluble in water and in physiological saline solutions. Clark, Cohen, and Gibbs (1925) give approximate figures for its solubility at pH 2·9 and 8·6; they also made careful and detailed potentiometric titrations to establish the dissociation constants of the leucobase. These dissociation constants we have used in the calculations below, but it has been necessary to obtain accurate values of the solubility of the leucobase over a wider range of acidities than in this early work.

This solubility has been estimated by three different methods: (*a*) by a direct colorimetric estimation of methylene blue after oxidation of a saturated leucobase solution, (*b*) by weighing the methylene blue precipitated when such a solution was treated with an excess of potassium dichromate, and (*c*) by a direct titration of the leucobase solution against 2, 6-dibromophenol indophenol in an atmosphere of nitrogen. Each value for the solubility at any given pH was checked by two of the three methods, method (*b*) being appropriate in acid solutions where the solubility is high, and method (*c*) at alkaline pH-values where the redox indicator is most reliable. The results are summarized in graphic form in fig. 1, which also shows the calculated concentrations of HB, H_2B^+, and H_3B^{++} present in an actual staining solution in which the embryos were immersed (assumed to be 0·5 ml. of RMB stock in 10 ml. of saline at 18° C).

It will be seen that from pH 9 down to pH 6 the concentration of leucobase in the staining solution is substantially constant; the solution is saturated, free precipitated leucobase being present, as the staining experiments show. Below pH 6 the amount of ionized H_2B^+ becomes appreciable; that of H_3B^{++} rises at and below pH 5. On the acid side of pH 5 the concentration of undissociated leucobase, HB, falls off very rapidly; the total soluble leucobase

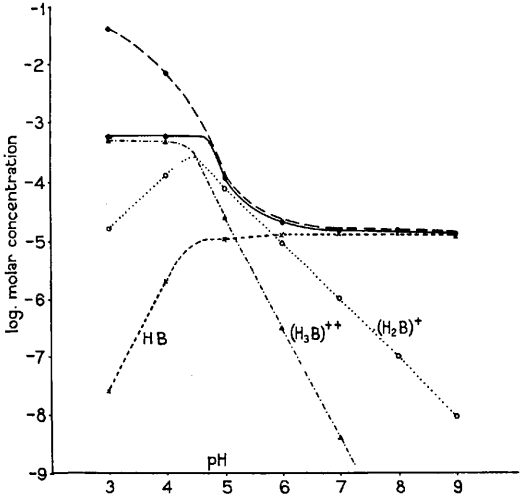


FIG. 1. The equilibrium of reduced methylene blue in aqueous solution.

•– – – –• experimentally determined solubility of leucomethylene blue.

•——— • total dissolved leucomethylene blue in a standard rongalit staining solution containing 6×10^{-4} *M* methylene blue. Above pH 4·5 precipitated leucobase is present.

HB
$(H_2B)^+$
$(H_3B)^{++}$
} concentrations of the three separate components present in the standard solution.

$(HB+H_2B^+ + H_3B^{++})$ increases until pH 4 is reached, below which value the whole of the reduced dye is present in true solution, principally as the divalent cation H_3B^{++}

2. *The effect of hydrogen ion concentration on staining.*

The first experiments were simply an extension of the new method to investigate the effect of pH on the uptake of dye under reducing conditions. Owing to the difficulty and expense of obtaining a large number of similar dogfish embryos, the work was done on pieces of earthworm body-wall, each

including a portion of the ventral nerve cord. Each experiment involved seven similar pieces of tissue taken from a single worm. The pieces were placed singly in 10 ml. of earthworm saline (Pantin, 1946), made up without phosphate; the pH of the seven solutions was adjusted to 3, 4, 5 . . . 9 by the addition of NaOH and HCl or by adding 2 ml. of standard buffer (Clark and Lubs, McIlvaine, or Kolthoff buffers were used for this purpose; see Clark, 1923). Whenever buffer solutions were not used, the pH was rechecked electrometrically at the end of each experiment; tests showed that there was no appreciable change during the process of uptake of the dye.

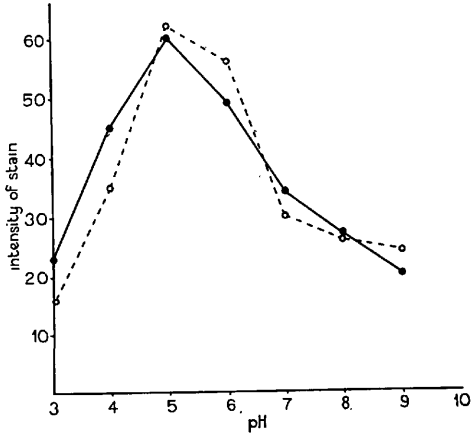


FIG. 2. Staining of earthworm tissues with rongalit methylene blue under anaerobic conditions. •———• nerve cells and fibres. o------o muscles. (For method of calculating ordinate, see text.)

At the end of a fixed period, identical for all seven specimens, but varying from 30 minutes to 5 hours in different experiments, each preparation was dropped into a solution of sodium perchlorate, which served to oxidize and fix it; it was then mounted in a gum acacia glycerine medium of refractive index 1·51–1·52 for examination.

Since little or no time was allowed for differentiation to occur, muscle and nerve were both stained; the seven preparations in each experiment were arranged in descending order of intensity of staining. To the best preparation was given a 'mark' of 7, the remainder being rated 6, 5 . . . down to 1.

The results of twenty-three experiments on 150 specimens are summarized in graphical form in fig. 2, which relates the 'total marks' in all experiments to the pH of impregnation, irrespective of the method of buffering and time of impregnation. It is clear that the maximum intensity of stain in both nerve and muscle occurred at pH 5. In fact, pieces stained at pH 3 and 9 were almost

colourless; neither the optimum pH nor the intensity of stain showed any systematic variation with the method of buffering employed.

In the above experiments we were not concerned with the differentiation of nervous tissue from muscle; the similarity of the curve of staining in the two tissues suggests that the main factor involved is the rate of penetration of the dye through the organism, which is most rapid at pH 5. We have shown that below this value the concentration of undissociated leucobase falls off; below pH 5, though the total solubility of leucomethylene blue increases rapidly, it is largely present as the ions H_2B^+ or H_3B^{++}.

When a whole organism is immersed in an oxygenated methylene blue saline solution, staining takes place in a very different manner. Penetration of the dye is very slow, except at points where the surface is damaged; from such regions a selective staining of nerve fibres takes place more or less rapidly, the non-nervous tissues being almost uncoloured. Along the peripheral nerve fibres the stain develops until it reaches the neurons in the central nervous system, through which it spreads in a similar selective manner, apparently through 'functional' contacts of each neuron with its physiologically linked neighbouring neurons and their fibres. The method is in fact a useful one for exploring the innervation of a peripheral structure. Since penetration of the dye in this way takes place by a different path, it seemed useful to investigate the effect of pH on this type of staining on the same material.

Pieces of earthworm body wall immersed in oxygenated saline containing methylene blue stain progressively from the cut edges; only the damaged muscles are stained appreciably, but nerve-staining extends centrally as described above. Fig. 3, obtained from a second series of twenty experiments with 140 specimens stained in this way and assessed on regions within the restricted area of satisfactory staining, shows that the best staining of the nerves occurred in alkaline solutions with an additional peak at pH 7; below pH 7 the intensity fell off until the preparation was almost colourless at pH 3. For the damaged muscles, the intensity of staining fell off progressively from pH 9 down to pH 3; once again there was no systematic difference that could be ascribed to different buffer solutions.

The very different pH optima shown by these experiments can be reconciled with that shown under reducing conditions if we assume that the final staining in the last experiment is a resultant of two distinct processes: (*a*) the penetration of the dyestuff along the nerve or muscle fibre, which does not involve the crossing of a cell boundary, and (*b*) the loss of dye from the stained nerve or muscle through the cell membrane into the surrounding unstained tissues. The latter process does demand the passage of the dye through a cell membrane, which we assume takes place almost entirely in the form of unionized leucomethylene blue. In the living cell there must always be an equilibrium between the oxidized and reduced form of the dyestuff; the amount of staining will be less if reduced dye molecules are constantly leaching away. This leaching out is most likely to occur at acid pH values provided that these are not so acid as to ionize the leucomethylene blue.

The pH of the neighbouring tissues is never likely to fall as low as that of an acid external medium; poor staining with oxidized methylene blue in acid media may be explained by loss of leucomethylene blue from stained cells if the permeability of the tissues to the leucobase increases on the acid side of neutrality. This result appears to contradict the earlier hypothesis, but a reference to the first experiment illustrated in fig. 2 supports this conclusion. The solubility of the leucobase is only slightly less at pH 9 than it

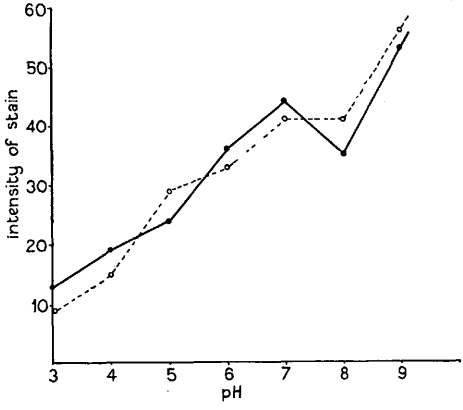


FIG. 3. Staining of earthworm tissues with methylene blue under fully oxygenated conditions; •———• nerve cells and fibres. o- - - - - - o muscles. (For method of calculating ordinate, see explanation to fig. 2. in text.)

is at pH 5; yet we see from fig. 2 that the staining (from RMB solution) falls off very greatly, not only below pH 5 where the leucobase ionizes, but also above pH 5 where it does not.

The reduced permeability of the tissues to leucomethylene blue as the pH rises above 5 required additional confirmation; a third series of experiments was designed to investigate this point. Pieces of earthworm were impregnated in RMB solution under anaerobic conditions at pH 5. At the end of a definite period of impregnation they were transferred to a series of tubes containing deoxygenated saline (without rongalit or dye) at pH values from 3 to 9. After a further period in these buffered saline solutions the specimens were removed and fixed in sodium perchlorate as before; one specimen, fixed immediately after it was taken out of the RMB solution at pH 5, served as a control.

The results of these experiments showed a rapid loss of leucomethylene blue into the saline solutions at all the lower pH values. At pH 9 and 8 there was almost no loss of stain, compared with the control, but at all values below pH 8 the loss became progressively greater. When the 'impregnation time'

and 'leaching-out time' were equal, tissues leached at pH 3 and 4 became completely colourless; if the impregnation time was rather less than the leaching-out time the preparation at pH 5 became colourless, and so on.

When the same experiment was tried with fully impregnated stained tissue leaching into *oxygenated* buffered saline solutions, only a very small loss of dye occurred even below pH 5.

It is important to realize that these tissues had originally been treated with saturated reduced methylene blue at pH 5; they had therefore accumulated a large amount of the dye throughout. In the earlier experiment reported (fig. 3), poor staining had been noted in acid oxygenated methylene blue at pH 5. Here, however, penetration of oxygenated methylene blue was limited to the 'exposed' nerve processes and cells; internal loss of leucobase could therefore occur to the very large volume of unstained neighbouring cells.

The rapid loss of reduced dye in solutions of low pH suggested the possibility of obtaining a very rough quantitative estimate of the amount of dye taken up. Pieces of earthworm were impregnated in deoxygenated RMB saline at pH 5; after 2, 3, and 5 hours they were transferred to fresh dye-free deoxygenated saline at pH 3. The solution obtained was allowed to oxidize and its dye content was estimated colorimetrically; from the wet weight of the pieces of worm used, an approximate estimate of the dye concentration in the tissue could be obtained. In seven experiments this was found to vary from $1{\cdot}5 \times 10^{-3}$ *M* to $2{\cdot}1 \times 10^{-3}$ *M*, showing no correlation with the time of impregnation; evidently equilibrium was attained in less than 2 hours. The average dye concentration ($1{\cdot}9 \times 10^{-3}$ *M*) in the wet tissue was about 17 times that in the original saline at pH 5 ($1{\cdot}1 \times 10^{-4}$ *M*), suggesting that even for leucomethylene blue there is a genuine accumulation of dye in the tissue. Clearly in the fully differentiated stained nerve fibre the concentration of oxidized dye must be enormously higher than this.

Experiments on earthworm fragments stained with RMB saline solutions in air (i.e. as in the normal rongalit staining process recommended by Pantin, 1946) gave inconclusive and highly variable results between pH 5 and pH 9; below pH 5 there was little or no staining. This inconclusiveness is not surprising; it is very difficult to achieve constant and reproducible conditions in dealing with a reducing solution in air, as anyone who has used the ordinary rongalit method on large specimens will appreciate. In addition a new factor enters into these experiments. The rate of oxidation of leucomethylene blue is a function of pH; it is relatively stable under acid conditions but the rate of oxidation, according to Clark, Cohen, and Gibbs (1925), is proportional to the fifth power of the OH-ion concentration. In the early stages of rongalit staining in air, the reduced dye will pass into the tissues as the leucobase, but at physiological pH values in the tissues it will be rapidly oxidized and will travel in the oxidized form along nerve fibres without crossing a cell membrane. Selective staining of nerve elements will therefore occur from the surface inwards, a result typical of the normal rongalit staining method.

It is clear that in the normal process of rongalit methylene blue staining,

one of the functions of the acid in the staining solution is to increase the permeability of the organism to the (reduced) dyestuff; another function may well be to depress the rate of oxidation of the leucobase in the saline medium.

Discussion and Conclusions

The experiments described have suggested the following general conclusions.

In pieces of excised tissue, and in whole organisms where surface damage has produced similar conditions, oxidized methylene blue stains nerve cells and fibres (and muscle cells, though less strongly), with increasing speed and intensity as the pH of the staining solution is raised. In such experiments the dye penetrates along intracellular pathways in the nervous tissue, not crossing cell boundaries except at functional (synaptic ?) contacts, which may well represent regions of alteration in properties of the membrane. A simple, though not necessarily correct, explanation of these facts could be made on the assumption that the free dye cation of the completely ionized methylene blue salt combined with negatively charged proteins (Singer, 1952).

Reduced methylene blue penetrates and stains such tissues, as well as undamaged whole organisms, much more readily, crossing cell-membrane barriers in the process. It is suggested that this penetration occurs in the form of uncharged molecules of leucomethylene blue. Below pH 5 the rate of penetration falls off very rapidly; this result agrees with a corresponding diminution in the concentration of uncharged leucomethylene blue, which ionizes at more acid values to give successively a singly and a doubly charged cation.

Unlike the oxidized dyestuff, reduced methylene blue is most rapidly taken up at this critical value of pH 5. Above this value penetration of leucomethylene blue falls off with increasing alkalinity. This is true even though the external concentration of the slightly soluble leucomethylene blue remains roughly constant above pH 5; under the conditions employed the solution was saturated. Between pH 6 and pH 9 the solubility of leucomethylene blue was estimated to vary only from $1{\cdot}17 \times 10^{-5}$ *M* to $1{\cdot}45 \times 10^{-5}$ *M*, a variation which was probably within the experimental error of the determination.

At pH values above 5, the rate of loss of reduced dye from previously impregnated fibres parallels the rate of uptake by unimpregnated ones. We are therefore not dealing with an affinity of the nerve fibre for the leucobase; the changes in rate of uptake and loss must reflect an effect of pH on the permeability properties of the cell membranes to the uncharged leucobase molecules.

There is little evidence that the positive ion of oxidized methylene blue can penetrate or leave undamaged living cells. Since, however, it must be in equilibrium with its reductant, the final colour of any stained preparation will depend not only on the affinity of the cell constituents for the oxidized dye, but also on the concentration of reductant which is in equilibrium with it; this will control movement of the reductant into and out of the cells in question. So long as the cells are intact, all these considerations will favour retention of

stain at high pH values. Under such conditions the ionization of the amino-groups of the proteins will be depressed, and their negative charge increased; also, the permeability of the cells to the small amount of leucomethylene blue in equilibrium within the stained cells will be reduced, limiting the escape of dye into other neighbouring cells.

The successful staining of intact organisms with methylene blue under aerobic conditions will be determined by similar complex considerations. Even when the bulk of the staining solution is fully oxygenated there will be, close to the surface of the organism, an appreciable concentration of reduced leucomethylene blue, which can penetrate the surface membranes. Once in the cells it will come into equilibrium with the oxidized cation which we assume combines with the cell proteins. Within the organism as a whole, oxidized methylene blue (i.e. stain) will accumulate (*a*) in regions in which the affinity for the cation is greatest, as with those proteins farthest on the alkaline side of their isoelectric point, and (*b*) in regions where the steady state equilibrium of the oxidant-reductant system is shifted most towards the former. Both of these characteristics may occur in living nerve cells; which of the two is the more important factor in the selective vital staining of nerve cells with methylene blue requires further investigation.

The conclusions outlined above are based largely on the experiments carried out in the present study, but they are in line with much of the earlier work with methylene blue as a vital stain. The contention of Irwin (1926) that the dye penetrates *Valonia* only in the form of oxidized lower methyl homologues appears unnecessary, but this view had already been criticized by M. M. Brooks (1927). It is pointed out by Brooks and Brooks (1941) that the dye is largely reduced in the cell protoplasm of *Valonia*, accumulating in the blue oxidized form in the central vacuole, so that our suggested mechanism of penetration may well apply to this alga. The fact that methylene blue penetrates *Valonia* faster from solutions at pH 8 than at more acid values (Brooks, 1926, 1927, 1929) agrees with our own data for the oxidized dye; in commenting on this result it is interesting to find that Brooks and Brooks (1941) say 'the difference in rate may be due to effects of pH on the plasma membrane rather than on the dye', but they do not appear to envisage penetration of the dye as the undissociated leucobase. There appears to be very little work on the effect of pH on permeability to such non-electrolytes. Davson (1939) has shown that haemolysis of rabbit red cells by glycerol is most rapid at pH 5, but Danielli (1943) emphasizes the abnormality of the conditions in these experiments, carried out in almost electrolyte-free solutions.

The most recent study of the effect of the medium on vital staining with methylene blue was reported by Heller, Thomas, and Davenport (1947), who worked on similar lines to those studied by Schabadasch (1936), but investigated staining by immersion instead of by injecting the dye into the blood circulation. Heller and others found the optimum pH to be about 5·6 in the oxygenated staining solution. This is nearer to our optimum for the reduced dyestuff but it may well be that even in Heller's experiments the actual

penetration was in the form of leucomethylene blue. The favourable effects of acetate, lactate, pyruvate, &c., reported by Schabadasch and Heller, reflect the probable importance of metabolic factors in the oxidation-reduction equilibrium, which we believe may be more particularly significant in the subsequent differentiation of the tissues than for the penetration of the stain.

One point in the present series of experiments has been only lightly touched upon. In wholly reducing anaerobic conditions leucomethylene blue accumulates in the tissues at a much higher concentration than in the surrounding medium. Stoichiometric combination of the dye cation with, or its adsorption on, an amphoteric tissue constituent as in the experiments of Pischinger (1927) and Singer (1952) cannot explain concentration of the uncharged leucomethylene blue; one is tempted to think of the process in terms of lipoid solubility. Though methylene blue is quoted by Brooks and Brooks (1941) as a lipoid insoluble dyestuff, it seems probable that they are referring to the oxidized form, which as a hydrated cation would be expected to have a low oil/water partition coefficient. The reverse is in fact true for the leucobase. A rough experiment with chloroform/water equilibration under anaerobic conditions showed a concentration ratio of the order of 25:1 in favour of the chloroform fraction.

We wish to acknowledge the help given by Miss L. Morris in the early stages of development of the staining technique. The later part was carried out during the tenure by one author (A. P.) of a graduate scholarship in the University of Bristol.

REFERENCES

Bialaszewicz, K., 1933. Arch. Intern. Physiol., **36**, 41.
Brooks, M. M., 1926. Amer. J. Physiol., **76**, 116.
—— 1927. Univ. Calif. Publ. Zool., **31**, 79.
—— 1929. Proc. Soc. exp. Biol. N.Y., **26**, 290.
—— 1929. Protoplasma, **7**, 131.
Brooks, S. C., and Brooks, M. M., 1941. *The permeability of living cells.* Berlin.
Clark, W. M., 1923. *The determination of hydrogen ions.* Baltimore (Williams and Wilkins).
Clark, W. M., Cohen, B., and Gibbs, H. D., 1925. Public Health Reports, **40**, 1131–1201.
Davson, H., 1939, quoted from Davson, H., and Danielli, J. F., 1943. *Permeability of natural membranes.* Cambridge.
Danielli, J. F., 1943, from Davson, H., and Danielli, J. F., 1943. Ibid.
Heller, R. E., Thomas, R. W., and Davenport, H. A., 1947. Stain. Tech., **22**, 111.
Heller, R. E., Starr, G. E., and Davenport, H. A., 1949. Ibid., **24**, 207.
Irwin, M., 1948. Amer. J. Physiol., **10**, 927.
Osterhout, W. J. V., 1925. J. gen. Physiol., **8**, 131.
Pantin, C. F. A., 1926. *Notes on microscopical technique for zoologists.* Cambridge (University Press).
Pischinger, A., 1927. Z. Zellforsch., **5**, 347.
Schabadasch, A., 1936. Bull. Hist. Appl., **13**, 5, 72, 137.
Singer, M., 1952. *Cytology I*, p. 211. Ed. Danielli and Bourne. New York (Academic Press).
Smith, J. E., 1947. Phil. Trans. B., **232**, 279.