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Factors Affecting Penetration of Methylene Blue and Trimethyl Thionine into Living Cells.*

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Since some confusion has arisen lately concerning the identity of the dye which penetrates into the vacuole of living plants of *Nitella* and *Valonia*, the writer has undertaken to analyze the causes of this apparent discrepancy.

Irwin[1, 2] has attempted to show by spectrophotometric determinations that the dye from solutions of methylene blue which penetrates the sap of these plants, is not methylene blue as originally stated by the writer[3, 4] but is rather some lower oxidation product, trimethyl thionine. This forms readily in alkaline solutions of methylene blue and penetrates as a free base, whereas methylene blue is completely dissociated and according to Irwin does not penetrate living cells except during injury.

The writer, on the other hand, also using the spectrophotometer to determine the identity of the dye found in the sap of these plants, found only methylene blue present.

An analysis of the differences in methods used by Irwin and the writer has been made. The following main points of divergence have been tested.

1. *pH value.* The writer used pH 8.83 while Irwin used 9.5 and 10.9. The use of the higher pH values in the external solution introduces 2 errors: first, precipitation of Mg (at pH 10.9) from sea water which produces a solution unbalanced with respect to cations. Such unbalanced solutions affect the permeability of all plant cells for which we have any data. The second effect of high pH value is the relatively rapid conversion of methylene blue into trimethyl thionine and related dyestuffs.

2. *Impurity of the dye.* The writer used Clark's methylene blue

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[1] Irwin, M., *J. Gen. Physiol.*, 1927, x, 927.

[2] Irwin, M., *J. Gen. Physiol.*, 1928, xii, 147; PROC. SOC. EXP. BIOL. AND MED., 1928, xxv, 563.

[3] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

[4] Brooks, M. M., *Univ. of Calif. Publica. in Zool.*, 1927, xxxi, 79; PROC. SOC. EXP. BIOL. AND MED., 1928, xxv, 704; *Proc. Nat. Acad. Sci.*, 1927, xiii, 821.

while Irwin used Merck's medicinal and French's. The spectrophotometric readings of all these dyes showed them to be pure methylene blue. But since the spectrophotometric method is not refined enough to show the presence of 5% or less of trimethyl thionine in a solution of methylene blue, according to Holmes,[5] impurities of this amount in presumably pure solutions of methylene blue would escape detection. For this reason, the spectrophotometric analyses of all these dyes failed to show whether there were any impurities present.

However, in comparing the rate of penetration into *Valonia* of samples of Merck's and Clark's dye, it was found that Clark's penetrates much slower than Merck's under similar experimental conditions. Also the absorption maximum of sap into which Merck's dye had penetrated was 662mμ, as compared with 667mμ, the maximum obtained from Clark's dye in sap. This shows that the methylene blue penetrating from Clark's dye is more nearly pure than that penetrating from Merck's. Irwin[2] also notes that Clark's penetrates slower than Merck's and produces an absorption maximum at a longer wave length than Merck's or French's.[2]

Since trimethyl thionine penetrates faster than methylene blue and colors the sap more rapidly, the dye which penetrates the slowest was considered the purest. This was Clark's.

3. *Concentration of dye in external solution.* Irwin used from 3 to 25 times the concentration of dye used by the writer, or .01% and .04% (which is equivalent to .00031 M and .00125 M) as compared with .000047 M and .000095 M used by the writer. There is a greater amount of trimethyl thionine present in higher concentrations of methylene blue than in lower ones. Since the rate of penetration of trimethyl thionine is faster than that of methylene blue, experiments have been made which show how this error can affect the results.

4. *Light.* Irwin's experiments were done in an incubator "with air holes" while the writer's were done in normal conditions of illumination.

5. *Injury.* It is possible that the high pH values and concentrations used by Irwin may have caused injury to her plants. Her observation of loss of turgor is a point in case. Where no morphological changes can be observed as in the writer's experiments, the only way at present of determining whether a plant has been permanently injured is by noting how long it survives after having been replaced in its normal environment. Since all the plants used

[5] Holmes, W. C., *Stain Technology*, 1927, ii, 71.

by the writer lived as long as the controls, no irreversible injury was manifest.

It has been shown that the apparent discrepancies in the findings of Irwin and those of the writer may be attributed to differences in methods. It seems best, therefore, in comparing results found by one investigator with those of another, to make sure that identical methods have been used, otherwise it is not justifiable to expect similar results. Differences creep in which are responsible for much confusion.

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Tissue Lactates and Blood Lactates as Affected by Muscular Exercise.

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In dogs anesthetized with amytal the following preparations were made: 3 fore limb muscles and 2 hind limb muscles were prepared for rapid excision, with care to avoid injury to said muscles or interference with their blood and nerve supply. A carotid artery was cannulized, and a brachial vein and the inferior vena cava below junction with the renal veins were exposed. The blood vessels leading to both kidneys were tied off. Stimulating electrodes were imbedded under the skin of the back at the levels of the first lumbar and last sacral vertebrae.[1]

Continuous records of arterial blood pressure and of oxygen consumption were made. At the end of a 15-minute basal period a sample of arterial blood was drawn and one fore limb muscle rapidly excised and thrown at once into liquid air. Artificial exercise (confined to the hind quarters by the location of the electrodes) was then induced for 15 minutes. At the end of the exercise period blood samples were drawn from the cannulized artery, from the inferior vena cava (representing the outflow from worked muscles) and from the brachial vein (representing the outflow from non-worked muscles). One hind limb muscle (worked) and one fore limb muscle (non-worked) were excised rapidly and thrown into liquid air.

At the end of a recovery period approximately one hour in length blood samples were again drawn, as above, and one previously

[1] Martin, E. G., Field, J., and Hall, V. E., PROC. SOC. EXP. BIOL. AND MED., 1928, xxv, 273.