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Improved Colorimetric Method for Determining Quantitatively the Indophenol Oxidase Content of Animal Tissues.

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The indophenol reaction employed depends upon the oxidation of a mixture of a-naphthol and dimethyl-paraphenylene-diamine-hydrochloride, in alkaline solution, into indophenol (blue) in accordance with the following equation:

$$(CH_3)_2N.C_6H_4.NH_3Cl+CO_{10}H_7.OH+O_2=(CH_3)_2N.C_6H_4.N=C_{10}H_6O+2H_2O+HCl$$

The HCl, which prevents the reaction, is neutralized by the alkali present. This oxidation occurs spontaneously at a very slow rate when the reacting substances are exposed to air, but may be accelerated twenty times or more when minced animal tissue is added. The determinations were always carried out in open flat-bottomed Petri dishes.

The substrate in use at the present time contains 0.072 per cent of a-naphthol (M/200), 0.08624 per cent of dimethyl-paraphenylene-diamine-hydrochloride (M/200) and 0.0625 per cent sodium carbonate in 19 per cent alcohol. In order to prevent spontaneous oxidation in the substrate prior to the beginning of the oxidase test, the reagents which make up the substrate must not be mixed until needed. By having stock solutions of: (1) 0.18 per cent a-naphthol in equal parts of distilled water and 95 per cent alcohol, (2) 0.216 per cent aqueous solution of dimethyl-paraphenylene-diamine-hydrochloride, and (3) 0.3125 per cent aqueous solution of sodium carbonate, the amount of substrate needed for a test may be obtained by pipetting 2 cc. of each of solutions number one and two, and 1 cc. of the carbonate solution into the Petri dish. The success of the reaction depends upon the freshness of the diamine-hydrochloride solution which is relatively unstable and oxidizes readily in the presence of air. It is stable in the powdered form and, therefore, the solution should be prepared fresh daily.

The tissue to be studied is ground thoroughly with an equal weight of quartz sand in a mortar for exactly 10 minutes. One gram of this minced tissue-sand mixture is now accurately weighed out in a flat-bottomed Petri dish 9.5 cm. in diameter. To this is added 5 cc. of the substrate as indicated above and the contents thoroughly mixed. This may be allowed to stand for any given time interval, but should be frequently stirred or preferably rocked to insure opti-

mum contact between tissue and substrate. Oxidation is more rapid when the tissue is merely moistened with the fluid rather than submerged. Thin films of substrate over the surface of the tissue particles are made possible by using small quantities of the substrate and flat-bottomed dishes. Vernon[1] has shown that this is not wholly a matter of better contact with atmospheric oxygen. The amount of indophenol formation induced by animal tissue is diminished when the reaction is carried out in a test tube although a continuous current of moisture-saturated oxygen was bubbling through.

After the desired period of the reaction, the contents of the Petri dish are made up to weight by adding 19 per cent alcohol. Ten cc. of 95 per cent alcohol are now added. This gives a final solution containing approximately 70 per cent alcohol. Further dilutions, when required, are made with 70 per cent alcohol so that the water-alcohol content will not vary from this point. The indophenol (blue), which is insoluble in water or the weaker alcohol of the substrate and, hence, adsorbed on the surface of the tissue particles, is dissolved by the stronger alcohol. At the same time the oxidase is inactivated, and the reaction brought to a standstill, save for a slight amount of spontaneous oxidation which continues. Fifteen minutes are allowed for the indophenol to become dissolved (under cover to prevent evaporation) and 10 minutes more for filtration. The filtrate should now be compared in a colorimeter against the standard. The time allowed for the solution of the indophenol and subsequent filtration is not so important, as the only oxidation now taking place is that which occurs spontaneously and is quite negligible. These readings, however, should be made on time when possible. Of greater importance is the time of the first dilution with alcohol which stops the reaction by inactivating the oxidase of the tissue. Even a delay of one minute may give a comparatively large error.

The standard is prepared by taking a definite measured quantity of the substrate, allowing it to stand until complete oxidation has occurred spontaneously and diluting it to 10 volumes with alcohol and water so that the final standard contains 70 per cent of alcohol. Due to the small amount of alcohol already in the substrate, 19 per cent, this is most easily accomplished by following the same procedure as used in diluting the test material, *viz.*, diluting the substrate with 2 volumes of 95 per cent alcohol and completing the dilution with 70 per cent alcohol. In order for the oxidation to be complete, a week or more is needed, but once the color has developed it remains unchanged for several months. Simultaneous development of color in the standard and unknown is quite impossible. In my

experience, catalyzing agents are likely to modify the shade of color developed.

Blank tests must be run under the same conditions, with the exception of omitting the tissue, to determine the amount of spontaneous oxidation in the time of any given experiment. These readings for spontaneous oxidation should be corrected, since the available substrate diminishes as the reaction proceeds. For example, after 2 hours oxidation in the case of skeletal muscle 75 per cent of the substrate was oxidized, five per cent was the value obtained for spontaneous oxidation in a blank test for the same time. The average amount of tissue oxidation was then only $^{75-5}/_{2}$ or 35 per cent, therefore, the average amount of substrate available for spontaneous oxidation would be 100 minus 35 or 65 per cent. Now the spontaneous oxidation of the substrate without the tissue, where 100 per cent of the substrate was available for oxidation, was 5 per cent of the total, but the amount subtracted where the two are acting together should not be 5 but only 5 per cent of 65 or 3.25 per cent. Such a correction is approximate and would express the true value only when there is a linear proportionality between the time and the amount of oxidation.

As accuracy in colorimetric methods depends upon having approximately the same intensity of color in the unknown as in the standard, it is obvious that a single standard cannot be used after oxidation periods of unequal length. Neither is a single standard adequate when a number of tissues are being tested although the time intervals are constant. Some tissues, such as skeletal muscle, are relatively poor in indophenol oxidase while heart muscle and kidney contain a comparatively large amount. Rather than have a number of standards, this difficulty may be overcome by further diluting the test material which has already been made up to 3 volumes by adding 10 cc. of 95 per cent alcohol. The total volume of tissue-substrate-alcohol mixture is now 15 cc. and contains 70 per cent of alcohol. An additional 10 cc. of 70 per cent of alcohol maintains the alcoholic concentration, but gives a dilution of 5 times the volume of the substrate, 20 cc. a dilution of 7 times, etc. The dilution best suited to any given test can easily be determined when the per cent of complete oxidation can be anticipated in advance. This can be done with sufficient accuracy in routine work. Dilution to 3 volumes should give a perfect match where thirty per cent of oxidation has occurred, 5 volumes for fifty per cent and so on.

Calculation:

$$\frac{\text{Reading of standard. X Dilution of unknown}}{\text{Reading of unknown X Dilution of standard}} = \text{Per cent of complete oxidation of substrate.}$$

[1] Vernon, H. M., *J. Physiol.*, 1911, xlii, 402.