TETRAZOLIUM BROMIDE AS A VALUABLE TOOL IN MICROBIOLOGICAL WORK

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IN the course of our studies on the reducing activities of bacteria of dairy importance, 2 : 3 : 5 triphenyl tetrazolium bromide* was found to be a very useful oxidation/reduction indicator with a variety of possible applications in microbiological research. The use of tetrazolium salts has been reported previously in connection with the testing of seed viability,[1,2] for the colorimetric estimation of reducing sugars and other reducing substances,[3,4] for vital staining[5] and for the *in vitro* studies of certain dehydrogenase activities.[6] However, its use in microbiological work has not received much attention.

Triphenyl tetrazolium bromide is soluble in water and forms a colourless solution, but in its reduced state (formazon) the indicator assumes an intense cherry red colour and is also insoluble in water. Formazon is, however, soluble in benzene, pyridine, glacial acetic acid, oleic acid, chloroform, butyl alhcohol, propyl alcohol and other organic solvents. Its E_0 has been reported to be 0·08 volts.[7] The reducing substances present in raw and heated milks and in autoclaved bacteriological media have no effect on this indicator (unlike methylene blue or resazurin) except under strongly alkaline conditions and at high temperatures. Any appreciable reduction of the indicator in culture media may, therefore, be ascribed to the reducing systems of bacteria during their growth and metabolic activities. The red dye can be extracted with a suitable solvent and measured quantitatively in a photo-electric colorimeter.

While studying the reducing activities of various organisms in milk and other media using tetrazolium bromide as an O/R indicator it was observed that the visible production of the red compound "formazon" started just after methylene blue and resazurin were completely reduced to their leucobases under comparable conditions (E_h of medium $-0·05$ to $+0·20$ V.) and the colour became intense at a stage corresponding to the complete decolourisation of Janus Green B (E_h of medium $-0·20$ to $-0·15$ V.). Preliminary work suggests that the reduction of the dye takes place mostly within the cell and partly on the cell surface and in the former case the red dye may diffuse into the medium along with organic solvents either in the normal course or as a result of the autolysis of some of the cells. The cell-free filtrate was not found to be capable of reducing the indicator. Like other dyes, tetrazolium (in concentrations of 0·1 per cent. and higher) was also found to exert some influence on the growth and acid production of the organisms in the early logarithmic stage of growth. The extent of inhibition or stimulation depends on the species and numbers of organisms as well as on the nature of the medium. The possibilities of using this indicator for the following aspects of microbiological research have been studied.

(*i*) *Taxonomical studies.*—Different species of bacteria showed significant differences in the rates of reduction of tetrazolium. The dye was found to be reduced most markedly by the lactic acid bacteria as a group but there was considerable variation from species to species within the same genus. For example, *Streptococcus liquefaciens* and *S. fæcalis* were the most powerful reducers while the heterofermentative streptococci showed the least reducing effect. Among the lactobacilli, *Lactobacillus lactis* and *L. bulgaricus* reduced the dye more strongly than other species. In the case of some species the red compound appeared to undergo further changes in colour on prolonged incubation of the culture. This differential behaviour of the organisms would appear to be helpful in their taxonomic classification and in the study of their metabolic activities. Sugar fermentation reactions and other biochemical tests commonly employed in classifying bacteria can be followed rapidly by observing the reduction of tetrazolium by the organisms in different media.

(*ii*) *Nutritional requirements of bacteria.*—The reduction of the indicator by growing cultures of bacteria was considerably influenced by the nutritional adequacy of the medium apart from the effect of cell population. By growing the organisms, in the presence or absence of various nutritional factors and then quantitatively measuring the reduced dye it has been possible to determine the specific nutritional requirements of different organisms within a short period of 6 to 8 hours. In the case of certain species, significant differences between their dye reducing capacities and their growth and acid production were also observed.

* Trade name: Grodex—May and Baker product.

(*iii*) *Microbiological assay of vitamins, amino acids, etc.*—An interesting application of the relation between the nutritional requirements of the organisms and their dye reducing abilities has been found in the use of this indicator for microbiological assay of riboflavin. An external source of riboflavin was found to be essential for the reduction of tetrazolium by *Lactobacillus plantarum*-89, *Streptococcus fœcalis*-190 and a few other organisms. The response of the test organism to graded doses of pure riboflavin was assessed in terms of the amount of formazon (intensity of red colour of the butanol extract) produced in the medium in about 6 to 8 hours and was found to be linear, like the response measured on the basis of acid production after 48 or 72 hours. The method has given encouraging results for the microbiological assay of riboflavin in milk. It is suggested that this principle of measuring the reducing activities of organisms as a basis for the assay of other vitmains, amino acids and other factors offers great possibilities.

(*iv*) *Quality control of milk.*—Dye reduction tests using methylene blue and resazurin have been widely employed in the quality control of milk. Tetrazolium, which does not impart any colour to milk initially, gradually colours it red as a result of its reduction to formozan by bacterial activity. It was found that poor quality milks with high bacterial numbers (plate counts over one to 10 million cells per ml.) became intensely red in 3 to 4 hours. The extent of reduction could be measured and standardised against suitable gradations of colour prepared by adding safranin and methyl orange to sterile milk. Although this indicator is not likely to be of much use for the rapid platform testing of milk, it appears to be particularly useful in quality improvement programmes for dramatically demonstrating to the producers the poor quality of milk and the need to improve their methods.

(*v*) *Other applications.*—The indicator is basic in character and can be used as a vital stain. In growing cultures of organisms like *Lactobacillus bulgaricus* in a medium containing the indicator, the metachromatic granules are stained intensely red. This also indicates the possibility of the use of tetrazolium for the quantitative determination of nuclear materials in the cell.

It was also observed that the addition of a small amount of the indicator (in non-toxic concentrations) to the agar medium resulted in the development of red pigmented colonies against a colourless background, thus making the enumeration of colonies easier. This method has been found particularly useful in counting cells by the 'Frost Little Plate' method since the small colonies developed in 4 to 6 hours are intensely red coloured and can be easily counted under the microscope.

Detailed results on some of the aspects referred to in the note will be published elsewhere.

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NEW THEORY OF SUNSPOTS

DR. S. CHANDRASEKHAR, Professor of Theoretical Astrophysics at the University of Chicago's Yerkes Observatory, was awarded the Bruce Medal, top American honour in Astronomy, presented annually by the Astronomical Society of the Pacific. Accepting the Medal, Prof. Chandrasekhar presented for the first time his new theory of the origin of sunspots.

The gist of the theory is that strong magnetic fields inhibit the movement of fluids by convection. Normally, convection currents are present in the body of the sun, whereby hot gas form far down moves up to the surface, gets cooled and then turns down again. This constant upwelling of hotter gas from below keeps the sun's surface bright. According to Prof. Chandrasekhar, strong magnetic fields on the sun's surface create a force which prevents the upwelling of the gas beneath the field. Since hot gas cannot come up to replace the cooled gas at the surface, the gas swirls around and cools off further, thus reducing its brightness and leading to the presence of dark sunspots.

The new theory is likely to offer a means of improving weather forecasting and lead to a better understanding of movements in the earth's atmosphere and of the gases within the sun and other stars.