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The Quantitative Determination of Dehydrogenase Activity in Cell Suspensions

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While investigating the action of nicotine on the enzyme systems of yeasts we attempted to use variations of the classical Thunberg technique to compare the behaviour of the dehydrogenases of a normal yeast with those of one which had been grown in the presence of nicotine. The methods proved unsuitable with the apparatus available because the suspended cells prevented reliable measurement of the residual colour with an ordinary photoelectric absorptiometer and some of the methylene blue was always firmly adsorbed on to the cells. If, on the other hand, the time required for complete decolorization be measured, it is difficult to draw conclusions from comparative inhibitions or activations because the concentration of the hydrogen acceptor becomes a limiting factor during the course of an experiment.

Triphenyltetrazolium chloride (TTC), which yields a water-insoluble red formozan on reduction (Kuhn & Jerschel, 1941; Straus, Cheronis & Straus, 1948), has advantages over methylene blue as hydrogen acceptor in that it can be present in excess throughout an experiment and it can be used in the presence of air. The colorimetric method of Kun & Abood (1949), which employs TTC for the determination of succinic dehydrogenase activity in mammalian tissue homogenates, depends on the solubility of the formozan in acetone. Their method cannot be applied to yeast suspensions since acetone fails to extract the colour from the cells. Even with mammalian tissues they found it necessary to prepare reference standards in order to correct for the unequal adsorptions of the dye by different tissues and Becker (1949), who investigated the suitability of TTC as a histochemical stain for dehydrogenase, observed that the formozan was deposited in and around the tissues in an irregular manner.

In the present paper a method is described which is suitable for the quantitative determination of the activity of various dehydrogenases in yeast suspensions.

EXPERIMENTAL

Colorimetry. All measurements were made with a Hilger Biochemical absorptiometer using a blue filter, OB2.

Yeast suspension. Except where otherwise stated, a suspension in distilled water of the washed cells of *Saccharomyces cerevisiae* was aerated for 2 hr. at 37°, centrifuged and the cells were resuspended in distilled water.

Triphenyltetrazolium bromide (TTB). A 0.5% (w/v) solution in distilled water of the commercial preparation 'Grodex' (May and Baker Ltd.) was freshly prepared or stored in a refrigerator.

Phosphate buffer. Clark & Lubs's standard buffer, 0.05M and pH 7.6.

Other chemicals. All materials used were of ordinary reagent purity.

RESULTS

In the preliminary experiments a suspension (1.0 ml.) of washed, but not aerated, yeast cells was mixed with phosphate buffer, pH 7.6 (2.5 ml.) and TTB solution (0.5 ml.) and the mixture was incubated at 37° until a distinct red colour had developed. Mixtures, so prepared, were mixed either with acetone (1–2.5 vol.) as in the method of Kun & Abood (1949) or with ethanol (1–2 vol.); or they were shaken with either toluene, xylene, chloroform, isobutanol or amyl alcohol. Similar attempts to extract the colour were made with mixed solvents (e.g. toluene with methanol or pyridine) and with the mixture rendered either acid or alkaline by the addition of sulphuric acid or sodium hydroxide. None of these methods was successful.

If the tubes containing the mixture with ethanol (1.5 vol.) were immersed in boiling water for 3 min., the colour was extracted completely from the cells,

but such a method has obvious disadvantages. Addition of glacial acetic acid (1.5 vol.) to the mixture dissolved the formozan which could then be extracted readily and completely by shaking with toluene. With smaller proportions of acetic acid the dye can be extracted from the cells, but stable emulsions result and it is difficult to separate the toluene layer.

Colorimetry of toluene solutions

Solutions in toluene of the formozan in known concentrations were prepared by extraction of solutions which contained measured quantities of TTB and to which a few crystals of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) had been added. The linear relation-

Fig. 1. Relationship between optical density and concentration of formozan (reduced triphenyltetrazolium bromide) in toluene. For details see text.

ship between optical density and the concentration of the dye in toluene is shown in Fig. 1.

Solutions of the formozan in either toluene or xylene are clear red, and the colour is stable for several hours, whereas the colour of solutions in the oxygen-containing solvents (alcohols, acetone, acetic acid) is dull orange-red and is somewhat less stable.

Measurement of dehydrogenase activity

Reaction mixtures (4 ml.) containing the cell suspension, buffer, substrate, TTB solution (0.5 ml.) and any other reagents are prepared in glass-stoppered tubes. Controls without substrate are also prepared. The mixtures are incubated at the desired temperature and, after a suitable interval, reaction is arrested by the addition of acetic acid

(6 ml.). The acidified mixtures are shaken with toluene (6–10 ml.), centrifuged if necessary, and the coloured toluene layers are compared in a photo-electric absorptiometer.

Effect of enzyme concentration

The colours developed in a series of mixtures, prepared as above, but which contained different amounts of non-aerated yeast cell suspension, were compared. The results, which demonstrate the linear relationship between the optical density of the toluene extracts and the concentration of the mixed dehydrogenases, are shown in Fig. 2. Similar results have been obtained with aerated yeast suspensions and either glucose or ethanol as substrate.

Fig. 2. Relationship between colour developed and quantity of yeast suspension. For details see text.

Microscopical examination of the cells remaining after an experiment confirmed that none of the colour had remained with the cells.

By means of the procedure, here described, we have been able to obtain consistent results on measuring the effects of nicotine on the activity of various dehydrogenases in living yeast cells. Consistent results were not obtained when we used other, previously available, methods.

SUMMARY

A colorimetric method is described for the quantitative determination of dehydrogenase activity in yeast and other cell suspensions. The method is especially useful when it is desired to measure the effects of either activators or inhibitors on dehydrogenase systems.

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