Reports

Tetrazolium Overlay Technique for Population Studies of Respiration Deficiency in Yeast

Attempts to apply tetrazolium salts to the diagnosis of respiration deficiency in yeast are based on Raut's observation (*1*) that a white colony found on a tetrazolium agar plate was respiration deficient (aer), whereas red colonies were respiration sufficient (AER). Laskowski (*2*) demonstrated, however, that growth in the presence of 2,3,5-triphenyltetrazolium chloride (TTC) induced AER cells to produce aer offspring. Recently, Nagai, Yanagishima, Hiraoka, and Takada applied TTC agar overlays to the study of populations of copper-sensitive and copper-resistant yeast cells after colonies had formed on media containing sublethal concentrations of copper (*3*). This technique allowed the use of a high concentration of TTC in a short test period during which no growth occurred.

This report describes a test procedure for the rapid, direct diagnosis of AER and aer colonies after overlay with TTC agar. Its rationale is based on the use of a relatively high concentration of TTC at a *p*H that yields deep red color development in AER colonies in a test period sufficiently short so that aer colonies are white. Diagnosis can thus be made in most strains without concern for color ambiguity, growth inhibition, or aer induction by the indicator.

The test medium consists of 1.5 percent Bacto agar in 0.067*M* phosphate buffer at *p*H 7.0, 0.1 percent with respect to TTC. Since the TTC is reduced chemically by autoclaving it with the agar, separate stock solutions are prepared and autoclaved. The final test agar is mixed under sterile conditions at 55°C and is stored in the liquid condition in an incubator at 50°C until it is used. For most purposes, sterilization may be omitted since the test period is short.

The test is performed by pouring 20 ml of TTC agar at 50°C over 3- to 4-day-old plates bearing 50 to 100 colonies per plate grown on a conventional peptone-yeast extract nutrient medium containing 1 percent glucose. Red and white colonies are scored routinely 3 hours after overlay.

Preliminary tests were made of several media and of procedural variables with a number of tester strains. Rate of formazan color development with AER strains increased with the TTC concentration up to 0.5 percent, the highest concentration tested. Increased *p*H also enhanced the rate of color development up to *p*H 7, with only slight further enhancement up to *p*H 9. The aer strains tested developed color at a very slow rate, and distinction between AER and aer colonies was possible over a considerable range of *p*H and TTC concentration. One-tenth percent TTC concentration and *p*H 7.0 were chosen for routine application because this concentration and *p*H yielded the greatest apparent differential in the rate of color development of AER and aer colonies. With this medium, at 1 hour after overlay, all essentially homogeneous AER colonies had developed sufficient colony color for a successful differentiation to be made from the white aer strains. At 2, 3, 6, and 24 hours, AER colony color became more intense. The aer colonies were almost all still white at 3 hours, but a few strains had developed faint pink color. At 24 hours, a number of aer strains showed light pink colony color, whereas AER colonies were very dark.

At all intervals between 1 and 24 hours, there was no overlap in colony color between essentially homogeneous representatives of 50 AER and 50 aer strains tested. The latter group included spontaneous variants, meiotic segregants, and variants induced by a number of chemical agents. One aberrant strain, which produced aer variants with a rate of TTC reduction comparable to the AER parental strain, was encountered. Occasional bacterial contaminants revealed themselves by intense color development within a few minutes. Pigmentation in adenine-dependent yeast strains did not interfere with TTC diagnosis.

After TTC overlay, several hundred red colonies and several hundred white colonies were picked from plates of essentially homogeneous strains and prepared mixtures. These were subjected to confirmatory test for the phenotype by streaking on lactate agar (*4*). Results were consistent with the TTC diagnosis with only a few apparent aberrancies. These cases are attributed to experimental error and the dissemination of cells inherent in an overlay procedure.

Applicability of TTC-overlay diagnosis to quantitative population study was tested on prepared mixtures of tetraploid AER and aer strains. The results obtained were consistent with calculations based on control plates and the dilutions. At low frequency of aer cells in the prepared mixtures, the number of colonies per plate was increased to 200. It was possible to estimate 3 percent of added aer cells after correction for the spontaneous frequency of aer cells on the control plates.

Some haploid strains are available which show a high spontaneous rate of AER to aer change during growth. Colonies formed on conventional nutrient media are therefore heterogeneous, the population equilibrium depending on the selective pressures of the growth conditions. A number of such strains were tested by the TTC overlay procedure. Heterogeneity was grossly apparent in some strains which yielded variegated colonies. In one strain, the entire heterogeneous colony yielded grossly homogeneous but lighter formazan color than that observed with homogeneous AER colonies. This case and the previously described aberrant aer strain which reduced TTC at a rapid rate would have been difficult to diagnose by formazan color alone. Diagnosis of the phenotype by the TTC overlay thus appears to have general but not universal applicability. Since both TTC reduction and respiration deficiency may be characterized by multienzyme sites not all of which are necessarily known or common to both, the application to the diagnosis of respiration deficiency is admittedly empirical.

However, the technique does fill a gap in current operations and sometimes offers certain advantages over differential plating on lactate agar (*4*). TTC overlay enables the direct estimation of a few percent of aer cells in a mixed population. Contact with physical or chemical aer inducers, leading to a viability difference of "damaged" AER cells on glucose and lactate agar, invalidates aer estimation by differential plate count unless the lactate plates are replicated from the glucose plates (*5*). Here the direct TTC overlay procedure has very evident

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advantages. When the problem involved is that of estimating low concentrations of AER cells (for example, 0.01 percent) in a predominantly aer population, the advantages of the selective lactate agar technique over TTC overlay become apparent (*6*).

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6. This work was supported in part by grant N17D from the American Cancer Society.

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Catabolism of Hexuronic Acids by Erwinia and Aerobacter

Only casual information is presently available on the metabolism of hexuronic acids by microorganisms (*1*); somewhat greater development of this field is evident with plant (*2*) and animal (*3*) materials. Our interest in this subject stems from an earlier study of the pectinolytic action of *Erwinia* soft-rot bacteria in which it was shown that pectin is broken down to galacturonic acid (*4*) and that the uronate is further catabolized to a mixture of end-products (*5*). For the past few years we have sought the initial step in the breakdown of galacturonate. We wish now to report that our investigations (*6*) indicate that the first detectable step in the catabolism of D-galacturonate and D-glucuronate by cell-free extracts of *Erwinia carotovora* and *Aerobacter cloacae* is a reduction, with either reduced triphosphopyridine nucleotide (TPNH) or reduced diphosphopyridine nucleotide (DPNH), to the corresponding hexonic acid, namely, L-galactonate and L-gulonate.

These reductions are carried out by substrate-induced enzymes. Glucose-grown intact cells oxidize glucose at once in manometric experiments, but act on galacturonate and glucuronate only after an induction period. On the other hand, galacturonate-grown intact cells of *Erwinia carotovora* respire galacturonate at once or after a brief induction phase; glucuronate is used very slowly. These galacturonate-grown cells are adapted at the same time to the oxidation of L-galactonate, but not to D-galactonate, D- or L-galactose, mucate, D- or L-lyxose, D-xylitol, L-arabitol, L-ascorbate, L-arabinose, or D-fucose. This sequential induction pattern suggests L-galactonate as a possible intermediate and reduces the likelihood that some often-suggested schemes (*1*) for galacturonic acid catabolism are operative in *E. carotovora*.

Glucuronate-grown intact cells of *Erwinia carotovora* oxidize glucuronate and galacturonate after a short induction phase; galacturonate- and glucuronate-grown cells of *Aerobacter cloacae* oxidize both uronic acids rapidly without a lag period; the last mentioned cells are sequentially induced to oxidize L-gulonate.

Cell-free extracts were prepared from both *Erwinia* and *Aerobacter* that had been grown on either galacturonate or glucuronate, by sonic oscillation and centrifugation. The supernatant was dialyzed overnight at 4°C against 0.05*M tris*-HCl buffer of *p*H 7.2 and centrifuged at 100,000 *g* for 90 minutes; the clear solution was used. These preparations contain an enzyme that catalyzes the reduction of both galacturonate and glucuronate by TPNH or DPNH. No reduction of D-mannuronate has ever been observed. The enzyme is provisionally named uronic reductase. When a preparation from galacturonate-grown *Erwinia* is used, DPNH reacts slightly faster with galacturonate than it does with glucuronate; when TPNH is the reductant, the reaction with glucuronate is about 5 times faster than it is with galacturonate under the conditions of our experiments. This difference is less distinct with preparations from cells of *Erwinia* grown on glucuronate, or *Aerobacter* grown on either uronate. The activity of the uronic reductase is very weak in extracts from glucose-grown cells of either species.

The end-products of the reduction of the uronates by both bacterial species, with either DPNH or TPNH, are nonreducing, nonlactonized acids: L-galactonate from galacturonate, and L-gulonate from glucuronate. By boiling for 5 minutes with 1*N* HCl, the acids are converted into the corresponding lactones which, on paper chromatograms, behave in every respect like L-galactono-γ-lactone after galacturonate reduction, or L-gulono-γ-lactone after glucuronate reduction. It is highly unlikely that the reaction proceeds by lactonization of the uronic acid followed by reduction to the corresponding L-hexonolactone; this opinion is based on (i) the failure of extracts of *Aerobacter cloacae* to reduce D-glucurono-γ-lactone with DPNH or TPNH, and (ii) the actual accumulation of the free L-hexonic acids taken together with the observed lack of delactonizing enzyme activity in the extracts for either of the L-hexono-γ-lactones.

The recent report by Payne (*1*) regarding the ability of dried cells of *Serratia marcescens* to form from glucuronic acid a substance tentatively identified as a "1,6-ester linked dihexuronic acid," prompted an examination by us of uronic reductase activity in that microorganism: an enzyme preparation from glucuronate-grown *Serratia* reduces glucuronate with TPNH or, somewhat more slowly, with DPNH. The relationship of the aforementioned reactions to the system described by Isherwood *et al.* (*2*) in pea mitochondria (which reduces the methyl ester of galacturonate, but not galacturonate, to L-galactono-γ-lactone) is under investigation, as are extensions of this general reaction to some of the less common uronic acids.

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Effects of Pentobarbital on Intermittently Reinforced Behavior

The behavioral effects of drugs have recently (*1–5*) been measured in terms of changes in the stable temporal patterns of responding that occur when behavior is reinforced intermittently. It has been shown that drugs may influence both the average rate and the pattern of emission of responses (*4*). The latter effects are of particular interest, since these patterns probably represent the operation of more subtle behavioral processes than are reflected in the average rate of response. In this report, a new measure of the temporal pattern of responding is used in order to compare the effects of a drug on temporal patterning with the effects on rate of response.

One form of intermittent reinforcement is designated as a fixed-interval