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## Determination of Housefly Succinic Dehydrogenase with Triphenyltetrazolium and Neotetrazolium Chloride

The tetrazolium salts are being used with increasing frequency as convenient indicators of biological redox reactions and enzymatic activity (*1*). However, certain peculiarities associated with the reduction of the various derivatives have been noted. Brodie and Gots (*2*) and Throneberry and Smith (*3*) have observed that the rate of reduction of 2,3,5,-triphenyltetrazolium chloride (TPTZ) is increased by incubation in a vacuum, as have other authors, and various workers have suggested that there is a direct competition between the indicator and naturally occurring aerobic hydrogen carrier systems, a decrease in dehydrogenase activity in air, an elevation of the redox potential of the system in air to an unfavorable level for reduction of the tetrazolium salt, an alternate mechanism of reduction of the indicator in air as opposed to the anaerobic mechanism, or a toxicity manifested by the indicator per se. The rapidity of reduction of the tetrazolium derivatives by such enzymes as succinic dehydrogenase varies considerably; Glock and Jensen (*4*) found neotetrazolium [*p,p'*-diphenylenebis-2-(3,5-diphenyltetrazolium chloride)] to be a more sensitive indicator of plant succinic dehydrogenase than TPTZ.

In the course of an investigation (*5*) to determine the inhibition by DDT and related compounds of muscle succinic dehydrogenase of the housefly, *Musca domestica* L., with TPTZ and neotetrazolium, an interesting variability in performance was observed. This difference was also demonstrated by a comparison of the effect of sodium malonate, which is a competitive but weak inhibitor of succinic dehydrogenase, and sodium cyanide, which inhibits the cytochrome carriers strongly but not succinic dehydrogenase (*6*).

The determinations were made by incubating aliquots of homogenized thoraxes (8 per milliliter) of female adult houseflies in ignition tubes with 0.05*M* phosphate buffer (*p*H 7.4 final concentration) and the inhibitor for 30 minutes at room temperature, then adding 0.067*M* sodium succinate and 200 μg of indicator in aqueous solution, bringing the volume to 3.0 ml, and incubating at 35°C until measurable reduction of the indicator was observed. In anaerobic determinations the tubes were placed in a 3-lit filter flask, which was evacuated through a stopcock attached to the side arm. The formazan was extracted from each incubation mixture with 3.5 ml of water-saturated *n*-butanol (*4*) by shaking 50 times, followed by brief centrifugation to separate the layers. The optical density of the formazan extract was determined spectrophotometrically at 480 (TPTZ) or 520 (neotetrazolium) mμ. The amount of indicator reduced was read from standard curves prepared with known amounts of formazan.

Table 1. Effect of two indicators on the inhibition of housefly-muscle succinic dehydrogenase by sodium cyanide and sodium malonate. TPTZ determination, incubated 115 minutes; neotetrazolium determination, incubated 30 minutes

| Inhibitor | Concn. (*M*) | Indicator reduced (μg) | | | |
|---|---|---|---|---|---|
| | | TPTZ | | Neotetrazolium | |
| | | Aerobic | Anaerobic | Aerobic | Anaerobic |
| | | 35.4 | 39.2 | 50.4 | 87.8 |
| Cyanide | $6.7 \times 10^{-4}$ | 0 | 3.9 | 19.4 | 26.5 |
| Cyanide | $1.7 \times 10^{-4}$ | 0 | 15.2 | 20.6 | 32.8 |
| Malonate | 0.05 | 6.3 | 9.6 | 2.3 | 5.5 |
| Malonate | 0.017 | 21.2 | 24.5 | 2.8 | 7.3 |

Table 2. Influence of cytochrome *c* on cyanide inhibition, and of increased substrate concentration on malonate inhibition of housefly-muscle succinic dehydrogenase ($5 \times 10^{-4}M$ $CaCl_2$ added throughout; incubated in a vacuum 45 minutes)

| Inhibitor | Concn. (*M*) | Reagent added | Concn. (*M*) | Indicator reduced (μg) | |
|---|---|---|---|---|---|
| | | | | TPTZ | Neotetrazolium |
| | | | | 36.5 | 143.4 |
| Cyanide | $3.3 \times 10^{-4}$ | | | 4.0 | 156.0 |
| Cyanide | $3.3 \times 10^{-4}$ | Cytochrome | $1.7 \times 10^{-5}$ | 11.7 | 143.4 |
| Malonate | 0.017 | | | 20.0 | 58.0 |
| Malonate | 0.017 | Succinate (2x) | 0.067 | 26.0 | 125.1 |

TPTZ reduction (Table 1) was found to be more strongly inhibited by cyanide and less strongly inhibited by malonate than neotetrazolium, both aerobically and anaerobically. Furthermore, as is shown in Table 2, cyanide inhibition of TPTZ reduction was somewhat lessened by added cytochrome *c* (*7*). The effect of malonate was partially counteracted by increasing the substrate concentration, as would be expected. The addition of calcium chloride appeared to stimulate reduction but did not alter the relative effect of the inhibitors. The action of cyanide and malonate on neotetrazolium reduction is in agreement with the hypothesis that this tetrazolium compound is reduced directly by succinic dehydrogenase, while the results with TPTZ may indicate the mediation of a cyanide-sensitive hydrogen carrier. It is also possible that the difference in reactivity is an artifact arising not from differences in mode of reduction by the enzyme but from differences in solubility or reactivity of the tetrazolium derivatives themselves, which influence the availability of the indicator.

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