Technical Papers

Propagation of Group A Coxsackie Viruses in Denervated Adult Mouse Muscle

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Evidence in the literature (*1, 2*) indicates that denervation of adult skeletal muscle results in decrease in activity of two enzymes: phosphorylase and phosphoglucomutase, toward the low levels found in embryonic and infant muscle. The concept that denervated muscle may resemble immature muscle in some aspects of its metabolism suggested that the metabolic change in denervated muscle might result in a return to the ability of the tissue to support the multiplication of the Group A Coxsackie viruses. These viruses previously have not been demonstrated to undergo multiplication in adult mice; Syverton *et al.* (*3*) have reported that following the simultaneous administration of cortisone and x-irradiation, a Group A, Type 4, Coxsackie virus was lethal for adult mice; however, no evidence was presented to prove that viral multiplication had occurred or to indicate that muscle was affected.

Five- to six-week-old white Swiss mice were subjected to unilateral sciatic nerve section in the thigh, and approximately 2 weeks later were inoculated intramuscularly into the calf of the denervated leg with a dilution of a Group A, Type 2, Coxsackie virus (N.I.H. strain 93). Three days after inoculation the gastrocnemius muscle was removed and the infectivity titer determined in 2-day-old mice. It has been found repeatedly that following inoculation of 10^3–10^4 LD_{50} (determined in 2-day-old mice) the infectivity titer of the muscle reached 10^{-5}–10^{-6}. In controls, consisting of normal adult mice, or of adult animals subjected to a sham operation, only traces or no virus remained on the third day. Strain 93 has been carried without difficulty through 50 serial passages in denervated adult mouse muscle, each passage being made by the injection of 0.02 ml of a 2.5% muscle suspension into the denervated calf. The infectivity titer of the 50th passage muscle was $10^{-6.1}$. Material from the 50th adult passage was typed serologically as strain 93.

For initiation of infection by the intramuscular route, an interval of at least 7 days after denervation and an inoculum of at least 3 logs of virus (LD_{50}'s in suckling mice) are required. Preliminary experiments indicate that the infection is confined to the denervated limb. Infection of the denervated muscle is occasionally initiated when large doses of virus are given intraperitoneally, but not when given orally.

Strains representing 7 additional types of Group A Coxsackie viruses have been tested, and evidence of multiplication has been obtained in all; no conclusive evidence of growth in controls has been found, and attempts to pass the viruses serially in sham-operated mice have been consistently negative. Two strains, representing Albany Type 3 and Type H_3, have been carried successfully through 7 serial passages in denervated adult mouse muscle, with virus present at theoretical dilutions of the original inoculum of 10^{-23}.

Although the hypothesis of the reversion of the muscle metabolism to that of immature muscle is attractive in regard to the ability of the denervated muscle to support the growth of the Group A Coxsackie viruses, the available data are not sufficient to warrant any conclusions on the mechanism of the phenomenon.

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Catalase Assay with Special Reference to Manometric Methods[1]

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The Michaelis-Menten concept of the mechanism of enzyme action has been very fruitful in correlating the rate of a catalyzed reaction with the concentration or "activity" of the enzyme (*1*). In the reaction

$$E + S \rightleftarrows ES \rightarrow E + \text{products}, \qquad (1)$$

where E is the free enzyme and S the substrate, the rate of breakdown of the intermediate complex, ES, is assumed to be rate-determining. Since in the presence of excess substrate all the enzyme is in the form of ES, the monomolecular breakdown of ES is linearly proportional to the total enzyme concentration. The concentration of the intermediate complex under these conditions is constant, and therefore the rate of the reaction obeys a zero order equation with respect to substrate concentration. These properties of a Michaelis-Menten type of enzyme provide the basis for the rationale for computing enzyme concentrations from a zero order slope.

The same rationale cannot be used for catalase,

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however. The substrate species reacts twice with the enzyme, first with the free form, then with the primary complex (*2–4*):

$$E + S \rightarrow ES \quad (2)$$
$$ES + S \rightarrow E + \text{products}. \quad (3)$$

The second step, a bimolecular reaction, is only partially rate-determining. Moreover, the kinetics of the destruction of hydrogen peroxide by an uninhibited catalase is well established as a readily integrable first order process with respect to the substrate concentration (*5, 6*):

$$ds/dt = -k_0(s), \quad (4)$$

where k_0 is the observed first order velocity constant (with the dimensions of sec^{-1}) for a given concentration of enzyme, and (s) is the substrate concentration. The value of k_0 is independent of substrate concentration (10^{-5} to 10^{-1} M) or time and is found to be linearly proportional to the enzyme concentration. Equation (4) may be written thus:

$$ds/dt = -k_s(e)(s), \quad (5)$$

where k_s is the specific reaction rate (with the dimensions of liter × $mole^{-1}$ × sec^{-1}) and (e) is the enzyme concentration. Since the molar concentration of the catalase can be determined spectrophotometrically (*6*), k_s can be readily calculated from Eqs. (4) and (5):

$$k_s = k_0/e. \quad (6)$$

Conversely, once k_s is known, Eq. (6) may be used to calculate (e) from the observed values of k_0. Equation (6) is valid for rates calculated from measurements of either the destruction of hydrogen peroxide or the oxygen evolution.

Despite the fact that these relations have been known for several years (*6*), the majority of papers published on catalase since 1947 (*7–13*) (with the exception of the series by Chance [*4*] and others [*2, 6, 14*]), have presented calculations of the concentration, degree of inhibition, or thermodynamic properties of catalase by means of "activity" measurements which bore no linear relation to the mass action laws governing the catalase-hydrogen peroxide reaction. For this reason, the data cannot be quantitatively compared nor the conclusions considered unequivocal. Quantitative methods for studying the rate of peroxide breakdown by catalase as a function of catalase concentration must yield data that meet the following criteria:

(*a*) The concentration of the active enzyme always must be linearly proportional only to a first order velocity constant (which does not contain the substrate or product concentration as a dimension), and (*b*) the first order velocity constant must be independent of substrate (or product) concentration and time. It follows that any velocity constant to be substituted in any of the thermodynamic equations must be calculated from Eq. (6).

For illustrative purposes and because of its extensive use, the manometric method of assaying catalase "activity" will be considered. The special problem of oxygen diffusion across the air-liquid interface makes the analysis of kinetics by the manometric method difficult. Precautions have been taken by several authors to compensate for this difficulty (*10, 15*), but in none of the experiments did the observed rate of oxygen evolution follow a strict first order equation. The initial lag in oxygen evolution commonly observed (*11, 16*) has no theoretical basis in the catalytic process (*2*). Reasons for the failures of these methods are discussed by Lewis and Whitman (*17*), Adney and Becker (*18*), and Roughton (*19*), where diffusion of insoluble gases across air-liquid interfaces is considered.

The usual manometric technique was employed in two studies (*16, 20*). The rate of evolution of oxygen from 2.5 ml of reaction mixture containing beef liver catalase (Worthington), varying concentrations of hydrogen peroxide (0.037–0.20 M) dissolved in 0.05 M borate buffer, pH 7.8, was followed in a Warburg-Barcroft manometer at 5° C. Figure 1 shows curves of oxygen evolution at three different rates of agitation at constant enzyme and initial substrate concentrations. Both the rate and the order of the rate of oxygen evolution increased with increasing rates of

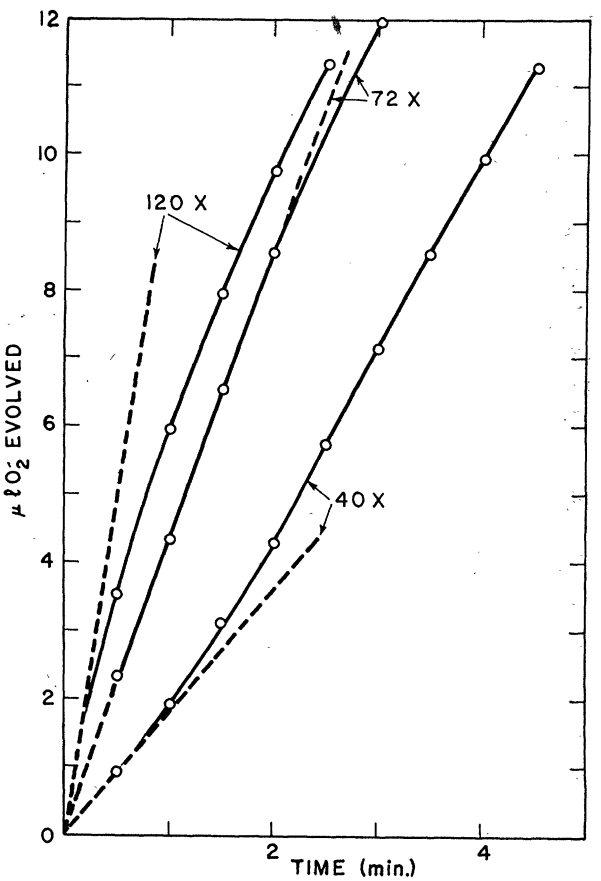

FIG. 1. Effect of varying rate of oscillation of Warburg cups from 40 to 120 times/min. Dashed lines are extrapolated initial rates. Stock solution of catalase diluted 1 : 200,000. Initial hydrogen peroxide concentration 0.073 M.

agitation. The initial lag, as expected, is most apparent at the lowest rate of agitation. This lag can be abolished by increasing the substrate concentration. Figure 2 illustrates the illusion of saturation of the enzyme by increasing the substrate concentration. Beyond a given concentration of substrate at a given rate of agitation, increasing the substrate concentration has no apparent effect on the rate of evolution of oxygen. The fallacy of the hypothesis of enzyme saturation is further supported by the additional finding that increasing the enzyme concentration under these same conditions increases the oxygen evolution at a rate almost linearly proportional to the enzyme concentration except at low enzyme concentrations (Fig. 3). In addition, the apparent saturation value of the substrate concentration increases with enzyme concentration. However, as the rate of agitation increases, the so-called "saturation value" of the substrate increases (Fig. 2). Control studies using titrimetric methods (*15, 20*) show that the agitation rate itself has no effect on the catalytic process. Therefore, the above phenomena are artifacts of the manometric method and are due to the peculiar compensatory action of the first order catalytic process and the limiting rate of transfer of oxygen across the air-liquid interface. Since the criteria for studying catalase kinetics have not been met, this particular manometric method must be considered unsatisfactory for catalase assay.

It is hoped that with an increased awareness of the kinetic properties of the catalase-hydrogen peroxide system, future investigators will employ methods that record the true rate of the reaction (or some linear function thereof) and will use rate constants consistent with the mass action laws. It is apparent from this study that, because the kinetics of the catalase-peroxide system are known, it becomes possible to specify rigorous criteria for quantitative assay methodology which permits adequate calculations of rate constants.

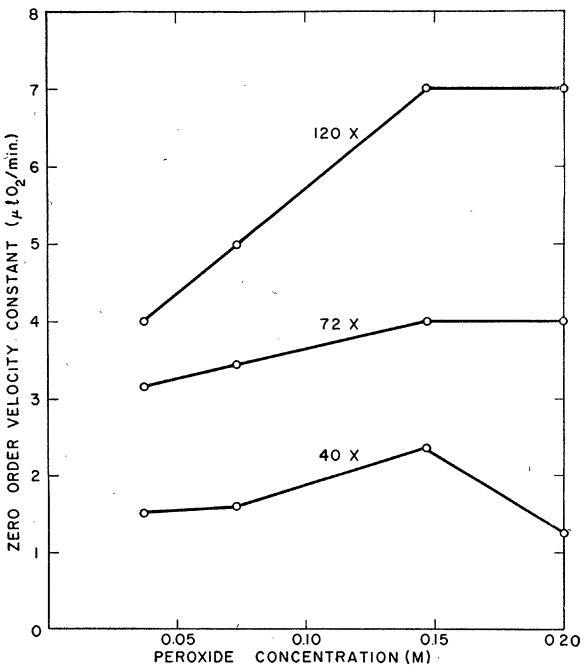

FIG. 2. Effect of varying substrate concentration and rate of agitation on rate of evolution of oxygen. Linear portion of slope used for rate calculation. Catalase stock solution diluted 1 : 250,000.

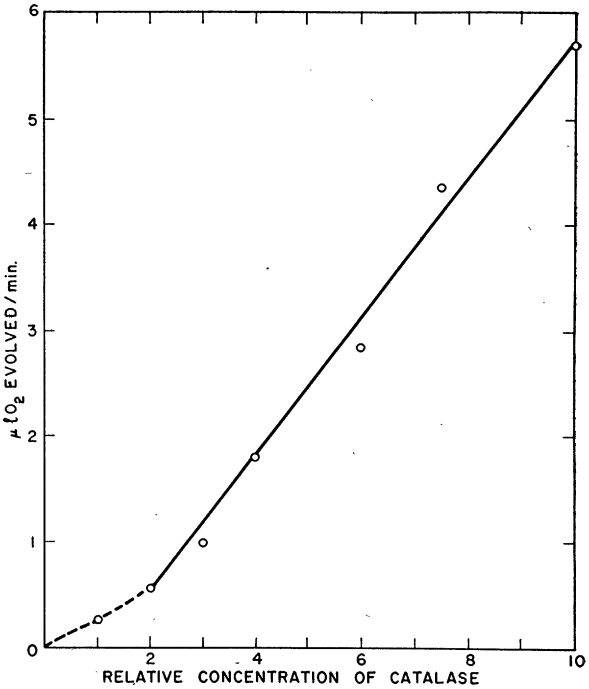

FIG. 3. Apparent linearity between rate of oxygen evolution and enzyme concentration. Rate of agitation 120 times/min. Initial peroxide concentration 0. 073 *M*. Catalase diluted 1 : 125,000 to 1 : 12,500. See text for details.

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