cient with respect to cytochrome oxidase (*8*). We believe that our direct spectroscopic study of the pigments of the intact, freely suspended, respiring tumor cells is somewhat more decisive than the various indirect assay methods that have led to the earlier conclusions.

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The State of Catalase in the Respiring Bacterial Cell[1]

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The physiological role of catalase has remained obscure because the two substances necessary for catalase action (a substrate such as peroxide and a hydrogen donor such as an alcohol, or nitrous or formic acid [*1*]) have not been proved to be present simultaneously in adequate concentrations in the respiring cell (*1*). In this paper we show the existence of both types of substances and, furthermore, show how the steady-state peroxide concentration can be regulated by the addition of suitable hydrogen donors, a point of some importance in the control of peroxide produced within the cell. The paper also provides the first demonstration of an enzyme-substrate compound in action *in vivo*.

By means of sensitive spectrophotometric methods (*2*) we can now study the spectra and kinetics of catalase and cytochrome in respiring suspensions of *Micrococcus lysodeikticus* (*3*).[2] We find catalase in the form of its primary hydrogen peroxide complex (complex I) (*4*) in the aerobic bacterial cells. The complex is identified by its specificity for donors such as nitrous and formic acid and by its absorption spectrum, as previously determined by *in vitro* studies of the pure crystalline enzyme (*5*).

When catalase is continuously supplied with hydrogen peroxide from an oxidase system, we have shown *in vitro* that the steady-state concentration of complex I may be considerably reduced by the addition of a donor (*6*). The same experiment is here repeated with intact respiring cells, as shown in Fig. 1, *A*. The recording spectrophotometer is responsive

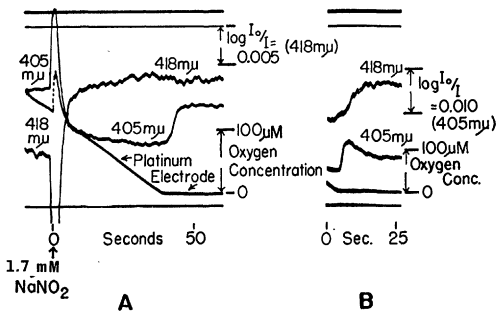

FIG. 1. *A*: Spectrophotometric recording of the addition of nitrite to a respiring suspension of *M. lysodeikticus*. The respiration of the bacteria is indicated by the linear decrease of the polarographic trace. The double beam spectrophotometer was set at 405 mμ and 418 mμ and records an increase of optical density as an upward deflection of the 418 mμ trace and a downward deflection of the 405 mμ trace (to avoid trace superposition). Cells suspended in a phosphate medium of pH = 7.0 with 2.7 mM glucose present; 1.7 mM nitrite added. (Expt 922b.) *B*: A similar record, but nitrite is omitted. (Expt 922a.)

to a change in the concentration of complex I at both 405 mμ (the peak of the complex I band) and 418 mμ (a wavelength at which the cytochromes of the bacteria do not interfere with the measurement of complex I). Both traces show an increase of optical density upon addition of 1.7 mM nitrite (or in other tests, a larger formate concentration), as would be expected when the steady-state concentration of complex I is reduced. (The absorption band of free catalase is higher than that of complex I [*5*, Fig. 3].) Upon exhaustion of the oxygen, the cytochromes are reduced, as shown by the decrease of optical density at 405 mμ. In the absence of added donor, complex I is present aerobically, and rapidly decomposes upon exhaustion of the oxygen, as clearly shown by the trace at 418 mμ in Fig. 1, *B*. These two experiments suggest that complex I is present in respiring cells and that adequate endogenous donor is present to cause its rapid decomposition when the cells become anaerobic.

The spectrum of the change in absorption caused by the addition of a catalase donor to aerobic cells is obtained by repeating the experiment of Fig. 1, *A* at various wavelengths or by using a sensitive recorder (*7*) as shown by Fig. 2, Curve *A*. This difference spectrum is identical to our *in vitro* result for complex I (*5*). For comparison, the cytochrome absorption is recorded in Curve *B*. Further proof of our conclusion is afforded by adding methyl hydrogen peroxide to the aerated cells, which greatly decreases the absorption (Fig. 2, *C*) as a result of further binding of catalase hematin as complex I of methyl hydrogen peroxide, in agreement with our *in vitro* studies (*5*, Fig. 4). A comparison of Curves *A* and *C* shows that about 1.6 molecules of hydrogen peroxide are bound to the 4 hematins of bacterial catalase *in vivo*, in accord with the *in vitro* calcula-

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[2] The fact that this bacterium has an unusually large catalase content means that a higher level of free peroxide concentration is required to reach the saturation value of complex I.

tion (*5*). This result strongly supports our conclusion that hydrogen peroxide is bound to catalase in the respiring cell.

Many other data support our identification of complex I; these include the gradation of the activity toward nitrite, formate, and ethanol, and the formation of the distinctive azide-catalase-peroxide complex.

By adding various formate concentrations to the respiring bacteria we find that roughly 2 mM formate halves the steady-state concentration of complex I. For this case, we have already shown that the steady-state hydrogen peroxide concentration can be computed (*6*). Our result for respiring *M. lysodeikticus* is about 10^{-8} *M* H_2O_2.

At such low peroxide concentrations, the "catalatic" decomposition of hydrogen peroxide into water and oxygen proceeds relatively slowly[3] because of the

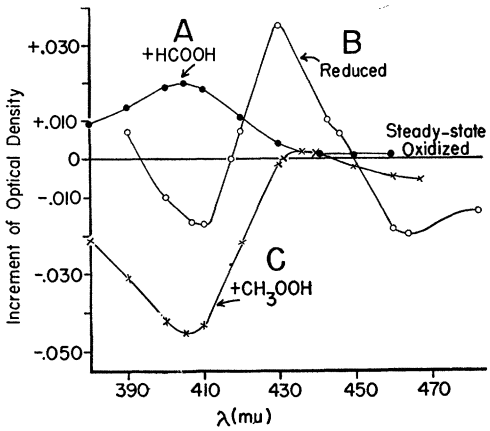

FIG. 2. Spectra representing changes in the optical density of a respiring suspension of *M. lysodeikticus*: (*A*) when 1.7 mM formate is added, (*B*) when the cytochromes are reduced by anaerobiosis, (*C*) when 67 μM methyl hydrogen peroxide is added. (Expt 924.)

peculiar mechanism of catalase action (*6, 8*). The addition of a suitable donor such as formate or nitrite causes catalase to act "peroxidatically" and thereby reduces the steady-state peroxide concentration. Thus the endogenous peroxide concentration may be regulated by the exogenous donor concentration.

On the basis of our *in vitro* studies of catalase action, Cole, Bond, and Fishler (*9*) have postulated that nitrite protects mice from x-radiation by reaction with complex I. And Hollaender has also shown the protection of bacterial cells from x-rays by formate, ethanol, and glycols (*10*). Our data on the *in vivo* interactions of catalase, hydrogen peroxide, and donor substances now give direct evidence in favor of the mechanism by which catalase may participate in the control of hydrogen peroxide and perhaps in protection from ionizing radiations.

[3] We estimate that about four times as much peroxide is decomposed "catalatically" as "peroxidatically" in the respiring cell (*6*).

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Schwarzschild-Villiger Effect in Microspectrophotometry

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Since at least 1903 (*1*), the experienced microscopist has been aware of the cause of and cures for lenticular glare (flare). This fault of refracting optical systems has been one of the main enemies of the cytologist in his constant battle for microscopic contrast and resolution. Cellular microspectrophotometry has been a natural outgrowth of cytology (*2, 3*). When the cytologist turns to this new technique in cellular studies, he naturally brings with him his thorough background in microscopical practice, which includes the routine rules for the control of lenticular glare.

These are (*4–7*): (*a*) the smaller the cone of illuminating light (i.e., the smaller the condenser aperture), the less the glare; (*b*) the smaller the area of the object illuminated, the less the glare.

The effect of glare in microdensitometry (in terms of [*b*]) came to the attention of the astrophysicists Schwarzschild and Villiger (*8*) in a study on the intensity distributions in the image of the solar disk. Naora has based a recent critique (*9*) of present methods of cellular microspectrophotometry on his familiarity with the Schwarzschild-Villiger effect. Although giving considerable attention to the dependence of glare on the magnitude of the area illuminated, he has apparently neglected the fact that, as the condenser aperture is reduced, glare is reduced and he thereby overestimates the magnitude of the glare error in the current technique.

Thus far, in most microspectrophotometric studies, small condenser apertures have been used, and for convenience of searching and maintaining adequate alignment the illuminated field has been kept as large as 30 μ in diameter. For example, Swift has explicitly prescribed such conditions for the expressed purpose of reducing glare to a low level (*10*). With clean optics, and with the refractive index of the specimen matched by the mounting medium (*3*), the above precautions limit the total *measured* glare intensity in