Enzymatic Removal of Oxygen for Polarography and Related Methods[1]

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Oxygen must be removed for most polarographic work, since it is itself reduced at the dropping mercury electrode (Fig. 1). This is usually done by the passage of an inert gas or by the addition of a reducing agent such as sulfite (*1*). We found that a combination of the enzymes glucose oxidase and catalase makes it possible to remove oxygen from solutions rapidly, quantitatively, and specifically by the following reactions:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O$$

$$\text{Net reaction: glucose} + O \longrightarrow \text{gluconic acid}$$

The enzymes[2] are eluted from the inert base on which they are adsorbed by shaking one part with 8 parts of 0.2 *M* phosphate buffer pH 7.0 or 0.2 *M* acetate buffer pH 6.0 for 10 min. The supernatant solution is used directly for oxygen removal in the ratio of 1 part/100 parts of test solution. Some preparations, which were found to be relatively deficient in catalase, were reinforced with this enzyme (crystalline beef liver catalase, Worthington) until the rates of disappearance of oxygen and hydrogen peroxide were equal. The final concentration of enzymes in the test solution was equivalent to 0.0017% nitrogen, about half of which is dialyzable, and the final concentration of the substrate, i.e., glucose, was 0.3%. The useful range of the enzymes extends from pH 5.0 to 8.5. They must be used in buffered solution, acetate or phosphate buffers in 0.05 *M* concentration being satisfactory for this purpose. KCl is preferable to KNO_3 as supporting electrolyte (*2*). Under these conditions oxygen was removed completely from solutions contained in open beakers within 5 min and the solutions remained oxygen free during the recording of the polarogram (Fig. 1), as the rate of oxygen removal is greater than the rate of diffusion of oxygen into the solution. Some of the special features of this method are considered below:

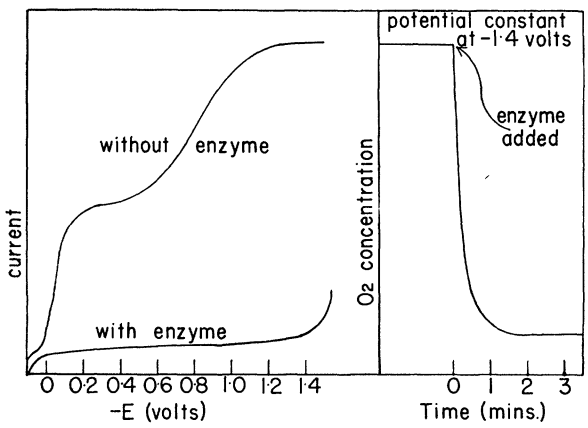

FIG. 1. Enzymatic removal of oxygen from buffered glucose solution.

TABLE 1

	$c \times 10^4$ moles/l	With enzymes: i_d (diffusion current) μamp	With enzymes: $i_d/c \times 10^{-3}$ μamp × l/mole	Without enzymes, O_2 removed with N_2: i_d (diffusion current) μamp	Without enzymes, O_2 removed with N_2: $i_d/c \times 10^{-3}$ μamp × l/mole
Cu*	3.0	1.92	6.40	—	—
	6.0	3.84	6.40	—	—
	10.0	6.48	6.48	6.45	6.45
Zn*	3.0	1.95	6.50	—	—
	6.0	3.87	6.45	3.96	6.60
	10.0	6.48	6.48	—	—
Cd†	3.0	2.49	8.30	2.50	8.35
	6.0	5.04	8.40	—	—
	10.0	8.34	8.34	—	—
$C_6H_5H_gOH$‡	0.5	0.16	3.20	—	—
	1.0	0.32	3.20	0.34	3.40
	1.5	0.48	3.20	0.50	3.34
	2.0	0.65	3.23	0.68	3.40
	2.5	0.80	3.21	0.86	3.44
	4.0	1.29	3.23	—	—

* Solution: acetate buffer pH 5.9, 0.05 *M*; KCl, 0.10 *M*; gluconic acid, 0.02 *M*; glucose, 0.3%.
† Solution: acetate buffer pH 5.9, 0.05 *M*; KCl, 0.10 *M*; glucose, 0.3%.
‡ Solution: phosphate buffer pH 7.0, 0.5 *M*; glucose, 0.3%.

(1) The substrate, the product, and the enzymes are polarographically irreducible under the conditions used.

(2) Since the catalyst is an enzyme, the reaction is specific for glucose and oxygen and no interference with the polarographic determination of other substances will occur, as might be the case with such nonspecific reducing agents as sulfite. The results with 4 different metals are shown in Table 1. A comparison of columns 3 and 5 shows that in the case of Cu^{++}, Zn^{++}, and Cd^{++} there is good agreement between the results obtained with and without the enzymes. Cu^{++} and Zn^{++}, which form complexes with the product of the reaction, were polarographed in excess gluconic acid. Phenylmercuric hydroxide appears to be bound by the enzymes and indeed slowly inactivates them. For this reason only one-third of the usual enzyme concentration was employed and a stream of nitrogen was passed over the solution during the experiment. This resulted in oxygen removal within 10 min and a constant, if somewhat lower, i_d/c. In all cases the half-wave potentials were found to be unaffected by the presence of the enzymes.

(3) The enzyme is an efficient maximum suppressor,

[1] This work was supported by grants from the Research Corporation and the Damon Runyon Cancer Fund.
[2] Obtained from Takamine Laboratories, Clifton, N. J., under the trade name of Dee-Gee.

for example, it suppressed the maximum on the copper wave completely.

(4) The enzymatic method is especially useful under conditions where conventional oxygen removal is either inconvenient or impossible.

(a) For polarographic determinations in very small volumes that cannot be conveniently handled in closed systems.

(b) For many amperometric titrations: closed systems or degassing after each addition of titrant can thus be obviated.[3] We have, for example, obtained stoichiometric results when solutions of glutathione (containing the usual amounts of enzyme and substrate) were titrated with phenylmercuric hydroxide at a potential of – 0.45 volt versus the S.C.E. (*3*). The titration was carried out in an open vessel and the oxygen introduced with each 0.2-ml addition of the titrant disappeared within about 1 min.

(c) For the removal of oxygen from protein solutions. This cannot be done satisfactorily by the usual methods, since such procedures as degassing or shaking in an inert atmosphere lead to foaming and denaturation and fail to remove oxygen completely (*4, 5*). The enzymatic method is therefore particularly suitable for this purpose and is proving very useful in current studies on the interaction between organic mercury compounds and proteins. In these experiments it is obviously desirable to keep the enzyme concentration to a minimum, so that it does not constitute more than a negligible fraction (less than 0.5%) of the total protein concentration. Such low enzyme concentrations do, of course, necessitate closed systems and the time for complete oxygen removal is somewhat longer.

[3] It should be noted, however, that the enzymes cannot be used in the presence of nonaqueous solvents, which are frequently employed in amperometric titrations.

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Comments and Communications

Sporabola

MY scientific urge, or perhaps only my idle curiosity, having been stimulated by the letter of Dr. P. H. Yancey (SCIENCE, **118**, 58 [1953]), I dipped into the right-hand top drawer of my desk where I keep the Oxford Pocket Dictionary because of its admirably convenient size, in spite of being myself a Cambridge graduate! I find there that "spore" is defined as "One of the germs by which flowerless plants are reproduced (Gk. *speirō*, sow)." Am I right in believing that, though the etymology is better, the definition is open to the same criticisms as those quoted by your correspondent from Alabama? I refrain from raising, except by mentioning, the general question of how far the scientist is justified in expecting from the nonscientific dictionary, definitions that will satisfy his criteria. Are not all scientific words a kind of condensate of experiment or observation and therefore not susceptible of precise definition, possibly even of complete comprehension, by the nonspecialist?

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Parental Age and Sex Ratio

MAY I be allowed to comment briefly on "The Dependence of the Secondary Sex Ratio in Humans on the Age of the Father," by Dr. Edward Novitski, which appeared in SCIENCE, **117**, 531 (1953). The data utilized by Dr. Novitski in his study are published by the U. S. Bureau of Vital Statistics, and give the sex distribution of offspring in five-year intervals. Dr. Novitski fits a multiple linear regression plane to these data, and overlooks major points relevant to such statistical technique.

(1) The data are not linear. For the youngest age group, fathers 19 years old or under, mothers 19 years old or under, the sex ratio of offspring is less than for the 20–29-year age groups. For fathers, the maximum sex ratio of offspring falls into the 25–30-year-age group; for mothers, it comes about three years earlier.

(2) The two inverted U-shaped curves of age of parent and sex ratio of offspring are almost identical with the ones reported by J. Yerushalmy [*Human Biol.*, **11**, 342 (1939)] on "Age of Parent and Stillbirth Rates," thus indicating that differences in abortion rates are a major factor in this age trend.

(3) Furthermore, age of husband and wife are known to be positively correlated; and even if the data used by Novitski were linear, an adjustment would have to be made to correct for this correlation factor. A. Ciocco (*Human Biol.*, **10**, 36–64 [1938]) states: "Some writers believe that the relation between age of parent and sex ratio is manifest for the fathers rather than for the mothers. Our data (also from the U. S. Bureau of Vital Statistics publication) cannot be used to support any such conclusion, since for both fathers and mothers, when the ages of either are kept constant, there is irregularity in the relationship between age of parent and the relative masculinity. . . ."