THE RELATION BETWEEN THE OXYGEN CONCENTRATION AND RATE OF REDUCTION OF METHYLENE BLUE BY MILK.

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After the discovery of the reducing power of milk[1] and the proof that its reducing action on methylene blue (decolorization) is not due to bacteria but to a reducing enzyme,[2] Bach,[3] in a series of researches, undertook the study of reducing enzymes in various tissues. Schardinger[1] had originally found that fresh cow's milk will reduce methylene blue in the presence of an aldehyde but not in its absence and not if the milk had previously been boiled. In one paper Bach[4] showed that nitrates could also be reduced to nitrites by Schardinger's enzyme of milk and he studied the effect (on reduction) of nitrate, aldehyde, and enzyme concentration, as well as the influence of temperature. As the nitrate is still further reduced, the quantitative results obtained by determining the amount of nitrate formed at successive intervals of time after mixing varying quantities of nitrate, or aldehyde, or milk, are not of much value. They do show, however, that rate of nitrite formation increases with, but is not proportional to nitrate concentration or aldehyde concentration. Bach found also that nitrite formation is proportional to enzyme concentration, at least in the early stages of the reduction. He did not study the effect of oxygen concentration in milk on the reduction of nitrate.

[1] Schardinger, F., *Z. Untersuch. Nahrungs-u. Genussmittel*, 1902, v, 1113; *Chem. Ztng.*, 1904, xxviii, 704.

[2] Trommsdorff, R., *Centralbl. Bacteriol., 1te Abt., Orig.*, 1909, xlix, 291.

[3] Bach, A., *Biochem. Z.*, 1911, xxxi, 443; 1911, xxxiii, 282; 1912, xxxviii, 154; 1913, lii, 412.

[4] Bach, A., *Biochem. Z.*, 1911, xxxiii, 282.

This paper deals with the reduction of methylene blue by milk and acetaldehyde under varying partial pressures of oxygen. The study was undertaken through the observation that milk will reduce oxyluciferin to luciferin, the light-producing substance of luminous animals, or methylene blue to leucomethylene blue, only if the milk and reducible material stand for some time.[5] The reduction does not occur in either case if the mixtures are continually shaken with air. It appears that time is necessary for using up the dissolved oxygen present. If the oxygen is removed from a milk-acetaldehyde-methylene blue mixture by exhaustion with an air pump, decolorization (reduction) occurs almost immediately. Without exhaustion of the air such a mixture must remain undisturbed for over an hour at room temperature before reduction occurs.

Preliminary experiments showed that the amount of reducing enzyme varied in different samples of milk. The addition of 1 cc. M acetaldehyde to 10 cc. of one sample of milk gave the quickest reducing action. Either more or less aldehyde increased the time. Methylene blue was added till a blue color in the milk was marked (0.1 cc. 0.01 M (= 0.319 per cent) methylene blue was used). With less methylene blue slightly less time is required. The standard mixture was therefore 10 cc. of milk and 1 cc. M acetaldehyde + 0.1 cc. 0.01 M methylene blue. If shaken with air (21 per cent oxygen) and set aside in a test-tube at 20°C., the blue color in one particular case completely disappeared, except at the surface in contact with air, in 43 minutes. If shaken with air containing only half the normal amount of oxygen, decolorization occurs in 23 minutes, about one-half the time. The times for decolorization have been determined for other concentrations of oxygen and are given in Fig. 1 where times for decolorization are plotted along ordinates and oxygen concentrations along the abscissæ.

The reduction in concentration of oxygen was accomplished by partially exhausting the air over the milk-acetaldehyde-methylene blue mixture in a large test-tube with an air pump, at the same time shaking the mixture vigorously. Tubes which have been brought into equilibrium with air in partial vacua can be removed to air under

[5] Harvey, E. N., *J. Gen. Physiol.*, 1918–19, i, 133.

atmospheric pressure and placed in a thermostat at 20° with safety because the diffusion of dissolved oxygen from the surface into the solution is very slow, if the tubes are undisturbed.

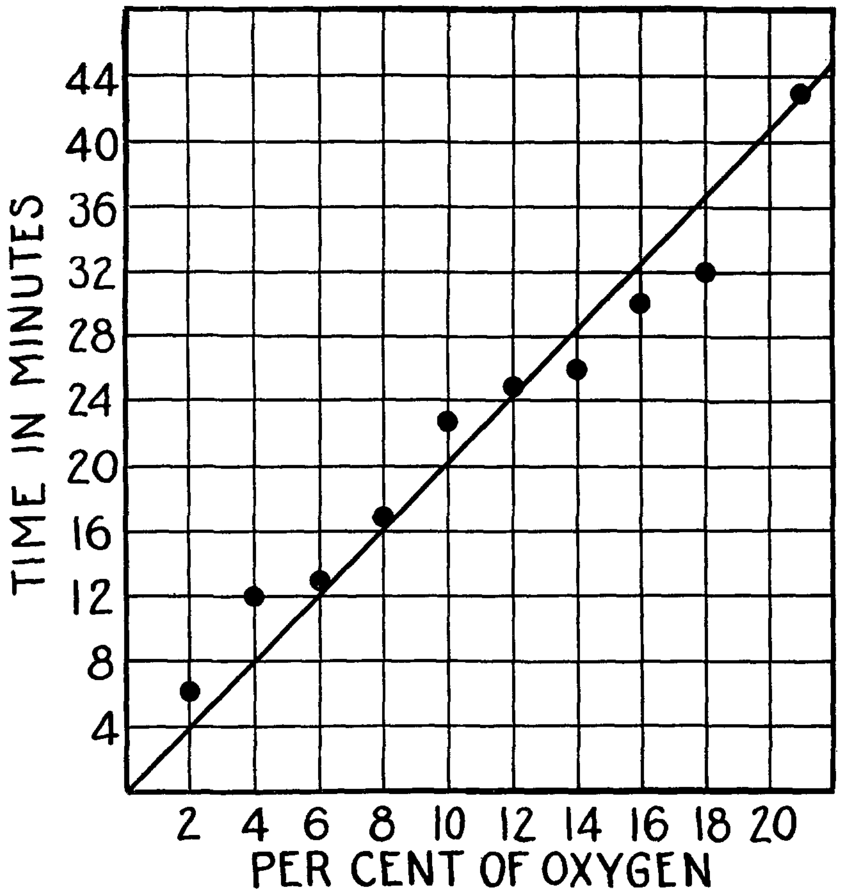

FIG. 1. Time necessary for reduction of methylene blue in varying concentrations of oxygen.

It will be noted from Fig. 1[6] that the curve is practically a straight line and the time for decolorization proportional to the oxygen concentration. It is obvious that this method can be used for determin-

[6] The results of this chart were obtained by Mr. J. P. Kelly, Princeton University, who undertook the determination at my suggestion.

ing the oxygen content in gaseous mixtures by use of the indicator methylene blue. The procedure differs somewhat from the indicator method recently described by Osterhout,[7] where organisms are placed in a hemocyanin solution (blue) and the time necessary for them to use up practically all of the oxygen is indicated by reduction to the colorless condition of the hemocyanin.

In using the milk-methylene blue method it is only necessary to shake a milk-acetaldehyde-methylene blue mixture with the gas to be analyzed and determine how long it takes for the blue color to disappear. The end-point can be quite accurately determined by comparison with a similar tube of milk containing no methylene blue. A control determination of the time necessary for decolorization of milk-acetaldehyde-methylene blue mixture shaken with air must be made under the same conditions. As air contains 21 per cent oxygen, if it takes 60 minutes to decolorize with air and 40 minutes to decolorize with the unknown gas, the latter must contain $\frac{40}{60}$ or $\frac{2}{3}$ of $21 = 14$ per cent of oxygen. Carbon dioxide in the gas up to 5 per cent does not affect the reducing action of milk.

The rate of decolorization of methylene blue by milk can be increased by raising the temperature or increasing the concentration of the reducing enzyme. This is easily done by evaporating the milk *in vacuo* to $\frac{1}{3}$ to $\frac{1}{4}$ its volume. The rate is roughly proportional to the concentration of the milk. Increase in temperature has the same marked accelerating action as on all chemical reactions, and it is important to maintain the temperature constant in all comparative work.

The reducing enzyme is unstable and cannot be preserved for any length of time by adding toluene, chloroform, or thymol to the milk. The addition of 2 per cent NaF to milk will prevent the growth of bacteria without affecting its reducing powers during a period of 2 months. Some samples of canned evaporated milk which I examined did not exhibit a reducing action. Since colloidal platinum and formic acid reduce methylene blue rapidly, imitating the milk-aldehyde reducing action,[8] it is likely that a protected platinum solution

[7] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 167.

[8] Bredig, G., and Sommer, F., *Z. physik. Chem.*, 1910, lxx, 34.

might take the place of milk as a more stable medium for oxygen determinations.

SUMMARY.

The rate of reduction of methylene blue by milk and acetaldehyde is proportional to the concentration of oxygen in the milk. This fact may be made the basis of a method of determining oxygen in gaseous mixtures.