of bound ions from the antibody molecules might account for the slow increase in neutralizing activity of unfractionated serum previously observed during early stages of incubation at low salt concentration.

Finally, the differences in the behavior of the virus-neutralizing activity of the various serum fractions with respect to changes in salt concentration of the antigen-antibody reaction mixture at pH 7, shows that the antibody molecules in the different fractions are different physical entities. This result is not surprising, since electrophoresis-convection separates proteins on the basis of differences in their electrophoretic properties.

This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service; in part by an institutional grant from the Damon Runyon Fund and the American Cancer Society; and in part by a departmental grant from the Division of Biology and Medicine, Atomic Energy Commission, Contract No. AT(11-1)-269.

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Communication No. 34 from the
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Received August 5th, 1955

The assay of soluble hydrogenase

CURTIS AND ORDAL[1] have shown that the soluble hydrogenase from *Micrococcus aerogenes* is inactivated by methylene blue, which is the dye most commonly used as hydrogen acceptor in the enzyme assay. Benzyl viologen is less inhibitory, while methyl violet proved least injurious of the dyes tested.

We have obtained analogous results for two other hydrogenation systems. Working with the soluble hydrogenase from *Desulphovibrio desulphuricans* (SADANA AND JAGANNATHAN[2]), we find that dilute solutions of the enzyme do not take up hydrogen in the presence of methylene blue. Concentrated solutions of the enzyme will transfer hydrogen to methylene blue for several minutes, followed usually by a sharp decline in activity. With benzyl viologen or methyl violet 3B we have observed little or no inactivation of the enzyme.

For comparison we have been studying the transfer of hydrogen to acceptors in the presence of one of the relatively simple organo-metallic complexes which, in aqueous solution, react with molecular hydrogen. The catalytically active complex is formed by addition of a large excess of potassium cyanide to cobaltous chloride solution in the absence of air (IGUCHI[3]). We find that H_2 uptake is inhibited by dyes in the increasing order methyl violet, benzyl viologen, methylene blue (Fig. 1).

There can be little doubt that the explanation of the inhibition is the same for both the hydrogenase and the cyanide systems. In each there are two opposing reactions:

(i) reaction of the catalyst with H_2;

(ii) reaction of the catalyst with oxidised dye.

The first is followed by donation of hydrogen to the dye, thus freeing the catalyst to undergo either (i) or (ii). The second leads to inactivation. Although it is a relatively slow reaction, it brings about a decline in the number of effective catalyst molecules. The degree of inhibition by each dye is a measure of the rate at which it can oxidise the catalyst and is determined not only by redox potential, but also by the mode of reaction.

This explanation suggests that the site in soluble hydrogenase which is capable of reacting directly with H_2 is readily accessible for collision not only with H_2, but also with any other solute. The active site is believed to consist of either one or two metal atoms. (The reasons are discussed in a forthcoming review.) On collision with an oxidant, such as methylene blue, an electron is transferred to the dye, while the metal atom passes to a higher oxidation state (*e.g.* $Co^{II} \rightarrow Co^{III}$). In the cobalt cyanide system the oxidation cannot be reversed, and this may well be true for hydrogenase.

With particulate hydrogenase, dyes have no inactivating effect. It is conceivable that the active site in particulate hydrogenase is screened by protein or lipid which, although it is readily permeable to diatomic gases, hinders the diffusion of larger molecules.

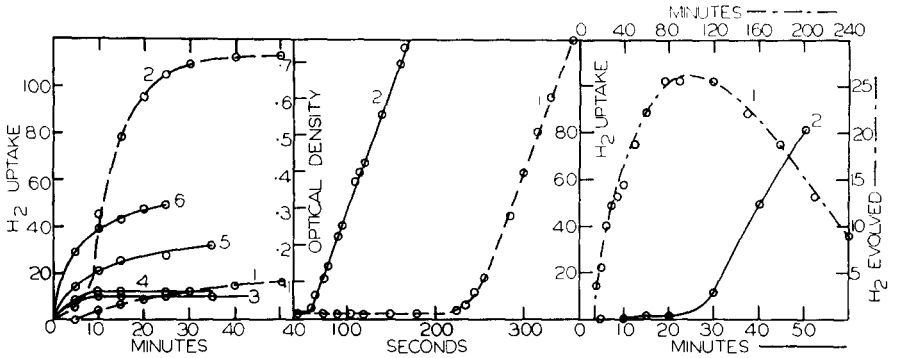

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Hydrogenase assay in the presence of methyl violet (curve 1) and benzyl viologen (curve 2) respectively. Under these conditions addition of methylene blue largely inactivates the enzyme. Curves 3, 4, 5 and 6 correspond respectively to the presence of methylene blue, benzyl viologen, methyl violet, and no dye, in the measurement of H_2 uptake by 10 micromoles Co^{++} in 200 micromoles KCN and tris(hydroxymethyl)aminomethane buffer at pH 7.8.

Fig. 2. Spectrophotometric assay (555 mμ) of hydrogenase with benzyl viologen as hydrogen acceptor, showing effect of dilution on the lag period. Curve 1: 0.05 ml enzyme; curve 2: 0.1 ml.

Fig. 3. Curve 1: example of a 20 min lag period in hydrogenase assay with benzyl viologen. Curve 2: H_2 evolution by hydrogenase in the presence of reduced methyl viologen.

From the above remarks it will be evident that an assay in which an oxidising dye, such as methylene blue, is added to the enzyme can be misleading. After addition of the dye, and before hydrogen consumption begins, there is generally a lag period, which might persist for a few seconds (Fig. 2) or can extend to 20 minutes or more (Fig. 3, curve 1). In the latter case, no hydrogen uptake would have been observed if methylene blue had been used, as the enzyme would have been completely inactivated before the lag period was over. Presumably most other hydrogen acceptors cause some inactivation during the lag period.

For preparations of the enzyme which have a lag period of the order of seconds (Fig. 2) instead of minutes, advantage may be taken of the relative slowness of reaction (ii) by using a rapid spectrophotometric assay in the presence of benzyl viologen (see, for example, PECK AND GEST[4]).

Highly active preparations can be assayed by the procedure of SADANA AND JAGANNATHAN[2], with benzyl viologen substituted for methylene blue. Alternatively, methyl violet may be used but its reduction is relatively slow (Fig. 1, curve 1). We have found it necessary to pass the H_2 over hot copper (650° C) to remove O_2 and through KOH (50% aqueous solution) to remove CO_2, and to keep the solutions at or near 0° C until the vessels have been flushed with H_2 and are ready to be brought to 30° C in the Warburg bath.

When the lag period is long, an assay which requires addition of an oxidising agent is best replaced by one based on the exchange reaction with deuterium (see, for example, KRASNA AND RITTENBERG[5]) or the evolution of H_2 in the presence of reduced methyl viologen. Compared with determination of H_2 consumption, the last method has the disadvantages that the reaction observed is slower, and is more critically dependent upon pH. We have found that it can be carried out under the following conditions: main compartment of Warburg vessel — 2.5 ml phosphate buffer (0.2 *M*) pH 6.4; 0.1 ml bovine serum albumin (0.2%); 0.1 to 0.5 ml enzyme solution; 0.1 ml sodium dithionite (0.0625 *M*) in buffer. Centre well — 0.1 ml pyrogallol (20%); 0.1 ml potassium carbonate (50%). Side-arm — 0.4 ml methyl viologen (0.04 *M*). The initial rate is taken as a measure of the enzyme activity (Fig. 3, curve 2). As the reducing potential of the system declines, the rate of gas evolution falls off and finally becomes negative.

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Received July 30th, 1955