The Inhibition of Hydrogenase by Carbon Monoxide and the Reversal of this Inhibition by Light*

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Received October 27, 1961

Carbon monoxide has been shown to inhibit competitively the hydrogenase of *Proteus vulgaris* and *Desulfovibrio desulfuricans*. The latter hydrogenase is inhibited at lower concentrations of carbon monoxide than the former. The inhibition is reversed by removing the carbon monoxide. The carbon monoxide inhibition of the hydrogenase of *Proteus vulgaris* can be reversed by strong visible light sources. Sodium hydrosulfite prevents this reversal. When the illumination is withdrawn the light effect persists, but it disappears when the gas phase which had been illuminated is removed and replaced with a new sample of gas.

The enzyme hydrogenase, which catalyzes the reversible activation of molecular hydrogen, has attracted the attention of many investigators because of the simplicity of its substrate, hydrogen. In 1943, Hoberman and Rittenberg showed that the enzyme in *Proteus vulgaris* was inhibited by carbon monoxide and that the inhibition was reversed by light. More recent evidence by Krasna and Rittenberg showing that the enzyme could be reversibly oxygenated (Fisher *et al.*, 1954) and that the reaction was inhibited by nitric oxide (Krasna and Rittenberg, 1954*a*) and various iron pentacyano compounds (Krasna and Rittenberg, 1955) supports the view that a ferrous complex is involved in the activity of hydrogenase.

In 1933, Kempner and Kubowitz showed that the production of H_2 from glucose by *Clostridium butyricum* was inhibited by carbon monoxide and the inhibition could be reversed by light. They obtained an action spectrum of the enzyme which Warburg (1949) interpreted as indicating that the enzyme which is inhibited by CO is a sulfhydryl complex of iron rather than a porphyrin complex.[1]

The inhibition of hydrogenase by carbon monoxide has been observed by many investigators, but, with the exception of Hoberman and Rittenberg (1943) and possibly Kempner and Kubowitz (1933), all have failed to demonstrate its reversibility by light. This was the case with the hydrogenase of *Azotobacter vinelandii* (Wilson and Wilson, 1943; Hyndman *et al.*, 1953), *Escherichia coli* (Joklik, 1950), *Clostridium butylicum* (Peck and Gest, 1957), and *Aerobacter aerogenes* (Tempereli *et al.*, 1960).

Since the reversibility of carbon monoxide inhibition of hydrogenase would give valuable information about the nature of the enzyme, we have investigated the effect of carbon monoxide on the hydrogenase of *Proteus vulgaris* and *Desulfovibrio desulfuricans* and the reversibility of this effect by light.

EXPERIMENTAL

For the experiments with *Proteus vulgaris*, whole cells were used which were grown as previously described (Krasna and Rittenberg, 1954b). The experiments with *Desulfovibrio desulfuricans* were carried out with a purified enzyme preparation [60–95% $(NH_4)_2SO_4$ fraction] (Krasna *et al.*, 1960).[2] Oxygen-free hydrogen was prepared by passage through a Deoxo purifier (Baker and Co., Inc., Newark, N.J.) and then over hot copper. Carbon monoxide–hydrogen mixtures (v/v) were prepared by mixing the appropriate quantities of H_2 and CO. The concentration of CO in mixtures containing less than 3% CO was determined by mass spectrometric analysis.

The hydrogenase was assayed by the deuterium exchange method (Fisher *et al.*, 1954; Krasna and Rittenberg, 1957) at 37°. The reaction flasks (Krasna and Rittenberg, 1954b) were of either 30 or 90 ml volume, depending on the number of gas samples to be removed from the flask. The D_2O concentration of the liquid phase was 20% and the amount of enzyme used was adjusted so that the rate of appearance of deuterium in the gas phase did not exceed 0.4% per hour. At this and lower rates of exchange, the rate of the reaction is proportional to enzyme concentration (Krasna and Rittenberg, 1957). The *p*H of the system was 6.7. The flasks were deoxygenated either by the addition of sodium hydrosulfite or by evacuation for 20 minutes while the solution was frozen in dry ice. When CO inhibitions in the dark were being studied, the flasks were wrapped with aluminum

* This work was supported by contracts to Columbia University from the Office of Naval Research, Department of the Navy (ONR 26602); from the Atomic Energy Commission (AT (30-1) 1803); and from the National Institutes of Health (E-2839). Reproduction of this article in whole or in part is permitted for any purpose of the United States Government. The deuterium oxide was obtained on allocation of the Atomic Energy Commission.

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[1] While it is reasonable to believe that the enzyme hydrogenase is involved in the sequence of reactions which produce hydrogen from glucose, they offered no evidence that it was hydrogenase rather than some other enzyme which was inhibited by CO and split by light.

[2] We are indebted to Dr. E. Riklis for preparing this enzyme.

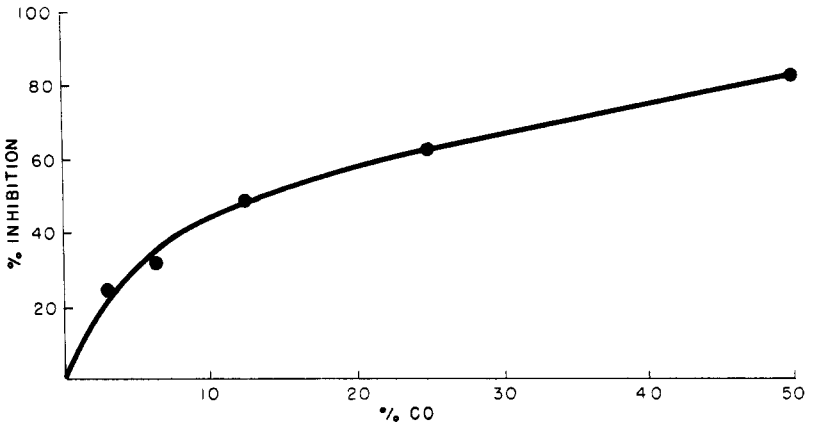

FIG. 1.—Inhibition of hydrogenase of *Proteus vulgaris* by carbon monoxide. The solution containing the *P. vulgaris* cells was deoxygenated by the addition of 3 mg/ml of sodium hydrosulfite. The hydrogen pressure was kept constant at 380 mm (3.48×10^{-4} M in solution, calculated from the solubility of hydrogen in water), and the presence of the carbon monoxide was varied.

foil. The inhibition by CO was always determined by comparing the rate of the enzyme under CO-H_2 to the rate of an identical amount of enzyme where N_2 replaced CO in the gas mixture.

For the experiments on light reversibility either a 1000 watt bulb (Westinghouse projection bulb #T12DFD) or a 2000 watt bulb (Westinghouse projection bulb #PH2M-T48-3AS/EDM) was used. The light was focused onto the reaction vessel with two Fresnel lenses, $14^3/_4$ in. diameter, 14 in. focal length. Between the light source and the first lens there was a large flat bottle filled with cold water to absorb the infrared.

RESULTS AND DISCUSSION

Inhibition by Carbon Monoxide in the Dark.—The effect of different concentrations of carbon monoxide in the dark on the hydrogenase activity of *Proteus vulgaris* is shown in Figure 1. The enzyme solution was deoxygenated by the addition of 3 mg/ml of sodium hydrosulfite. With 80% CO, the enzyme was more than 90% inhibited. Identical results were obtained with 5 mg/ml of sodium hydrosulfite. The inhibition could be reversed by removing the CO and filling the flasks with hydrogen. In Figure 2 the reciprocal of the rate of

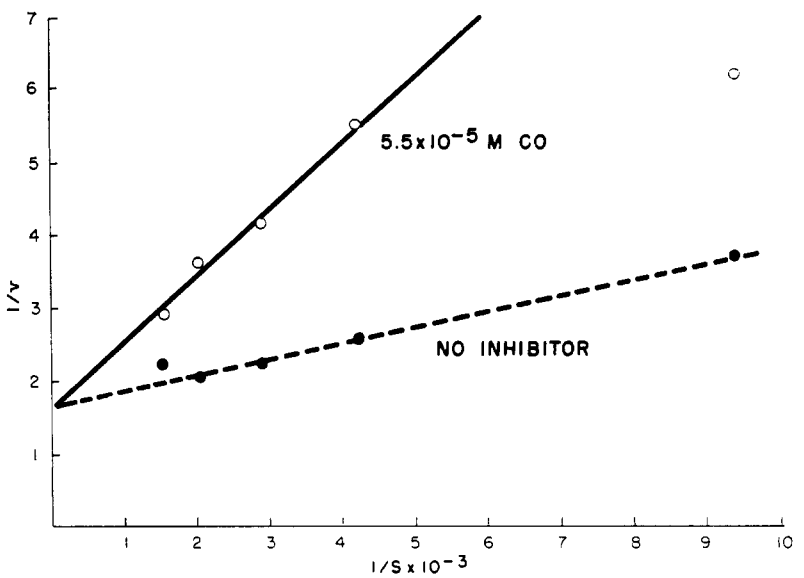

FIG. 2.—Determination of K_m and K_i for the inhibiton of the hydrogenase of *Proteus vulgaris* by carbon monoxide. The solution containing the *P. vulgaris* cells was deoxygenated by the addition of 3 mg/ml of sodium hydrosulfite. The velocity of the reaction, v, is expressed as the change in atom % deuterium in the gas phase per hour (see footnote 3). The carbon monoxide concentration was kept constant at 5.5×10^{-5} M (P_{CO} = 54 mm).

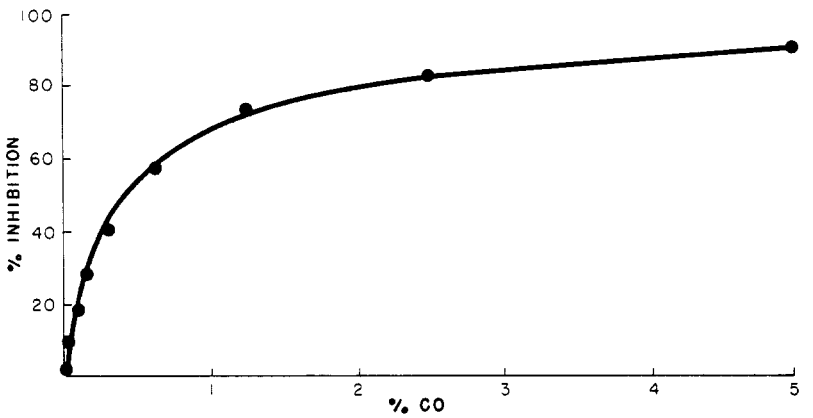

FIG. 3.—Inhibition of hydrogenase of *Desulfovibrio desulfuricans* by carbon monoxide. The partial pressure of hydrogen was kept constant at 700 mm.

exchange[3] is plotted against the reciprocal of the hydrogen concentration in the absence and in the presence of 5.5×10^{-5} M carbon monoxide (P_{CO} = 54 mm). The data show that carbon monoxide is a competitive inhibitor of hydrogenase. From these data the K_m of hydrogenase for hydrogen was calculated to be 1.4×10^{-4} and the K_i for carbon monoxide, 1.8×10^{-5}. Similar values were obtained when the data of Figure 1 were plotted according to Dixon (1953). The enzyme has a greater affinity for carbon monoxide than for hydrogen.

In Figure 3 is shown the effect of various concentrations of CO on the purified hydrogenase of *Desulfovibrio desulfuricans*. In this case the enzyme was deoxygenated with 5 mg/ml of sodium hydrosulfite. The inhibition could be reversed by the removal of the CO. The hydrogenase of *Desulfovibrio desulfuricans* is inhibited at much lower concentrations of carbon monoxide than the hydrogenase of *Proteus vulgaris*. With the *Desulfovibrio* hydrogenase, 0.3% CO (3×10^{-6} M in solution) caused a 50% inhibition, while with the *Proteus* hydrogenase about 30% CO was required for a comparable inhibition. The hydrogenase of *Desulfovibrio desulfuricans* is more sensitive to inhibition by oxygen (Krasna *et al.*, 1960).

The data indicate that the enzyme combines reversibly with CO; *i.e.*, $E + CO \rightleftharpoons E \cdot CO$. When the results obtained in Figures 1 and 3 were plotted according to Dixon (1953), *i.e.*, the concentration of carbon monoxide vs. the reciprocal of the velocity, a straight line was obtained. A linear relationship was not obtained if the reciprocal of the velocity was plotted *vs.* the square or the square root of the carbon monoxide concentration. This shows that only one molecule of carbon monoxide combines with one enzyme molecule. Since the rate of the reaction is directly proportional to the amount of free enzyme, E, the data of Figures 1 and 3 represent the binding of the enzyme with CO.

The effect of *p*H on the CO inhibition of the hydrogenase of *Desulfovibrio desulfuricans* is shown in Figure 4. The inhibition in each case was determined by comparing the rate to that in a control flask at the same *p*H but containing no CO. The

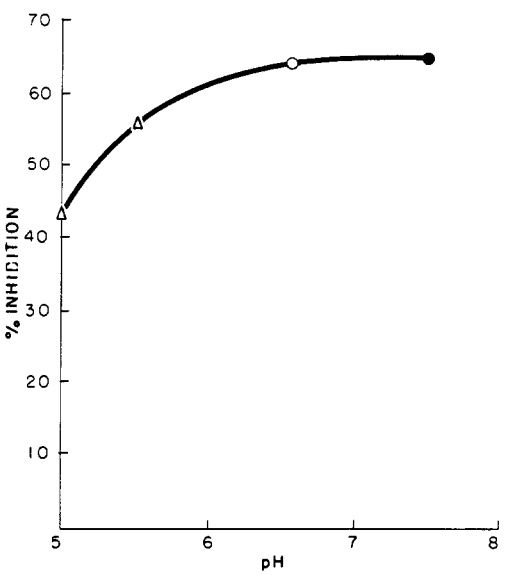

FIG. 4.—Effect of *p*H on CO inhibition of hydrogenase of *Desulfovibrio desulfuricans*.—The gas phase contained 0.15% CO. The enzyme was deoxygenated with 3 mg/ml of sodium hydrosulfite. The inhibition at each *p*H was determined by comparing to a control flask at the same *p*H but containing no carbon monoxide. All buffers were 0.15 M. △, acetate; ○, phosphate; ●, tris(hydroxymethyl)aminomethane.

[3] The activity of the hydrogenase is determined by measuring the change in atom % deuterium in the gas phase per hour. When the hydrogen pressure in the various experimental flasks is the same, this value of atom % D can be used directly to express the rate of the reaction. This value is used in Figure 1 and in the subsequent figures and tables. In Figure 2, however, the hydrogen pressure is varied and the measured atom % D per hour does not give the real rate of the reaction since the HD produced in the exchange reaction catalyzed by the enzyme is diluted to different extents at different hydrogen pressures. In order to correct for this dilution factor all the measured values of atom % D per hour in this experiment were normalized to a standard pressure of 380 mm by multiplying each value by the ratio, partial pressure of H_2/380. These corrected rates are used in Figure 2.

*p*H optimum for this hydrogenase is 5.5 (Krasna *et al.*, 1960). The CO inhibition becomes greater as the *p*H is increased; as the *p*H decreases the binding of CO to the enzyme relative to H_2 falls off. Since *p*H has no effect on either the substrate or the inhibitor, the effect must be on the enzyme.

Reversal of CO Inhibition by Light.—When the CO inhibition was carried out as described in the previous section, with the enzyme being deoxygenated by the addition of sodium hydrosulfite, all attempts to demonstrate light reversibility failed. Varying the light intensity, CO concentration, and *p*H were ineffective. However, when as in the experiments of Hoberman and Rittenberg (1943) the hydrogenase of *Proteus vulgaris* was deoxygenated by prolonged evacuation of the flask without addition of sodium hydrosulfite, the CO inhibition was reversed by light. Figure 5 shows a typical experiment with *Proteus vulgaris* in which light reversibility was demonstrated. After activation of the reaction in 25% N_2–75% H_2, the flask was evacuated, filled with 25% CO–75% H_2, and shaken in the dark. The exchange was inhibited 78%. When the flask was then illuminated the inhibition decreased to 43%. The rate did not increase immediately when the light was turned on, and a lag phase of about $^1/_2$ to 1 hour was usually observed.

Similar results were obtained when the flask was illuminated as soon as it was filled with 25% CO–75% H_2. In this case the flask in the light was inhibited only 33% while a flask kept in the dark was inhibited 67%. The light source in these experiments was a 1000-watt bulb, and somewhat greater reversibility was obtained with a 2000-watt bulb. Similar results were obtained with a 1500-watt carbon arc.

The effect of light intensity on the reversibility of the CO inhibition is shown by the data in Table I. In the dark, the enzyme was inhibited 74% and the inhibition decreased as the intensity of the illumination was increased. When 150- or 300-watt bulbs were used, no light reversibility was detected.

It is interesting that although the hydrogenase was inhibited by carbon monoxide in the presence of sodium hydrosulfite (the CO inhibition in the

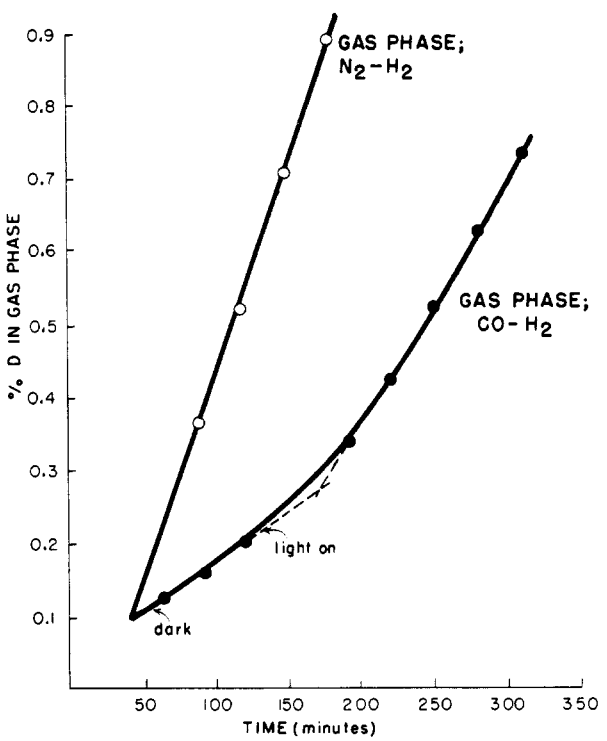

FIG. 5.—Reversal of CO inhibition by light. The source of hydrogenase was cells of *Proteus vulgaris*. A 90-ml flask containing the cells was deoxygenated by prolonged evacuation (see Experimental) and filled with 25% N_2–75% H_2. The flask was shaken at 37° and the rate of exchange was determined and was 0.341% D/hour. The flask was then evacuated and filled with 25% CO–75% H_2 and shaken at 37° in the dark. The rate was 0.077% D/hour (78% inhibition). At 132 minutes the flask was illuminated with a 1000-watt bulb and the rate of the exchange increased to 0.20% D/hour (43% inhibition).

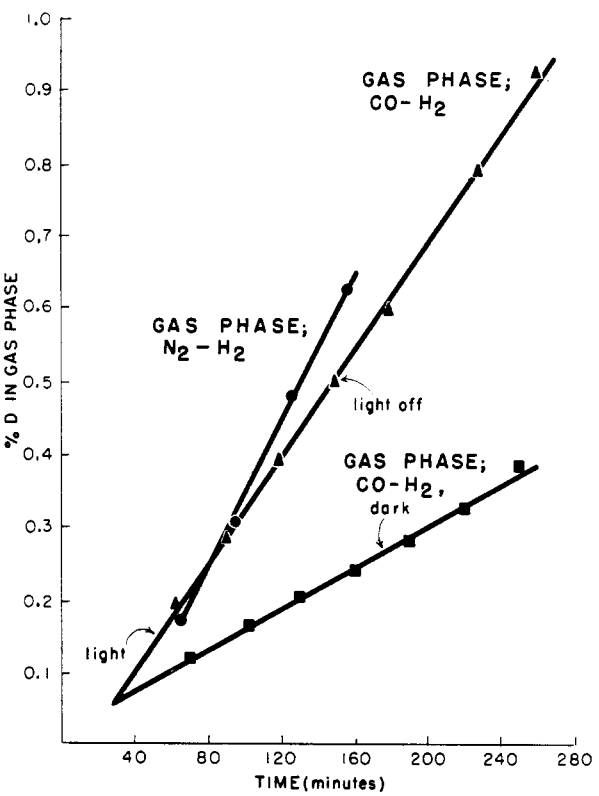

FIG. 6.—Effect of removing illumination on light reversibility of CO inhibition of hydrogenase. The source of hydrogenase was cells of *Proteus vulgaris*. Two flasks containing cells were deoxygenated by prolonged evacuation (see Experimental) and filled with 25% N_2–75% H_2. The flasks were shaken at 37° and the rate of exchange was determined. The rate was the same in both flasks, 0.348% D/hour. The flasks were then evacuated and filled with 25% CO–75% H_2 and shaken again at 37°. One flask (dark) was wrapped in aluminum foil and the other (light) was illuminated from a 1000-watt bulb. The flask in the dark had a rate of 0.091% D/hour (75% inhibition) and the flask in the light had a rate of 0.227% D/hour (34% inhibition). At 150 minutes the light was turned off.

TABLE I

EFFECT OF LIGHT INTENSITY ON CO REVERSIBILITY

Proteus vulgaris cells were used. Two flasks containing cells were deoxygenated by prolonged evacuation and filled with 25% N_2–75% H_2. The flasks were shaken at 37° and the rate of exchange was determined. The flasks were then evacuated and filled with 25% CO–75% H_2 and shaken at 37°. One flask was kept in the dark throughout the experiment (dark). The other flask was illuminated at first with a 1000-watt bulb which was supplied with 95 volts from a Variac (half light intensity). The voltage to the bulb was then increased to 110 volts (full light intensity). The light intensity at 95 volts was half that at 110 volts.

		Activity (% D/hr)	% Inhibition
N_2–H_2		0.330	0
CO–H_2	dark	0.087	74
	half light intensity	0.116	65
	full light intensity	0.163	51

dark was 10–20% greater in the absence of hydrosulfite) no light reversibility could be demonstrated if hydrosulfite was present. If the enzyme was deoxygenated with 0.14 mg/ml[4] of sodium hydrosulfite rather than 3–5 mg/ml, light reversibility was observed, although less than in the absence of hydrosulfite.

The hydrogenase of *Desulfovibrio desulfuricans* is extremely sensitive to oxygen (Krasna *et al.*, 1960) and could not be activated without sodium hydrosulfite.

[4] This amount of sodium hydrosulfite should be sufficient to react with the oxygen present in 5 ml H_2O in equilibrium with air and not give an excess of hydrosulfite.

Effect of Withdrawing Illumination.—Under conditions where light reversibility was observed (*Proteus vulgaris* cells in the absence of sodium hydrosulfite) it was of interest to see whether the light effect would be removed when the illumination was removed. Such an experiment is shown in Figure 6. In the dark the enzyme was inhibited 75% and in light 34%. When the light was shut off at 150 minutes, the rate of the reaction continued unchanged and did *not* decrease to that of the enzyme in the dark. If, however, after removal of the illumination, the gas in the flask was removed and the flask filled with a new sample of CO-H_2, the rate in the dark after light was inhibited to the same extent as previously in the dark, *i.e.*, the light effect was removed (see Table II). These results were surprising since, in general, light effects are removed upon withdrawal of illumination.

TABLE II

EFFECT OF REMOVING ILLUMINATION AND CHANGING GAS ON LIGHT REVERSIBILITY OF CO-INHIBITED HYDROGENASE

The whole experiment was carried out with *Proteus vulgaris* in the same flask. The flask was first filled with 25% N_2–75% H_2 (1), then evacuated and filled with 25% CO–75% H_2 in the dark (2), then evacuated and filled with 25% CO–75% H_2 in the light (3), and finally evacuated and filled with 25% CO–75% H_2 in the dark (4).

		Activity (% D/hr)	% Inhibition
(1)	N_2–H_2	0.520	0
(2)	dark	0.159	70
(3)	CO–H_2 light	0.250	52
(4)	dark after light	0.171	68

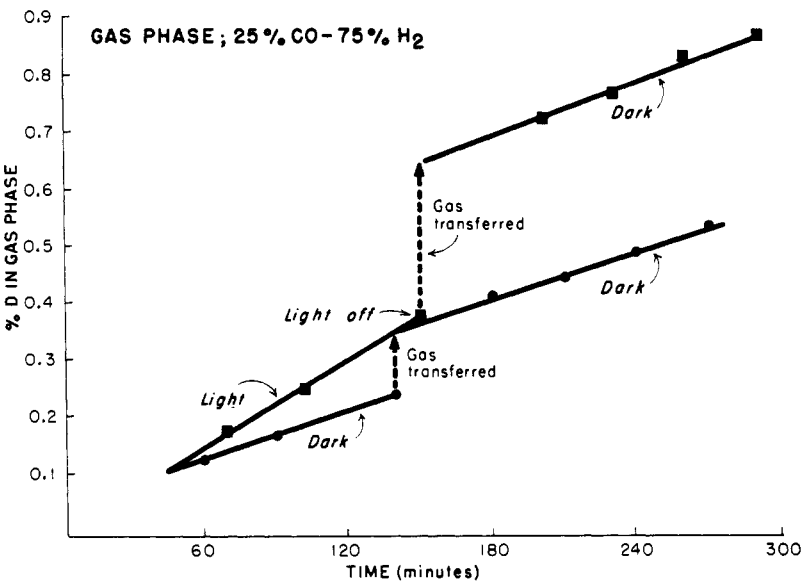

FIG. 7.—Effect of transferring illuminated CO-H_2 gas. *Proteus vulgaris* cells were the source of hydrogenase. Two 90-ml flasks were first shaken with 25% N_2-75% H_2 and had an exchange rate of 0.478% D/hour. The flasks were then evacuated and filled with 25% CO–75% H_2. One flask was shaken in the dark and had a rate of 0.091% D/hour (81% inhibition), and the other was shaken in the light and had a rate of 0.156% D/hour (68% inhibition). While the 90-ml flasks were being shaken, two 30-ml flasks were prepared with 25% N_2–75% H_2 and had a rate of 0.378% D/hour. At 150 minutes the light was turned off, the shaking stopped, and the gas from the two large flasks transferred with a Toeppler pump to the two small flasks and the latter two flasks shaken in the dark. The flask with the gas which had been in the dark all along had a rate of 0.101% D/hour (73% inhibiton), and the flask with the gas which had previously been in the light had a rate of 0.111% D/hour (71% inhibition).

Analysis of the CO-H_2 gas mixture in the mass spectrometer for all masses between 16 and 212 before and after illumination showed no difference between the two samples. Further evidence that the gas is not changed by illumination is given by the following experiment (see Fig. 7). Two flasks were filled with 25% CO–75% H_2, and one was shaken in the dark and the other in the light. The reaction in the flask in the dark was inhibited 81% and that in the light 68%. The gas from these flasks was then transferred by a Toeppler pump to two other flasks containing active enzyme, and the latter two flasks were then shaken in the dark. The rates in the second two flasks were identical; the gas which previously had been exposed to light gave the same inhibition as the gas which had been in the dark all along. It is clear that the gas which had been exposed to light is no different from the gas kept in the dark, and the continued activation upon cessation of illumination is not due to any change in the gas composition.

The failure of the enzyme·CO complex, which becomes catalytically active on illumination, to revert to the inhibited state on the cessation of illumination is unexpected. These observations are consistent with the following explanation. Light converts the CO-inhibited enzyme to a metastable state in which the enzyme contains CO but in which the catalytic center is active, *i.e.*, the center is not blocked by the bound CO, *i.e.*,

$$E + CO \rightleftharpoons E\cdot CO \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} E^*\cdot CO$$

evacuation

In this equation E·CO represents the inhibited enzyme in the dark while E^*·CO represents the catalytically active metastable state. E·CO and E^*·CO are interconvertible only in the presence of light, for our data show that the light-activated enzyme does not revert in the dark to the inhibited form. When CO is removed by evacuation the metastable enzyme·CO complex collapses into the normal state of the uninhibited enzyme. The enzyme is now in a configuration in which CO can be bound to the enzyme in such a manner as to block the site which activates hydrogen. In this regard the findings of Gibson (1959) are of interest. He found that the dissociation of the hemoglobin-CO complex by light yielded a form of hemoglobin that reacts more rapidly with oxygen than normal hemoglobin. Further experiments are in progress to clarify this phenomenon.

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