# *THE ACTIVATION OF THE HYDROGENASE OF PROTEUS VULGARIS BY VISIBLE LIGHT**

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We have previously shown[1] that the hydrogenase of *Proteus vulgaris* is inhibited competitively by carbon monoxide, and that the enzyme has a greater affinity for carbon monoxide than for hydrogen. We showed that the activity of the CO-inhibited enzyme could be substantially increased by exposing the inhibited enzyme to strong visible light sources, suggesting that light reversed the CO inhibition as is the case with many enzymes. The inhibition of hydrogenase by carbon monoxide has been observed by many investigators, but, with the exception of Hoberman and Rittenberg,[2] and Kempner and Kubowitz,[3] all have failed to demonstrate its reversibility by light.

Two observations were made in our earlier study that were quite puzzling, and no satisfactory explanation could be found for them. The first was that although light greatly increased the activity of the CO-inhibited enzyme, the activity did not reach that of the completely uninhibited enzyme in the absence of CO, i.e., although light appeared to reverse the CO inhibition, this reversal was never complete. In these experiments the CO inhibition in the dark was of the order of 70 per cent. The second observation that could not be explained was the finding that the effect of visible light on increasing the activity of the CO-inhibited enzyme persisted long after the source of illumination was removed. In general, reversal of CO inhibition of enzymes by light disappears when the light source is removed.

The experiments described here were undertaken to study these two problems and to elucidate the mechanism by which light increases the activity of CO-inhibited hydrogenase in *Proteus vulgaris*. We will show that visible light does not appreciably reverse the CO inhibition but strongly stimulates the activity of the residual active enzyme. Visible light strongly activates the hydrogenase in *Proteus vulgaris* in an irreversible manner, even in the absence of carbon monoxide.

*Experimental.*—The experimental details were essentially the same as previously described.[1] The deuterium exchange assay was carried out with 10 per cent $D_2O$ rather than with 20 per cent $D_2O$, and at times the tritium exchange assay[4] was also employed. In all cases, the rate of exchange was in the range where the rate of the reaction was proportional to enzyme concentration.[5] The flasks containing the cells were deoxygenated either by the addition of sodium hydrosulfite (5 mg/ml) or by evacuation for 20 minutes while the solution was frozen in dry ice. In the latter case, the flasks were filled with $H_2$ and shaken at 37°C to completely activate the hydrogenase. The flasks were then evacuated and filled either with 25 per cent CO–75 per cent $H_2$ or 25 per cent $N_2$–75 per cent $H_2$ and placed in the light or dark, and the rate of exchange was measured. When deoxygenation was accomplished by the addition of hydrosulfite, the two-hour preincubation was omitted.

The light source was a DWY 650-watt high-silica halogen lamp (color temperature = 3400° K) mounted in a Sylvania Sun Gun of the type employed for home

movies. (These halogen lamps are available from Sylvania Electric Products, Inc., and from General Electric Company.) The light was focused onto the reaction vessel with two 5-cm-diameter lenses of 5-cm focal length. The first lens was mounted 13 cm from the light source and the second lens was 17 cm from the reaction flask. The two lenses were separated by 14 cm, and a large flat pyrex bottle filled with cold water, to absorb the infrared radiation, was mounted between the two lenses. The entire light system was mounted on an optical bench 50 cm long which could be easily moved. This optical system illuminated the reaction flask with a light intensity of about 15,000 ft-c. When lower light intensities were desired, neutral density filters or metal screening was placed in the light path. The reaction flasks were shaken in a constant temperature water bath at 37°C.

*Results.*—The effect of visible light on the activity of the hydrogenase of *Proteus vulgaris* in the presence and absence of CO is shown in Figure 1. The two upper curves show the results with cells in 25 per cent $N_2$–75 per cent $H_2$. It is clear that light strongly activates the uninhibited hydrogenase, the activity in light in this instance being 110 per cent greater than in the dark. The two lower curves show the results with cells in 25 per cent CO–75 per cent $H_2$. Again, it is clear that light greatly stimulates the activity of hydrogenase, the stimulation here being about 120 per cent. In this instance, 25 per cent CO inhibited the hydrogenase 50 per cent. When the experiment was carried out with half the quantity of cells in a $N_2$-$H_2$ atmosphere, the rates in light and dark were the same as with twice the quantity of cells in CO-$H_2$. Thus, light does not reverse the CO inhibition but stimulates the residual active hydrogenase to increased activity.

This would explain our earlier finding[1] that although light stimulated the activity of the CO-inhibited enzyme, the activity never reached that of the control in the absence of CO. In these experiments, the CO inhibition in the dark was about 70 per cent with only 30 per cent active enzyme remaining. Even if the residual activity were to double in the light as reported here, the activity would only reach 60 per cent of the uninhibited control.

Although the light stimulation of hydrogenase is a reproducible phenomenon

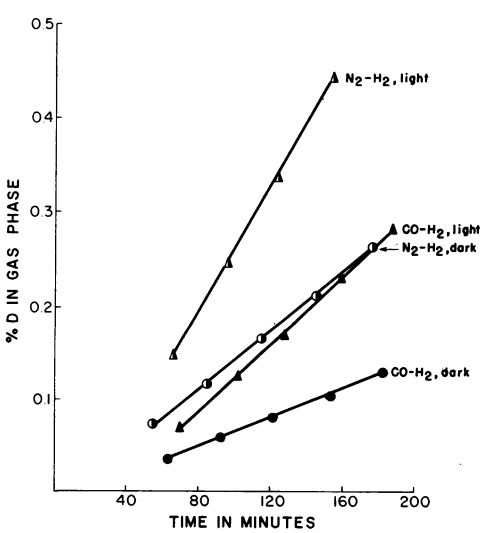


FIG. 1.—Effect of light on the hydrogenase activity of *Proteus vulgaris* in the presence and absence of carbon monoxide. The flasks containing 0.04 ml cells in 10% $D_2O$ were evacuated and filled with either 25% CO-75% $H_2$ or 25% $N_2$–75% $H_2$ after a preincubation for 2 hr in $H_2$. Two of the flasks were illuminated at 15,000 ft-c while the other two were kept in the dark.

▲ = $N_2$-$H_2$, light; rate = 0.207% D/hr
◑ = $N_2$-$H_2$, dark; rate = 0.099% D/hr
▲ = CO-$H_2$, light; rate = 0.113% D/hr
● = CO-$H_2$, dark; rate = 0.049% D/hr

TABLE 1

EFFECT OF LIGHT INTENSITY ON STIMULATION OF HYDROGENASE ACTIVITY

| Light intensity (ft-c) | Exchange rate (% D/hr) | Activation (%) |
|---|---|---|
| *Gas phase:* 25% $N_2$–75% $H_2$ | | |
| Dark | 0.166 | — |
| 3,750 | 0.218 | 27 |
| 7,500 | 0.237 | 43 |
| 15,000 | 0.368 | 122 |
| *Gas phase:* 25% CO–75% $H_2$ | | |
| Dark | 0.156 | — |
| 3,750 | 0.213 | 37 |
| 7,500 | 0.225 | 45 |
| 15,000 | 0.331 | 112 |

The cells were activated by evacuating for 20 min and then preincubating in $H_2$ for 2 hr. Three identical light sources were used in each experiment and the lower intensities were obtained by placing metal screens or neutral density filters in the path of the light source.

with different batches of cells, the precise extent of stimulation is not constant. The stimulation illustrated in Figure 1 is 110 per cent, but in other experiments we have found stimulations ranging from 12 per cent to 150 per cent depending on the particular batch of cells. We cannot, at present, offer any cogent explanation for this variability and are investigating the effect of culture conditions on the light stimulation.

The results in Table 1 show that light stimulation is dependent on the light intensity though not in a completely linear fashion. We have not yet tried light intensities much greater than 15,000 ft-c. At all light intensities employed, there was no appreciable difference in the light stimulation in the absence or presence of CO.

It was of interest to determine whether the activation of hydrogenase by light required the constant presence of a source of illumination or whether the activation persisted even after the light was turned off. Table 2 summarizes the results of this experiment, which was carried out both in the presence and absence of CO. Twice as many cells were used in the presence of CO to achieve the same rate as in the absence of CO. First, all flasks were shaken in the dark to determine the control rate. These rates are listed in the table under Phase 1 and are seen to be approximately the same. The flasks were then evacuated and filled with the appropriate gas, and one flask in each experiment was placed in the light. This is Phase 2, and it is clear that there is about a twofold increase in activity in the light. After about a $2^1/_2$-hour exposure to the light, the flask that was in the light was placed in the dark and one of the flasks that had been kept in the dark was then placed in the light. The rates during this interval are listed under Phase 3. It is clear that removing the light source does not cause any change in the activation whether the gas phase contained CO or not. As expected, the flask placed in the light during Phase 3 showed the light effect predicted. As a control, one flask was kept in the dark during the entire experiment and showed a constant rate of exchange.

Since light is stimulating the activity of the uninhibited hydrogenase rather than reversing the CO inhibition, it is not too surprising that the stimulation continues after the illumination is terminated. If it were due to a CO reversal, it would be expected that the light stimulation would disappear when the light is removed. The continued stimulation of the hydrogenase after the light is removed would

TABLE 2

EFFECT OF REMOVING LIGHT ON ACTIVATION OF HYDROGENASE

| Phase 1 | | Phase 2 | | Phase 3 | |
|---|---|---|---|---|---|
| Condition | Rate (% D/hr) | Condition | Rate (% D/hr) | Condition | Rate (% D/hr) |
| *Gas phase:* | 25% $N_2$–75% $H_2$ | | | | |
| Dark | 0.179 | Dark | 0.180 | Dark | 0.180 |
| Dark | 0.169 | Light | 0.310 | Dark | 0.310 |
| Dark | 0.175 | Dark | 0.182 | Light | 0.331 |
| *Gas phase:* | 25% CO–75% $H_2$ | | | | |
| Dark | 0.172 | Dark | 0.173 | Dark | 0.173 |
| Dark | 0.156 | Light | 0.320 | Dark | 0.320 |
| Dark | 0.164 | Dark | 0.168 | Light | 0.300 |

The cells were activated by evacuating for 20 min and then preincubating in $H_2$ for 2 hr. Twice as many cells were used in the presence of CO in order to achieve the same rate as in $N_2$–$H_2$. The flasks were then evacuated and filled either with 25% $N_2$–75% $H_2$ or 25% CO–75% $H_2$ and shaken for $2^1/_2$ hr in the dark during Phase 1. At the end of this period of time, the flasks were reevacuated and filled with the same gas as in Phase 1. In each experiment, two flasks were placed in the dark and one in the light and shaken for 160 min. This is Phase 2. After being shaken for 160 min, the flask that was in the light was transferred to the dark and one of the flasks that was in the dark was placed in the light and shaking was continued for an additional 2 hr. This is Phase 3. The flasks were *not* evacuated between Phase 2 and Phase 3. The light intensity was 15,000 ft-c.

suggest that light causes some change in the enzyme which is not reversed when the light is turned off. If the activation of the enzyme is indeed irreversible, the effect should persist even if the gas phase present during the illumination is removed and replaced by a fresh sample of the same gas. The results of such an experiment are shown in Table 3 where the experimental conditions were the same as those described in Table 2 except that the flasks were evacuated and refilled with new samples of gas as Phase 2 was changed to Phase 3; i.e., when the flask in the light was placed in the dark, it was evacuated and refilled with a new sample of gas before being placed in the dark. It is clear from the results in Table 3 that the light stimulation persists both in the presence and absence of CO when the light source is removed and the gas phase changed. This establishes that light irreversibly stimulates the activity of hydrogenase.

These experiments showing that light irreversibly stimulates the hydrogenase of *Proteus vulgaris* were all carried out with the enzyme under hydrogen at all times. It was of interest to see whether this stimulation also persisted if the cells were exposed to air after being activated by light. We therefore prepared two flasks with cells under $H_2$ and placed one in the dark and one in the light and measured the rates of exchange. In light, the activity was more than twice that in the dark. The hydrogen was removed from both flasks and the flasks were left exposed to air overnight. They were then evacuated, filled with hydrogen, and the exchange

TABLE 3

EFFECT OF REMOVING LIGHT AND CHANGING GAS ON LIGHT ACTIVATION OF HYDROGENASE

| Phase 1 | | Phase 2 | | Phase 3 | |
|---|---|---|---|---|---|
| Condition | Rate (% D/hr) | Condition | Rate (% D/hr) | Condition | Rate (% D/hr) |
| *Gas phase:* | 25% $N_2$–75% $H_2$ | | | | |
| Dark | 0.121 | Dark | 0.123 | Dark | 0.122 |
| Dark | 0.123 | Light | 0.261 | Dark | 0.267 |
| Dark | 0.122 | Dark | 0.117 | Light | 0.274 |
| *Gas phase:* | 25% CO–75% $H_2$ | | | | |
| Dark | 0.132 | Dark | 0.131 | Dark | 0.131 |
| Dark | 0.129 | Light | 0.295 | Dark | 0.291 |
| Dark | 0.130 | Dark | 0.129 | Light | 0.296 |

The experimental conditions were the same as those described for the experiment in Table 2 *except* that the flasks were evacuated and refilled with new gas whenever one phase was changed to another.

rates measured in the dark after activation in the usual manner. The flask which had been exposed to light was still twice as active as the flask which was not illuminated. Therefore, the activation brought about by light is not reversed by exposure to air which oxygenates the hydrogenase.

In all the experiments reported here, the cells were activated by deoxygenation by prolonged evacuation followed by a two-hour preincubation in $H_2$. This effectively activated the hydrogenase and we were able to show activation by light. However, this means of deoxygenation is very time-consuming, especially if one uses cell-free extracts. It has previously been shown[6] that hydrogenase can be effectively deoxygenated rapidly by the addition of sodium hydrosulfite. We therefore repeated all the experiments described here with cells that were deoxygenated by the addition of 5 mg/ml of sodium hydrosulfite. In the presence of hydrosulfite, the hydrogenase was activated by light both in the presence and absence of CO to the same extent as in the absence of hydrosulfite. Here, too, the light activation persisted even after the light was shut off and when the gas phase was removed and replaced with a new sample of gas.

*Discussion.*—We have shown that the light stimulation of CO-inhibited hydrogenase previously reported[1] is not due to a reversal of the CO inhibition by light but rather to a light activation of the residual uninhibited enzyme. In the absence of CO, light increases the activity of hydrogenase by about 100 per cent and this activation persists even after the light is turned off. In the present experiments, we find that the light activation persists after the light is turned off even if the original gas phase is removed and replaced with a new sample of gas. In our previous experiments with the CO-inhibited hydrogenase, we found that the light effect is lost after the light is turned off if the gas phase is changed at the same time. We now find a light activation in the presence of sodium hydrosulfite whereas previously we could not demonstrate any light effect if hydrosulfite was present. We cannot offer any satisfactory explanation for these different results except to suggest that some changes of which we are not aware may have taken place in the cell culture in the intervening years.

Light effects on hydrogenase activity have been previously reported in photosynthetic organisms but all these effects have been shown not to be due to a direct action on hydrogenase. Bose, Gest, and Ormerod[7] reported that light stimulated the reduction of ferricyanide with $H_2$ in whole cells and cell-free extracts of the photosynthetic bacterium *Rhodospirillum rubrum*. This effect of light could only be demonstrated at low concentrations of ferricyanide and was shown to be due to a permeability barrier to ferricyanide which was overcome in the light by the formation of ATP in photophosphorylation. Hanson reported[8] that in *R. rubrum*, light stimulates the reduction of low concentrations of ferricyanide by $H_2$ only in the presence of calcium ions, and suggested that calcium renders the cell membrane impermeable to ferricyanide and that light alters the membrane permeability. In *Scenedesmus*, light inhibits hydrogenase activity[9] and this inhibition was shown to be due to the production of oxygen by photosynthesis and the inhibition of hydrogenase by the oxygen produced.

This report is the first demonstration of the activation of the hydrogenase of a nonphotosynthetic organism by visible light. Since the assay employed does not require the presence of hydrogen acceptors, it is unlikely that we are dealing with a

permeability phenomenon. Preliminary experiments indicate that the light activation is also observed with cell-free extracts which would argue against any permeability phenomenon. The fact that the light stimulation is irreversible may indicate that light is causing some conformational change in the enzyme which increases its catalytic activity. Experiments are now in progress to elucidate the mechanism of the light activation.

*Summary.*—The hydrogenase activity of *Proteus vulgaris* can be increased about twofold by exposing the cells to intense visible light sources. This activation of hydrogenase is dependent on the light intensity and persists even after the light is turned off and the gas phase replaced with a new sample of gas. The previously reported light activation of CO-inhibited hydrogenase is not due to a reversal of the CO inhibition but rather to an activation of the residual active enzyme by light.

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