was prepared by mixing 8100 cc. of purified dioxane with 5400 cc. of carbon dioxide-free distilled water; both ingredients were at 23–24° and the mixing operation was performed at that temperature.

**Rate Measurements.**—For fast reactions (reactions of piperidine and sodium thiophenoxide with 2,4-dinitrochlorobenzene and 4-chloro-3-nitrobenzotrifluoride), the reacting solution was made up in a volumetric flask immersed in the thermostat using ingredients all at thermostat temperature. Zero time was not accurately recorded. The flask was briefly removed from the thermostat, quickly shaken and returned. Samples (8 or 9 per run) were withdrawn by a fast-delivery pipet and discharged into an excess of dilute sulfuric acid, the time of release from the pipet being recorded. In the case of runs at 0°, the pipet was chilled by ice before being used and between withdrawals of samples.

For the other reactions, the reacting solution was made up in a volumetric flask at 23° and then dispensed by means of a pipetting device into nitrogen-filled, rubber-stoppered test tubes (9 or 10 per run). All the tubes were placed in the thermostat at once. Reaction was stopped by addition of an excess of dilute sulfuric acid to the reacting solution in each tube, the time of addition being recorded. This technique required corrections to be made for thermal expansion or contraction of the solution.

Thermostats were constant to ±0.02°, and their temperatures were checked against thermometers certified by the Bureau of Standards.

The extent of reaction in all samples was estimated by potentiometric titration of the chloride ion liberated, using a silver electrode *vs.* glass electrode cell. Since thiophenol interfered with this cell, it was necessary to extract each sample from thiophenoxide runs with carbon tetrachloride and then ether before titration; the titrations were then fairly satisfactory, though the precision in these measurements was less than in the others.

In all runs the initial concentration of the aryl chloride was about 0.015 *M*. In runs with phenoxide or thiophenoxide, the initial concentration of the nucleophilic reagent equalled that of the aryl chloride; in runs with piperidine or hydroxide (except as noted in Table I), it was double. Rate coefficients were calculated from the slope of the plot of $1/(a - x)$ *vs.* time, the slope being, respectively, $k$ and $2k$ for the two cases mentioned above. (Appropriate logarithmic expressions were used in calculation of runs 3 and 4 of Table I). The plots were linear without regular curvature or much scatter of points, and covered as little as 40% reaction to as much as 80% reaction. Slopes were determined by the method of least squares with each point weighted according to the fourth power of the per cent. of aryl chloride unreacted.

Duplicate runs were made for each reaction at each temperature (except for runs 3 and 4 in Table I) and the two determinations agreed within an average deviation of one per cent. except for the reaction with phenoxide ion in methanol (1.7%) and for runs with thiophenoxide ion (2.5% to 6%).

The following rate coefficients have not already been given in the tables or text (all in units of l. mole$^{-1}$ min.$^{-1}$): 2,4-dinitrochlorobenzene with sodium hydroxide at 45.05°, 0.419 with sodium phenoxide at 0°, 0.0733; with piperidine at 0°, 0.874; 4-chloro-3-nitrobenzotrifluoride with sodium thiophenoxide at 0°, 2.62.

**Product Identification.**—The following expected products were isolated from samples of the reacting solutions which were allowed to stand a long time unquenched: 2,4-dinitrophenol, m.p. 113–114°; 2,4-dinitrodiphenyl ether, m.p. 69.5–70°; N-2,4-dinitrophenylpiperidine, m.p. 91–92.5°; 2,4-dinitrodiphenyl sulfide, m.p. 121°; 4-chloro-2-nitrodiphenyl sulfide, m.p. 83–84° (lit.[31] 86°); 2-nitro-4-trifluoromethyldiphenyl sulfide, m.p. 71–72°.[32]

(31) J. D. Loudon and N. Shulman, *J. Chem. Soc.*, 1618 (1938).

(32) G. W. Stacy and C. R. Bresson have recently prepared and characterized 2-nitro-4-trifluoromethyldiphenyl sulfide; they found m.p. 72.5–73.5° (private communication).

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# The Mechanism of Action of the Enzyme Hydrogenase[1]

BY ALVIN I. KRASNA[2] AND D. RITTENBERG[3]

RECEIVED DECEMBER 31, 1953

Both whole cells and cell-free extracts of *Proteus vulgaris* catalyze the exchange reaction between heavy water and hydrogen and the conversion of para to normal hydrogen. Both reactions follow first-order kinetics and are equally affected by the inhibitors, oxygen and cyanide. While cells, suspended in $H_2O$, catalyze the conversion of para- to orthohydrogen, no conversion occurs if $D_2O$ is substituted for $H_2O$. These experiments are all in accord with the following mechanism for the mode of action of hydrogenase: $H_2 + E \rightleftarrows H{:}E^- + H^+$, where E represents the enzyme. We postulate that $H{:}E^-$ is the active reducing agent.

The enzyme hydrogenase has been found in several bacterial species and is presumably present whenever hydrogen gas is either utilized or produced by them. The similarity between the action of this enzyme and platinum hydrogenation catalysts was pointed out by Green and Stickland[4,5] who showed that the extent of reduction of N,N′-dimethyl, γ,γ′-dipyridyl ($E_0' = -0.446$) is the same with *B. coli* and colloidal platinum. It was suggested that the reaction was

$$H_2 \rightleftarrows 2H\cdot \quad (1)$$

$$H\cdot \rightleftarrows H^+ + e \quad (2)$$

In 1934, Farkas, Farkas and Yudkin[6] showed that the hydrogenase in *B. coli* is capable of catalyzing the exchange reaction between $D_2O$ and molecular hydrogen. This exchange reaction has been studied in detail in *Proteus vulgaris* by Hoberman and Rittenberg[7] and Farkas and Fischer.[8]

When *B. coli* was suspended in $H_2O$ in an atmosphere of ortho deuterium,[9] Farkas[10] observed that

(1) This work was aided by a contract between the Office of Naval Research, Department of the Navy, and Columbia University (ONR 26602).

(2) National Science Foundation Predoctoral Fellow, 1953–1954. This report is from a dissertation to be submitted by Alvin I. Krasna in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(3) On leave, 1953–1954; The Institute for Advanced Study, Princeton, N. J.

(4) D. E. Green and L. H. Stickland, *Biochem. J.*, **28**, 898 (1934).

(5) L. H. Stickland, *Nature*, **133**, 573 (1934).

(6) A. Farkas, L. Farkas and J. Yudkin, *Proc. Roy. Soc.* (*London*), **B115**, 373 (1934).

(7) H. Hoberman and D. Rittenberg, *J. Biol. Chem.*, **147**, 211 (1943).

(8) L. Farkas and E. Fischer, *ibid.*, **167**, 787 (1947).

(9) A discussion of the various nuclear spin isomers of hydrogen and deuterium can be found in "Orthohydrogen, Parahydrogen and Heavy Hydrogen" by Farkas.[11] In this paper, the term normal hydrogen denotes the equilibrium mixture at room temperature of ortho- and parahydrogen (25% para, 75% ortho) and the term normal deuterium denotes the equilibrium mixture at room temperature of ortho and para deuterium (67% ortho, 33% para). The term parahydrogen is used for hydrogen containing more than 25% parahydrogen. $H_2$(o) represents orthohydrogen, $H_2$(p), parahydrogen and $H_2$(n), normal hydrogen. The reaction which converts $H_2$(p) to $H_2$(n) we shall call the conversion reaction; the reaction between hydrogen gas and deuterium oxide or between deuterium gas and normal water we shall call the exchange reaction.

(10) A. Farkas, *Trans. Faraday Soc.*, **32**, 922 (1936).

the ortho-para conversion was negligible compared with the exchange reaction.

The experiments described in this paper were undertaken to study the mechanism of the activation of hydrogen by the hydrogenase of *Proteus vulgaris* by comparing the ortho-para hydrogen conversion and the deuterium exchange reaction.

## Experimental

**Materials.**—*Proteus vulgaris*, which has a very active hydrogenase,[7] was chosen for study. The bacteria were grown in a broth of the following composition: $Na_2HPO_4$, 7.93 g.; $KH_2PO_4$, 1.47 g.; glucose, 5.0 g.; nutrient broth (Difco), 10.0 g.; NaCl, 5.0 g.; Casamino acids (Difco), 5.0 g.; $FeSO_4 \cdot 7H_2O$, 0.005 g. and $H_2O$ to 1 liter. Bacteria from an agar slant were transferred three times through broth and then inoculated into fifteen liters of broth. They were grown at 37° for 12 to 20 hours and then centrifuged in a Sharples Super-centrifuge. The bacterial paste was washed twice by centrifugation and suspended in distilled water. Aliquots of this suspension were used for the experiments.

Cell-free extracts were prepared by treating heavy bacterial suspensions with sonic vibration in a nine kilocycle Raytheon Oscillator for 20 minutes. The cell-debris was removed by centrifugation at 13,000 × *g*.

The bacterial suspension and the cell-free extract were stored in the cold under hydrogen. The concentration of the bacterial suspension and the cell-free extract was estimated from Kjeldahl nitrogen values.

Oxygen-free hydrogen was prepared by passage over hot copper. 50–65% parahydrogen was prepared by adsorbing purified hydrogen on degassed coconut charcoal at 63 to 77°K.[11]; after a time sufficient to establish spin equilibrium, the hydrogen was desorbed.

**Methods.**—The concentration of parahydrogen was determined by the thermal conductivity method of Bonhoeffer and Harteck[11,12] in which the resistance of a thermal conductivity cell containing the gas sample is measured. The cell was immersed in liquid nitrogen at its boiling point and its filament was heated by a direct current. The hydrogen sample was introduced into the conductivity cell to a pressure of 30.0 ± 0.1 mm. The resistance of the conductivity cell was determined by making it one leg of a Wheatstone bridge. The bridge was balanced by a slide wire. Known samples showed that the reading of the slide wire R, at balance, was proportional to the concentration of parahydrogen. The reproducibility of the determinations was ±1% in parahydrogen. Deuterium analyses were carried out with a mass spectrometer.

All reactions were carried out in 30-ml. flasks described by Hoberman and Rittenberg (see Fig. 2 of ref. 7). The liquid volume in the flask was always adjusted to 5 ml. When the flask was to be filled with gas, it was cooled in ice-water for ten minutes, evacuated for ten minutes and then filled with normal hydrogen, parahydrogen or deuterium[13] to a pressure of 300–500 mm. The flasks were shaken on a rotatory shaker[14] at 100 r.p.m. at room temperature and aliquots of gas removed at intervals for analysis.

Except when noted, the exchange experiments were carried out by adding 0.5 ml. of 99.9% $D_2O$ to the cell suspension or extract and making up the volume to 5 ml. with 0.15 *M* phosphate buffer, *p*H 6.7. The gas phase was normal hydrogen. For the ortho-para hydrogen conversion experiments, no heavy water was added and the gas phase was parahydrogen.

## Results

**Conversion of Parahydrogen by *Proteus Vulgaris*.**—A cell suspension was shaken for 14 hours in an atmosphere of parahydrogen. Cells which had been killed by heating at 100° for 20 minutes served as a control. The results are given in Table I.

TABLE I

CONVERSION OF PARAHYDROGEN BY *Proteus vulgaris*

The flasks contained 1.0 ml. of a bacterial suspension (1.88 mg. N/ml.).

| Sample | *R* | $\Delta R$[a] | % Conversion of parahydrogen |
|---|---|---|---|
| Normal hydrogen | 6.98 | .... | ... |
| Parahydrogen | 8.45 | 1.47 | ... |
| Parahydrogen in contact with bacteria for 14 hr. | 6.85 | −0.13 | 109 |
| Parahydrogen in contact with dead bacteria 14 hr. | 8.15 | 1.17 | 20 |
| Parahydrogen in contact with distilled water for 14 hr. | 8.19 | 1.21 | 18 |

[a] $\Delta R$ is the difference between the value of *R* of the conductivity cell when filled with the gas sample and when filled with normal hydrogen.

It is evident that *Proteus vulgaris* contains an enzyme system capable of catalyzing the conversion of para to normal hydrogen. The small conversion observed with the dead cells is due to the nuclear moment of the protons in the water molecules[11] since this conversion was also observed with distilled water. In all subsequent experiments in which the reaction time was not more than five hours, no appreciable conversion occurred with dead cells.

To eliminate the possibility that the decrease in the resistance observed with the active cells is due to the production of some gas other than hydrogen, the thermal conductivity of the gas at the end of the experiment was measured at room temperature. At this temperature the thermal conductivities of normal and of parahydrogen are the same. The presence of other gases would alter the thermal conductivity since this property is a function of the mass of the molecules present. In each case the thermal conductivity of the gas was the same as that of pure hydrogen. The gas was also analyzed in the mass spectrometer. No ions other than those which could arise from hydrogen were detected.

**Kinetics.**—The results for a typical experiment in which the parahydrogen conversion by a cell suspension was studied is shown in Fig. 1. The conversion follows first-order kinetics; $k$ is 6.3 × $10^{-3}$ per minute.

The results for a typical exchange experiment with a cell suspension is shown in Fig. 2. Here $\log (C_\infty - C)$ is plotted against time where $C$ is the atom per cent. excess deuterium in the gas at the indicated time intervals and $C_\infty$ is the atom per cent. excess deuterium in the gas at equilibrium. The equilibrium value $C_\infty$ found after 24 hours, is 3.30 atom per cent. excess deuterium. This is the same as the equilibrium value resulting from catalysis by active platinum. The exchange reaction also is first order; $k$ in this case is 2.4 × $10^{-3}$ per minute.

The reaction rates of these two experiments should not be compared since different cell preparations were used. In general, the values of $k$ in different experiments are not comparable since

(11) A. Farkas, "Orthohydrogen, Parahydrogen and Heavy Hydrogen," Cambridge Univ. Press, London, 1935.

(12) K. F. Bonhoeffer and P. Harteck, *Z. physik. Chem.*, **B4**, 113 (1929).

(13) The deuterium oxide and deuterium gas were obtained on allocation from the Atomic Energy Commission.

(14) Shaker used was Model No. 3623, Arthur H. Thomas Company, Philadelphia, Pa.

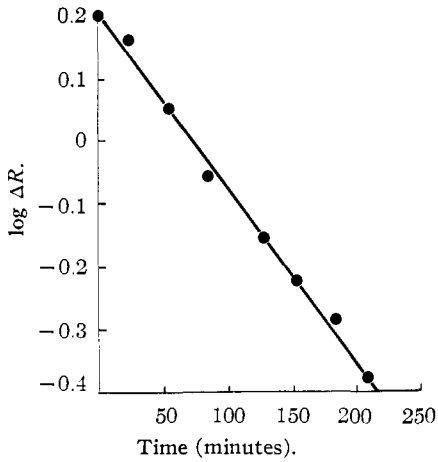


Fig. 1.—Catalysis of the ortho–para hydrogen conversion by a cell suspension of *Proteus vulgaris*. The flask contained 1.0 ml. of a cell suspension (1.88 mg. N/ml.). $\Delta R$ is the difference between $R$ of the conductivity cell when filled with the gas sample and when filled with normal hydrogen. The value of the rate constant, $k$, calculated from the slope, is $6.3 \times 10^{-3}$ per minute.

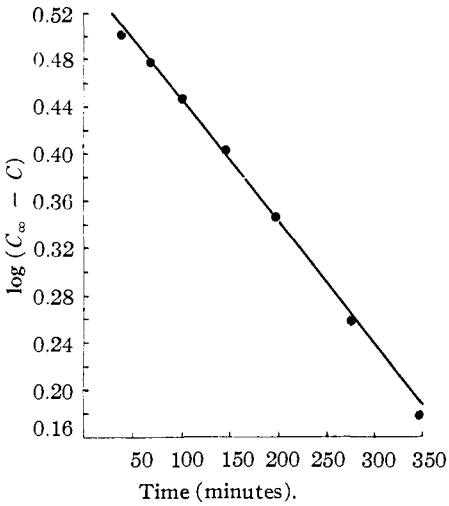


Fig. 2.—Catalysis of the exchange reaction between hydrogen and heavy water by a cell suspension of *Proteus vulgaris*. The flask contained 1.0 ml. of a cell suspension (1.32 mg. N/ml.). $C$ is the atom per cent. excess deuterium in the gas at the indicated time intervals and $C_\infty$ is the atom per cent. excess deuterium in the gas at equilibrium. The value of the rate constant, $k$, calculated from the slope, is $2.4 \times 10^{-3}$ per minute.

many different cell preparations were used and the activity of a single preparation did not remain constant over a long period of storage. Only values of $k$ in an individual experiment where the same cell preparation was used at the same time should be compared.

**Effect of Bacterial Concentration on the Reaction Rate.**—The effect of the bacterial concentration on the rate of the exchange and ortho–para conversion reactions is shown in Fig. 3. Similar curves were obtained by Hoberman and Rittenberg[7] and Farkas and Fischer[8] for the exchange reaction.

Both reactions involve (1) solution of the gaseous

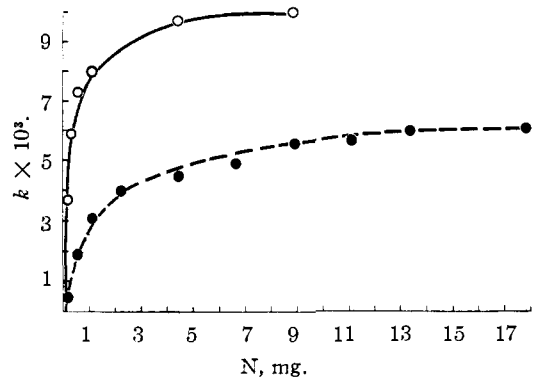


Fig. 3.—Effect of bacterial concentration on the rate of the exchange and ortho–para conversion reactions; ●, exchange reaction; O, ortho–para conversion reaction. The values of the rate constants $k$ were determined as in Figs. 1 and 2.

hydrogen in the medium and (2) activation of the dissolved hydrogen by the enzyme present in the cells. At low bacterial concentration, the rate of reaction is proportional to the concentration of the bacteria and the rate limiting step is the activation of the dissolved hydrogen by the enzyme. At high concentrations, the rate is independent of the bacterial concentration and the rate limiting step is the solution of the hydrogen in the water.

Further evidence that the rate limiting step with high bacterial concentrations is the solution of hydrogen in the water was afforded by the observation that at these concentrations the rate was dependent on the speed of shaking (see Table II). Addition of glass beads to increase the surface of the liquid also increased the reaction rate. The reaction rate remained the same when the heavy water concentration was 10, 30, 50 and 70% (see Table III).

TABLE II

EFFECT OF SPEED OF SHAKING ON THE RATE OF THE EXCHANGE REACTION AT HIGH BACTERIAL CONCENTRATION

Each flask contained 2 ml. of a bacterial suspension (6.2 mg. N/ml.).

| Speed of shaking, r.p.m. | 0 | 82 | 125 | 200 |
|---|---|---|---|---|
| Rate constant ($k \times 10^3$) | 4.7 | 5.6 | 8.2 | 12.4 |

TABLE III

EFFECT OF $D_2O$ CONCENTRATION ON THE RATE OF THE EXCHANGE REACTION AT HIGH BACTERIAL CONCENTRATION

Each flask contained 1.5 ml. of a cell suspension (3.5 mg. N/ml.).

| $D_2O$ concn., % | 10 | 30 | 50 | 70 |
|---|---|---|---|---|
| Rate constant ($k \times 10^3$) | 7.6 | 5.9 | 5.9 | 7.7 |

With cell-free extracts, a lag phase occurred before the full activity appeared. This could be eliminated by incubation under hydrogen or, most efficiently, by the addition of sodium hydrosulfite. In all experiments with cell-free extracts, 25 mg. of sodium hydrosulfite was routinely added.

The effect of the concentration of the cell-free extract on the rate of exchange and ortho–para conversion is the same as that observed with bacterial suspensions (see Fig. 4).

Since we were unable to prepare an extract which

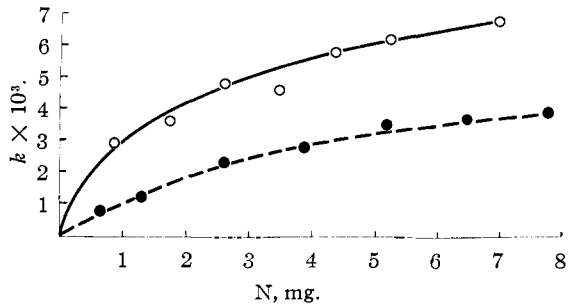


Fig. 4.—Effect of extract concentration on the rate of the exchange and ortho–para conversion reactions: ●, exchange reaction; O, ortho–para conversion reaction. The values of the rate constants $k$ were determined as in Figs. 1 and 2.

was concentrated enough to give the maximum rate as determined for the bacteria, this rate was estimated from a plot of $1/k$ *vs.* 1/(concentration) (see Fig. 5). The intercept gave maximum values of $k$ for the extract, and these were the same as those determined experimentally for the bacteria. At high extract concentration, the limiting rate also is that of solution of hydrogen in water. This rules out diffusion across the cell membrane as the rate-limiting step with the bacteria.

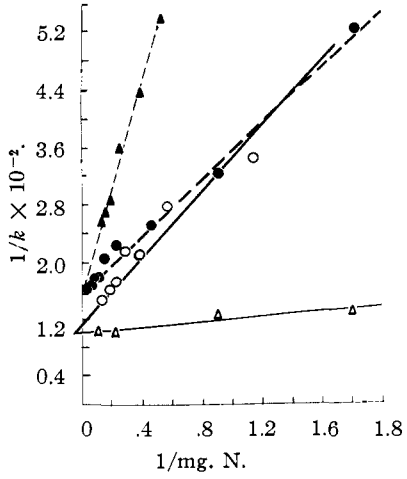


Fig. 5.—Plot of $1/k$ *vs.* 1/concentration for the exchange and conversion reactions in both whole cells and cell-free extracts. These values were taken from Figs. 3 and 4: ●, exchange by whole cells, $k_{max.} = 6.5 \times 10^{-3}$; ▲, exchange by cell free extracts, $k_{max.} = 6.3 \times 10^{-3}$; Δ, conversion by whole cells, $k_{max.} = 10.0 \times 10^{-3}$; O, conversion by cell free extracts, $k_{max.} = 9.0 \times 10^{-3}$.

When diffusion into or within the solution is the rate-limiting step (the rates of the chemical reaction, conversion and exchange, being extremely rapid), the maximum rates observed for both reactions might be expected to be identical. Actually, the maximum rate of the conversion reaction ($k_{max.} = 10 \times 10^{-3}$) is distinctly larger than the maximum rate for the exchange reaction ($k_{max.} = 6.5 \times 10^{-3}$). We are unable to explain this difference.

**Effect of Oxygen and Cyanide on the Exchange and Conversion Reactions.**—When oxygen was bubbled through the bacterial suspension for 24 hours at room temperature, both the exchange and conversion were inhibited 60%.[7] When these oxygen inactivated suspensions were then kept under hydrogen for 24 hours, almost complete restoration of both activities occurred (see Table IV). Addition of $2 \times 10^{-3}$ *M* sodium cyanide to the active suspension inhibited both reactions only about 15% when measured immediately; however, if the suspension was then kept under hydrogen for 24 hours, both reactions were inhibited 50%. Addition of $2 \times 10^{-3}$ *M* cyanide to an aerated suspension prevented the reversibility of the inhibition by incubation under hydrogen or by the addition of sodium hydrosulfite. In all cases tested, the conversion and exchange reaction were affected in a similar way.

TABLE IV

EFFECT OF OXYGEN AND CYANIDE ON THE EXCHANGE AND CONVERSION REACTION

Each system contained 2.0 ml. of a cell suspension (0.55 mg. N/ml.).

| System | Exchange $k \times 10^3$ | Exchange Inhibition, % | Conversion $k \times 10^3$ | Conversion Inhibition, % |
|---|---|---|---|---|
| Unaerated cells[a] | 1.9 | .. | 7.8 | .. |
| Aerated cells[b] | 0.76 | 60 | 3.5 | 55 |
| Aerated cells under hydrogen for 24 hr. | 1.8 | 5 | 7.2 | 8 |
| Cyanide[c] + unaerated cells | 1.7 | 11 | 6.3 | 19 |
| Cyanide + unaerated cells under hydrogen for 24 hr. | 0.83 | 56 | 4.7 | 40 |
| Cyanide + aerated cells | 0.76 | 60 | 3.1 | 60 |
| Cyanide + aerated cells under hydrogen for 24 hr. | 0.76 | 60 | 3.9 | 50 |
| Cyanide + aerated cells + sodium hydrosulfite | 0.83 | 56 | ... | .. |

[a] Unaerated cells refers to cells which had been stored under hydrogen. [b] Aerated cells refers to cells through which oxygen had been bubbled for 24 hours. [c] The cyanide concentration in these experiments was $2 \times 10^{-3}$ *M*.

**Parahydrogen Conversion by Cells in Heavy Water.**—In the conversion experiments, the liquid phase was always $H_2O$ and the gas phase consisted of two species, ortho- and parahydrogen. The resistance of the conductivity cell directly determined the concentration of parahydrogen. When the reaction is carried out in $D_2O$, the exchange reaction takes place and the gas phase contains five species, parahydrogen, orthohydrogen, HD and the equilibrium mixture of ortho and paradeuterium.[9] All these gases affect the thermal conductivity and to measure the change in parahydrogen concentration only, the effect on $R$ of the deuterium in the gas phase must be taken into account.

Two flasks containing equal amounts of bacterial suspension and phosphate buffer salts in 5 ml. of 100% $D_2O$, *p*H 6.6 (0.15 *M*) were used for an experiment. One was filled with normal hydrogen and the other with parahydrogen. The flask initially filled with normal hydrogen contained as the experiment proceeded $H_2$(n), HD and $D_2$(n) while the one initially filled with parahydrogen contained $H_2$(p), $H_2$(o), HD and $D_2$(n). Even at equal isotope concentrations the values of $R$ for the gas samples will be different (see Table V); however the

TABLE V

PARAHYDROGEN CONVERSION BY CELLS IN $D_2O$ AND $H_2O$

The cells for the $D_2O$ experiment were centrifuged, washed twice with $D_2O$ and then suspended in $D_2O$. Each flask contained 0.5 ml. of a cell suspension (3.85 mg. N/ml.).

| | Cells in $D_2O$ | | | | | | | | Cells in $H_2O$, para $H_2$ in gas phase | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gas phase | | | | | | | | | | |
| Time, min. (1) | Normal hydrogen D, % (2) | Normal hydrogen R (3) | Parahydrogen D, % (4) | Parahydrogen R (5) | R calcd.[a] normal (6) | $\Delta R$ (7) (5)-(6) | Mole fraction $H_2$, (8) | $P^c$ (9) | R (10) | $\Delta R^b$ (11) | $P^c$ (12) |
| 0 | 0.0 | 5.55 | 0 | 7.40 | 5.55 | 1.85 | 100 | 100 | 7.40 | 1.85 | 100 |
| 33 | 5.78 | 5.06 | 5.56 | 6.78 | 5.08 | 1.70 | 91.8 | 100 | 7.00 | 1.45 | 78 |
| 65 | 11.6 | 4.80 | 11.75 | 6.21 | 4.78 | 1.43 | 83.0 | 93 | 6.73 | 1.18 | 64 |
| 97 | 24.9 | 4.14 | 22.3 | 5.49 | 4.25 | 1.24 | 69.8 | 96 | 6.50 | 0.95 | 51 |
| 129 | 28.9 | 4.00 | 28.2 | 5.04 | 3.96 | 1.08 | 62.0 | 94 | 6.25 | 0.70 | 38 |
| 192 | 41.8 | 3.10 | 42.4 | 3.90 | 3.15 | 0.75 | 45.4 | 90 | 6.00 | 0.45 | 24 |

[a] The values in this column are the values of $R$ of a gas sample having the same concentration of $H_2$, HD and $D_2$, but in which the $H_2$ and $D_2$ were $H_2$(n) and $D_2$(n) rather than $H_2$(p) and $D_2$(n). These were obtained from a graph in which the data in column 2 were plotted *versus* column 3. [b] $\Delta R$ is the difference between $R$ of the conductivity cell when filled with the gas sample and when filled with normal hydrogen. [c] $P$ is the relative parahydrogen concentration (see text).

difference in the value of $R$ for the gases of the two flasks at equal isotope concentration, $\Delta R$ (see Table V, column 7) is a measure of the parahydrogen concentration. Parallel to the determinations of $R$ samples of gas were analyzed in the mass spectrometer for deuterium concentration and mole fraction $H_2$, $[H_2/(H_2 + HD + D_2)]$ (see columns 2, 4 and 8, Table V). For comparison, the results obtained with parahydrogen and the same amount of bacterial suspension in 100% $H_2O$ are included (see columns 10, 11 and 12, Table V).

As the deuterium concentration of the gas phase rises, the mole fraction of $H_2$ decreases; $H_2$ molecules are replaced by HD and $D_2$. If we define relative parahydrogen concentration, $P$, as

$$P = \frac{(H_2(p) - 25)}{(H_2(p) - 25)_{t=0}} 100$$

$P$ can be calculated from the formula[10]

$$P = \frac{100\Delta R}{(\Delta R_{t=0})\,(\text{mole fraction } H_2)}$$

where $\Delta R_{t=0}$ is the difference in resistance between para and normal hydrogen at zero time. The results of these calculations are given in Table V, column 9. They show that conversion of parahydrogen does not take place when the bacteria are suspended in $D_2O$, while it does when they are suspended in $H_2O$.

## Discussion

Reactions 1 and 2 have been postulated to account for the activity of hydrogenase.[4,5] The similarity between the kinetics of the exchange and the conversion reactions and the effect of oxygen and cyanide on these two reactions indicates that the same mechanism is operating in both reactions. However, reaction 1 must inevitably lead to the conversion of para to normal hydrogen regardless of the nature of the liquid phase, for recombination of the atomic hydrogen must yield normal hydrogen. Since hydrogenase does not catalyze the conversion of para- to normal hydrogen in $D_2O$ (see Table V)[10] we conclude that reaction 1 does not occur. Lastly, this formulation of the reactions is unsatisfactory since it fails to assign any role to the enzyme.

We can explain all the known effects of hydrogenase by assuming the following: let E represent the enzyme, then

$$H_2 + E \rightleftharpoons H{:}E^- + H^+ \qquad (3)$$

This reaction[15] will explain the ortho–para conversion in $H_2O$, for the back reaction will produce $H_2$(n). In $D_2O$, however, the $H^+$ is diluted with the potentially large amount of $D^+$ from the $D_2O$. The back reaction produces HD, not $H_2$; $H_2$(n)

$$H{:}E^- + D^+ \longrightarrow HD + E \qquad (4)$$

cannot be formed by this reaction. Reactions 3 and 4 will remove $H_2$ but will not change the $H_2$(p) concentration in the remaining gas. It will also account for the exchange reaction since the $H^+$ ion, at least, is in rapid equilibrium with the water. If the hydrogen of $H{:}E^-$ is not immediately exchangeable with water, this postulated mechanism requires that the rate of the conversion reaction be twice that of the exchange reaction, disregarding differences in zero point energy. This follows for, whenever a hydrogen molecule interacts with the enzyme and reforms, the nuclear spins will be completely randomized, *i.e.*, every time a $H_2$(p) reacts with the enzyme it will yield $H_2$(n). However when a hydrogen molecule interacts with the enzyme and $D_2O$ it can at the most, in one elementary reaction, return to the gas phase as HD, *i.e.*, only one half of the hydrogen molecule will come to equilibrium with the water. Therefore, each molecule of hydrogen must react at least twice with the enzyme before it comes to equilibrium with water.

Under those conditions in which the rate of the reaction is determined by enzyme concentration (see initial parts of curves in Figs. 3 and 4), the rate of the conversion is in fact about three times that of the exchange reaction.

According to our proposed mechanism, the reduction of the various substrates, carried out by hydrogenase, is effected by the hydride of the enzyme ($H{:}E^-$). For the reduction of fumarate the reaction would be

$$H{:}E^- + \text{fumarate} + H^+ \longrightarrow \text{succinate} + E \qquad (5)$$

In support of this formulation are the results of Farkas and Schneidmesser[16] who found, in a system containing *B. coli*, $H_2O$, $D_2$ gas and fumarate, that a deuterosuccinate was formed. The hydrogen atoms added to the double bond contained 20% of

(15) There are several equivalent methods of formulating this reaction. For example if we write the enzyme as E:OH then $E{:}OH + H_2 \rightleftarrows H{:}E + H_2O$.

(16) L. Farkas and B. Schneidmesser, *J. Biol. Chem.*, **167**, 807 (1947).

the deuterium concentration of the gas phase. It is clear that the activated hydrogen produced by hydrogenase is not in immediate equilibrium with the water. This is consistent with our view of the hydrogenation process since we postulate that only one out of the two hydrogen atoms of the hydrogen molecule which interacts with the enzyme is immediately exchangeable with water. The amount of deuterium which will appear in the reduced compound will depend on the relative rates of reactions 3 and 5.

Hoberman and Rittenberg[7] have adduced evidence that hydrogenase is a ferrous protein complex and Waring and Werkman[17] have shown that hydrogenase activity is absent in bacteria grown in an iron-free medium.

There exist certain analogies to this enzyme. For example $Co_2(CO)_8$ undergoes hydrogenolysis to form a hydride. The reaction is reversible. Since

$$Co_2(CO)_8 + H_2 \rightleftarrows 2HCo(CO)_4 \quad (6)$$

the hydrogen of $HCo(CO)_4$ is acidic and presumably, rapidly exchangeable with the hydrogen of water, the reaction should catalyze the exchange of hydrogen in the gas phase with hydrogen of the water. In addition, the hydride will reduce methylene blue.[18]

Any organism which possesses hydrogenase activity should be capable of carrying out both the conversion of parahydrogen and the exchange reaction. We have found that suspensions of Scenedesmus D-3 and Euglena,[19] which have been kept under hydrogen so as to activate the hydrogenase system,[20] catalyze both these reactions.

(17) W. S. Waring and C. H. Werkman, *Arch. Biochem.*, **4**, 75 (1944).

(18) H. J. Emeléus and J. S. Anderson, "Modern Aspects of Inorganic Chemistry," 2nd edition, D. Van Nostrand Co., New York, N. Y., 1952, p. 426.

(19) Kindly supplied by Dr. Seymour Hutner of the Haskins Laboratories, N. Y. C.

(20) H. Gaffron, *J. Gen. Physiol.*, **26**, 195 (1942).

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# The Oxygenation Reaction of Hexaarylethanes.[1] I. The Stoichiometry of the Reaction of Hexaphenylethane

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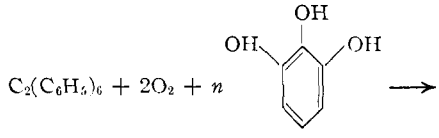
The stoichiometry of the reaction of hexaphenylethane (I) with oxygen has been investigated in the presence and absence of pyrogallol by using numerous solid samples of identical history. In bromobenzene solution, in the absence of pyrogallol, with simultaneous dissolution of sample and reaction, consumption of oxygen decreases with increasing temperature and decreasing pressure of oxygen and, with air as reagent, appears to reach a limiting value in the vicinity of 25–30°. With samples of different history this limiting value was found to approach but never to exceed that expected on the basis of equation 1. Even under these conditions, however, bis-trityl peroxide (II) is not the sole product. The consumption of oxygen in the presence of pyrogallol was found to fall short of that expected on the basis of equation 2 in all experiments where dissolution of I and exposure to oxygen were simultaneous, regardless of variation in temperature, oxygen pressure, solvent and auxiliary reagents. The results of one set of experiments suggest that agreement is obtained if dissolution precedes reaction.

## Introduction

That the reaction of hexaphenylethane (I) and related substances with oxygen is not a simple one has been recognized for some time. Thus, quantitative yields of bis-trityl peroxide[2] (II) and its congeners[3] have not been obtained. Furthermore, although Gomberg and his co-workers[4] as well as numerous other workers[5] employed oxygen absorption as a means of analyzing I and its congeners, several workers have reported that adherence to the stoichiometry exemplified by equation 1 is not exact and depends on such variables as nature of the solvent,[2b] structure of the ethane,[3] pressure of oxygen,[6,7] temperature[6] and ethane concentration.[6] Pyrogallol is particularly effective[7,8] in altering the stoichiometry and Ziegler and his associates have collected considerable evidence[9] in support of equation 2. Oxygen consumption falling short of that expected from 2 has been

$$C_2(C_6H_5)_6 + O_2 \longrightarrow C_2(C_6H_5)_6 \cdot O_2 \quad (1)$$




$$2(C_6H_5)_3COOH + \text{unknown products} \quad (2)$$

observed, however,[10] and the suggestion made that this might be due to dissolution of I and oxygenation being simultaneous. In addition, it has been established[11] that triphenylmethyl can initiate the autoxidation of readily oxidized aldehydes and olefins.

Ziegler and his associates have deduced from the kinetics[12] of the oxygenation of I that the reaction

(1) Presented in preliminary form before the Division of Organic Chemistry at the 123rd Meeting of the American Chemical Society, Los Angeles, March, 1953. *Cf.* p. 54 M of the Abstracts of Papers.

(2) (a) M. Gomberg, *Ber.*, **34**, 2726 (1901); (b) M. Gomberg and L. H. Cone, *ibid.*, **37**, 3538 (1904).

(3) J. B. Conant and M. W. Evans, THIS JOURNAL, **51**, 1925 (1929).

(4) M. Gomberg and C. S. Schoepfle, *ibid.*, **39**, 1652 (1917).

(5) (a) P. Walden, "Chemie der Freien Radikale," S. Hirzel, Leipzig, 1924, p. 87; (b) H. E. Bent, G. R. Cuthbertson, M. Dorfman and R. E. Leary, THIS JOURNAL, **58**, 165 (1936).

(6) K. Ziegler, L. Ewald and Ph. Orth, *Ann.*, **479**, 277 (1930).

(7) W. E. Bachmann and F. V. Wiselogle, *J. Org. Chem.*, **1**, 364 (1937).

(8) (a) H. W. Scherp, THIS JOURNAL, **58**, 576 (1936); (b) K. Ziegler and G. Ganicke, *Ann.*, **551**, 220 (1942).

(9) K. Ziegler, *Ann.*, **551**, 135 (1942).

(10) H. E. Bent and J. E. Cline, THIS JOURNAL, **58**, 1626 (1936).

(11) K. Ziegler and L. Ewald, *Ann.*, **504**, 162 (1933).

(12) *Cf.* W. A. Waters, "Chemistry of Free Radicals," Oxford University Press, London, 1948, pp. 45–49.