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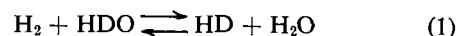
Catalysis of the H₂-HTO Exchange by Hydrogenase. A New Assay for Hydrogenase*

S. R. Anand and Alvin I. Krasna

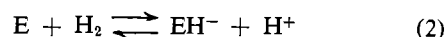
ABSTRACT: A method is described for the assay of hydrogenase activity by its ability to catalyze the reaction $\text{H}_2 + \text{HTO} \rightleftharpoons \text{HT} + \text{H}_2\text{O}$. The assay measures the rate of appearance of HT in the gas phase by determining its radioactivity in an ionization chamber. The effects of sample size, filling gas pressure, and enzyme concentration are studied.

The enzyme hydrogenase, which activates molecular hydrogen, can be assayed by three general methods. The most commonly used procedure measures the rate of reduction of some acceptor, organic or inorganic, by hydrogen gas. The reduction is followed by measuring the rate of hydrogen uptake or the change in concentration of the acceptor. Some of the acceptors often used are: methylene blue (Stephenson and Stickland, 1931), benzyl viologen (Krasna and Rittenberg, 1956), oxygen (Wilson *et al.*, 1942), nitrate (Krasna and Rittenberg, 1954a), ferricyanide (Hyndman *et al.*, 1953), and fumarate (Farkas and Fischer, 1947). A second method measures the rate of hydrogen evolution from compounds which have a lower oxidation-reduction potential than hydrogen, such as reduced methyl viologen (Tamiya *et al.*, 1955), reduced benzyl viologen (Krasna and Rittenberg, 1956), and malate (Gest *et al.*, 1962). Both these methods measure the resultant of at least two consecutive reactions, *i.e.*, the activation of molecular hydrogen and the transfer of the activated hydrogen to the electron acceptor. Therefore, the rate of reduction of an acceptor is not necessarily a direct measure of the activation of hydrogen, since other enzyme systems or electron carriers may be involved in the utilization of the activated hydrogen for the chemical reduction. This has been clearly demonstrated by many authors (Krasna and Rittenberg, 1956; Hoberman and Ritten-

berg, 1943; Curtis and Ordal, 1954; Whitely and Ordal, 1955).
A third assay method for hydrogenase which we have used extensively depends on its ability to catalyze the exchange reactions between hydrogen and heavy water, *i.e.*



This reaction was first used by Farkas *et al.* (1934) and studied later in great detail by Hoberman and Rittenberg (1943). Hydrogenase was also shown (Krasna and Rittenberg, 1954b) to catalyze the conversion of para- to orthohydrogen and, on the basis of this and the deuterium exchange, the mechanism of activation of hydrogen by hydrogenase was postulated to be a heterolytic split of hydrogen to a hydride and a proton, *i.e.*



where E represents the enzyme. These two exchange reactions do not require the addition of any electron acceptors or cofactors and, therefore, directly measure the rate of reaction of hydrogen with the enzyme.

The deuterium exchange assay is very rapid and accurate but requires the use of a mass spectrometer which is not always available. The parahydrogen assay is slow and not very sensitive. We therefore investigated the use of tritium exchange as an assay for hydrogenase. Recently (Goldsby, 1961; Gingras *et al.*, 1963) hydrogenase has been assayed by studying the

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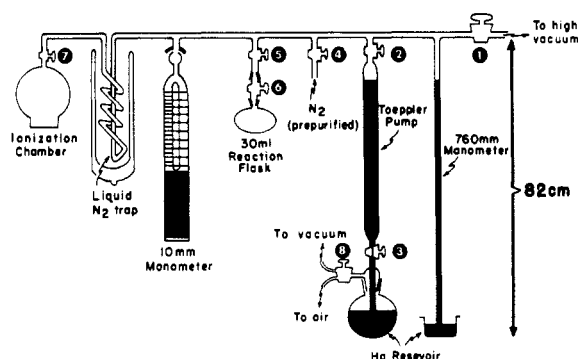
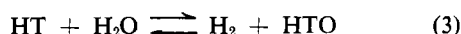


FIGURE 1: Vacuum system for determination of tritium radioactivity in an ionization chamber.

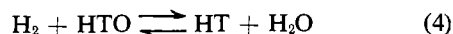
exchange reaction



With tritium gas as a tracer in the gas phase, samples of the reaction mixture were removed at different time intervals, the reaction was stopped by methanol, and the solution was counted directly in a liquid scintillation counter. This method would seem to be rather slow and cumbersome, requiring removal of liquid samples anaerobically, addition of methanol and other reagents for scintillation counting, followed by the counting procedure itself. It does not appear to be suitable for the rapid routine assay of a large number of samples.

A more convenient and rapid assay suitable for routine use would be the analysis of the tritium content of the gas phase rather than the liquid phase, similar to what is done in the deuterium assay. Black and Taylor (1943) determined the equilibrium constant for reaction 3 in the vapor phase catalyzed by platinum-charcoal catalysts. They determined the tritium content of both HT and HTO vapor by counting the gas sample in a Geiger-Mueller tube with alcohol, water vapor, hydrogen, and argon as the counting mixture.

This report describes a new assay for hydrogenase which measures its catalysis of the exchange reaction



The reaction is followed by measuring the tritium content of the gas phase without disturbing the liquid phase. The tritium content of the gas phase is determined in an ionization chamber, and the signal produced is amplified in a vibrating-reed electrometer. A comparison is made between the kinetics of this reaction and that of reaction 1 to determine the isotope effect in the reaction of hydrogen with water as catalyzed by hydrogenase.

Experimental Section

The source of hydrogenase in this study was whole cells of either *Proteus vulgaris* or *Scenedesmus obliquus*

393. *Proteus* cells were grown in a complex media as previously described (Krasna and Rittenberg, 1954b) and *Scenedesmus* cells were grown photosynthetically on CO_2 and inorganic salts (Hartman and Krasna, 1963). The platinum oxide and platinum tetrachloride were purchased from Baker and Co., Newark, N. J. The D_2O was purchased from the Liquid Carbonic Division of General Dynamics Corp., San Carlos, Calif., and the T_2O from New England Nuclear Corp., Boston, Mass. Oxygen-free hydrogen was prepared by passing commercial prepurified hydrogen over hot copper.

The exchange reactions were carried out in 30-ml flasks similar to those described by Hoberman and Rittenberg (1943). The flask contained 0.5 ml of D_2O (>99.5%) or HTO of appropriate specific activity, cells of *Proteus* or *Scenedesmus* in a volume of 0.1 to 2 ml, and 0.15 M PO_4 buffer, pH 6.7, to make the final volume 5 ml. The flask was cooled in ice water for 10 min, evacuated for 10 min with a mercury diffusion pump, and then filled with oxygen-free hydrogen. The flasks were shaken at 25° and gas samples were removed at about 0.5-hr intervals for analysis of deuterium or tritium content. Deuterium analyses were carried out on a mass spectrometer by measuring the HD (mass 3) content of the gas phase.

Tritium analyses were carried out by measuring its radioactivity in a gas ionization chamber with prepurified nitrogen as the filling gas. The use of an ionization chamber for the assay of radioactive gases is described by Tolbert *et al.* (1956). The ionization chamber (275-ml volume) was of the Cary-Tolbert type and was purchased from Applied Physics Corp., Pasadena, Calif. The outside wall of the ionization chamber was kept at a negative voltage of 90 v with a dry cell battery. The current developed in the ionization chamber was fed through a 10^{12} ohm resistor to a Model 31 vibrating reed electrometer (Applied Physics Corp.) which was critically damped for this resistor. The electrometer was equipped with a shorting switch to ground to adjust the zero of the instrument. The output of the electrometer was fed to a 10-mv Elektronik 15 potentiometer recorder (Minneapolis-Honeywell Reg. Co., Brown Instrument Division, Philadelphia, Pa.) so that the equilibrium voltage could be measured.

The design of the vacuum system used with the ionization chamber was dictated by two important considerations. First, the gas sample as it is removed from the reaction flask is saturated with water vapor which contains large amounts of tritium relative to that expected in the hydrogen gas. Therefore, all traces of water vapor must be removed before the gas sample is introduced into the ionization chamber. Second, since we are measuring the quantity of tritium in a gas sample rather than its per cent abundance, the amount of sample introduced into the ionization chamber must always be the same, *i.e.*, in a fixed volume, its pressure must be controlled very accurately and reproducibly.

A sketch of the most importance features of the vacuum system is shown in Figure 1. Stopcock 1 connects the vacuum system to the vacuum manifold

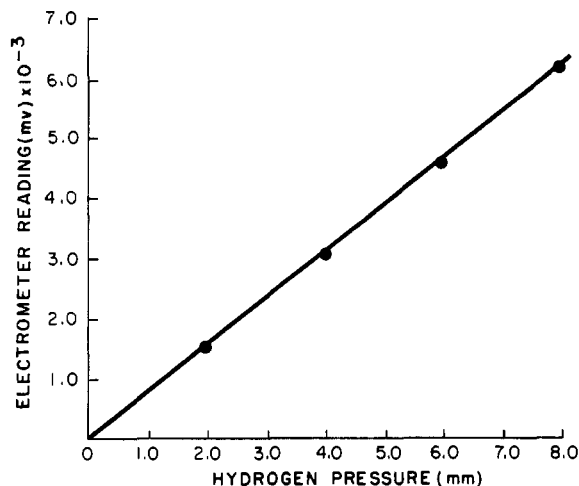


FIGURE 2: Relationship between the pressure of the hydrogen sample and the electrometer reading. The flask contained platinum catalyst in buffer and the HTO concentration was 1 mcurie/ml. The filling N_2 gas was adjusted to a pressure of 300 mm.

containing a liquid N_2 trap to condense all radioactive water, a vacuum gauge, a mercury diffusion pump, and a mechanical pump. The complete system achieves a vacuum better than 10^{-6} mm. The volume of the glass system with all stopcocks closed is about 185 ml. The Toeppler pump is used to adjust the pressure of the gas sample to any desired value which is measured on the 10-mm manometer to ± 0.05 mm (Model C-1300, Roger Gilmont Instruments, Inc., Great Neck, N. Y.). The coiled liquid nitrogen trap is essential to freeze out all the water vapor in the system. Stopcock 7 connects directly to the ionization chamber and is used in place of the metal valve supplied with the chamber. The 760-mm manometer is to measure the pressure of the filling N_2 gas. The volume between stopcocks 5 and 6 (ca. 0.7 ml) is the sampling volume. Though the reaction flasks could be evacuated and filled with hydrogen on this vacuum system, it is usually done on an auxiliary system which can evacuate seven flasks at once.

A typical sample analysis is carried out as follows. The vacuum system is always kept under vacuum with stopcocks 1, 2, and 7 open. When the vacuum is 2μ or less, the system is ready for use. The reaction flask is attached to the $10/30$ female joint at stopcock 5 and stopcock 5 is then opened until the pressure again falls to 2μ (ca. 60 sec). Stopcocks 1, 5, and 7 are closed, and the sample is introduced into the system by opening stopcock 6 and closing it again and then opening stopcock 5 and closing it again. The reaction flask is then returned to the shaking apparatus. To efficiently condense all the water vapor, the gas is expanded and condensed three times by alternately opening and closing stopcock 8. Finally, the height of the Toeppler column is adjusted with stopcock 3 so that the pressure on the 10-mm manometer reads 8 mm exactly. (This pressure can be varied as desired; see later.) At this point, stop-

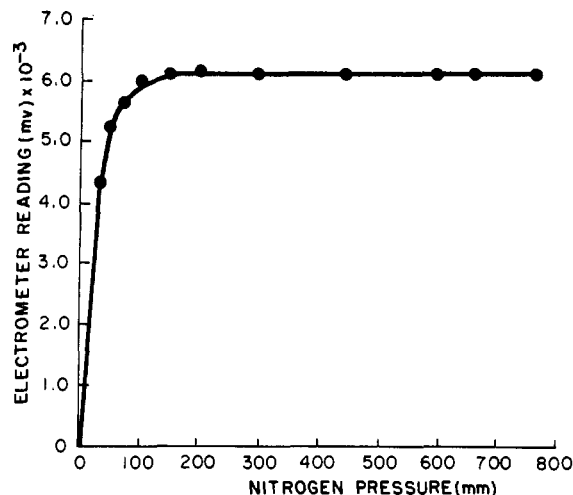


FIGURE 3: Relationship between the pressure of the nitrogen filling gas and the electrometer reading. The flask contents were the same as used in Figure 2. The hydrogen sample pressure was 8 mm in all cases.

cock 2 is closed so that the volume of the system is constant, and the sample is introduced into the ionization chamber by opening stopcock 7. Prepurified nitrogen is then admitted through stopcock 4 until the pressure on the 760-mm manometer reaches 500 mm or any desired value (see later). Stopcocks 4 and 7 are then closed, and the glass system is evacuated by opening stopcock 1. The current developed in the ionization chamber is determined, and the chamber is then evacuated by opening stopcock 7.

With the sample in the ionization chamber, the electrometer is first shorted to get a zero reading. Then the 10^{12} ohm resistor is switched into the circuit and the voltage developed is plotted on the recorder. If desired, the zero reading can again be determined followed by another sample reading.

In the initial experiments, air was used as the filling gas and gave erratic results. Use of prepurified nitrogen instead of air corrected the difficulty, which may suggest that the moisture in air is not being condensed rapidly enough and is entering the ionization chamber. A sample of H_2 in contact with HTO gave the same reading as a dry sample of H_2 , showing that the removal of water vapor from the sample is complete. The reading for H_2 gas itself has been subtracted from all values reported.

The radioactivity of the tritium gas is obtained in units of millivolts, and all the results are plotted in these units.

The method used to measure the current in the ionization chamber with a high resistance is quite rapid. A much slower method is to use no resistance but measure the rate of charge buildup in the condenser of the electrometer. When this method was tried it gave results identical with those of the high resistance leak method, but because of the rapidity of

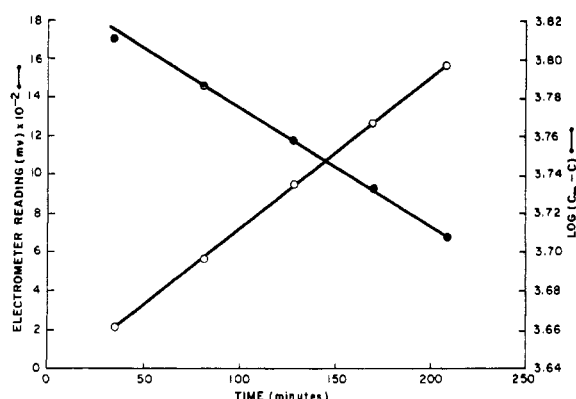


FIGURE 4: Catalysis of tritium exchange reaction by the hydrogenase of *Proteus vulgaris*. The data are plotted both as zero order (O—O; left-hand ordinate) and as first order (●—●; right-hand ordinate). C is the millivolt value at the indicated time and C_{∞} is the millivolt value at equilibrium. The zero-order rate is 498 mv/hr and the first-order rate constant, k_{exp} , is 1.49×10^{-3} .

this latter method it was used in this investigation.

When the tritium content of a water sample was desired, the water was converted to hydrogen gas by reduction with zinc dust (San Pietro, 1957) and the gas was analyzed as described above.

Results

Determination of Optimum Conditions for Tritium Counting. To find the optimum conditions for tritium counting, PtCl_4 or PtO_2 (10–15 mg) was shaken with the buffer containing HTO in an atmosphere of hydrogen gas for 24 hr. Equilibrium is reached under these conditions, and the tritium content of the gas phase is the maximum obtainable with any given HTO concentration. The quantity of tritiated water used should be the smallest amount necessary to give a reasonable value in the gas phase when it contains only 1% of that expected at equilibrium. By determining the equilibrium gas value with water of differing tritium concentrations, it was decided to routinely use a concentration of 1 mcurie/ml of tritium in the liquid phase.

Figure 2 shows the linearity between the pressure of the hydrogen sample and the electrometer reading in millivolts. Removal of a single sample from the reaction flask as described in the Experimental Section gives a sufficient quantity of gas so that its pressure can be adjusted to give 8 mm even after six samples are removed from the reaction flask. This pressure was routinely used.

The relationship between the pressure of the pre-purified N_2 gas used for filling the ionization chamber and the electrometer reading is plotted in Figure 3. It is evident that any pressure above 150–200 mm will yield reproducible results. For routine analysis, N_2 is added to a final pressure of ca. 300 mm, which does not have to be measured very accurately.

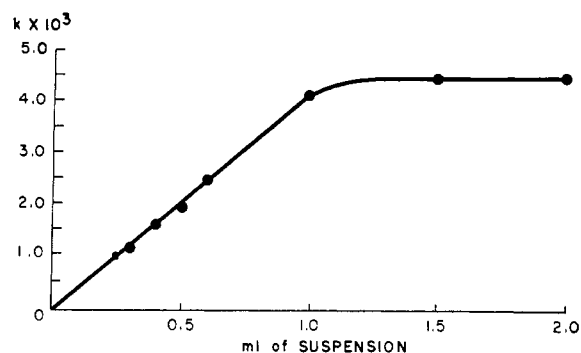


FIGURE 5: Effect of hydrogenase concentration on the rate of the tritium exchange. *Scenedesmus* cells were used in this experiment at a concentration of 0.166 ml of wet packed cells per ml of suspension. The rate is expressed as the first-order rate constant, k_{exp} .

From the above experiments, the following conditions were then routinely used for all tritium assays: tritium concentration in liquid phase of 1.0 mcurie/ml, pressure of 8 mm of hydrogen gas for each analysis, filling ionization chamber with prepurified N_2 to a final pressure of ca. 300 mm.

Equilibrium Constant for the Exchange Reaction. The equilibrium constant for the reaction studied, eq 4, is

$$K = \frac{(\text{HT})/(\text{H}_2)}{(\text{HTO})/(\text{H}_2\text{O})} \quad (5)$$

Since the actual mole fraction of tritium present in a solution containing 1 mcurie/ml is of the order of 10^{-7} , the mole fraction of H_2 and H_2O can be taken as unity and the equilibrium constant can be taken as

$$K = \frac{(\text{HT})}{(\text{HTO})} \quad (6)$$

Samples which had reached equilibrium were analyzed for the tritium content of the hydrogen gas and of the water by converting the water to hydrogen with Zn dust. Both hydrogen samples were analyzed at the same gas pressure under identical conditions. With 1 mcurie/ml in the liquid phase, an average of six experiments gave the following values: H_2 gas = 6681 mv, H_2 gas from H_2O = 40,668 mv, $K_{25^\circ} = 0.164$. With 2 mcuries/ml of tritium the values were: H_2 gas = 13,335 mv, H_2 gas from H_2O = 79,710 mv, $K_{25^\circ} = 0.167$. These values for the equilibrium constant were obtained whether the exchange was catalyzed by platinum, *Proteus vulgaris*, or *Scenedesmus obliquus*. In a study of this exchange reaction between HT gas and water vapor catalyzed by platinum-charcoal catalysts where the tritium was detected in a Geiger-Mueller tube. Black and Taylor (1943) obtained the same value for the equilibrium constant.

Catalysis of the Exchange Reaction by Hydrogenase. The kinetics for a typical tritium exchange reaction

TABLE I: Comparison of Zero- and First-Order Rate Constants for Deuterium and Tritium Exchange Reactions.

	Proteus Concn (ml)	D Exchange		T Exchange		T Exchange/D Exchange	
		Zero Order (% D/hr)	First Order ($k_{\text{exp}} \times 10^3$)	Zero Order (mv/hr)	First Order ($k_{\text{exp}} \times 10^3$)	Zero Order (mv/% D)	First Order
1	0.04	0.147	0.81	297	0.81	2020	1.00
2	0.06	0.216	1.19	419	1.12	1940	0.94
3	0.08	0.258	1.49	498	1.49	1930	1.00

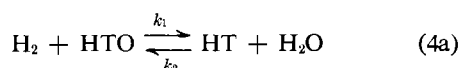
catalyzed by the hydrogenase of *Proteus vulgaris* is shown in Figure 4. The results are plotted both as a zero-order plot, millivolts vs. time, and as a first-order plot, $\log (C_\infty - C)$ vs. time, where C is the millivolt value at any time and C_∞ is the millivolt value at equilibrium. Though the reaction is first order, if the enzyme concentration is low so that the over-all rate is low and we are measuring initial rates, then the zero-order rate is a convenient measure of the enzyme concentration.

The effect of hydrogenase concentration on the rate of the tritium exchange reaction is shown in Figure 5. Similar curves are obtained for the deuterium exchange and parahydrogen conversion (Krasna and Rittenberg, 1954b). The nonlinearity at high cell concentration is due to the limiting rate of diffusion of hydrogen into and out of the liquid phase (Krasna and Rittenberg, 1954b).

Comparison of the Hydrogenase-Catalyzed Exchange Reaction of H_2 with HDO and HTO. To determine the isotope effect in the hydrogenase-catalyzed exchange reaction, the exchange was carried out at 25° with the same quantity of *Proteus* cells in both 18% HDO and in HTO containing 1 mcurie/ml. For each reaction, both the zero-order rate constant and the first-order rate constant were determined, and the values are given in Table I. The ratio of the zero-order rate constants at different cell concentrations is a constant, and a deuterium exchange rate in 18% HDO of 1 atom % D per hour is equal to a tritium exchange rate of 1960 mv/hr when the liquid phase contains 1 mcurie/ml. These two values are defined as one unit of hydrogenase activity (Krasna *et al.*, 1960).

The first-order rate constants, k_{exp} , for both reactions are the same, but since this rate constant measures both the forward and reverse rates of the exchange reactions it is necessary to know the separate rate constants for the forward and reverse steps of the exchange reaction in order to determine the isotope effect. These individual rate constants can be evaluated from the determined k_{exp} values in the following manner. The equations are written with T as the isotope but apply as well with D as the isotope.

Consider the reaction



which is a second-order reaction. Since the HTO and H_2O concentrations do not change during the course of the reaction we can include these concentrations in the rate constants and consider the reaction as a pseudo-first-order reaction, *i.e.*



where

$$k_1' = k_1(HTO) \quad (8)$$

$$k_2' = k_2(H_2O) \quad (9)$$

Let the concentration of H_2 at any time, t , be represented by (C_a) and the concentration of HT at any time, t , be represented by (C). Then the total hydrogen concentration of both species can be represented by $C_0 = C_a + C$. The rate of appearance of HT will be

$$\frac{d(C)}{dt} = k_1'(C_a) - k_2'(C) \quad (10)$$

or

$$\frac{d(C)}{dt} = k_1'(C_0 - C) - k_2'(C) \quad (11)$$

At equilibrium, the concentration of HT is represented by (C_∞) and, since the net rate at equilibrium is zero, it can be shown that

$$k_2' = k_1' \frac{(C_0 - C_\infty)}{(C_\infty)} \quad (12)$$

Substituting this value for k_2' in eq 11 gives

$$\frac{d(C)}{(C_\infty - C)} = k_1' \frac{(C_0)}{(C_\infty)} dt \quad (13)$$

which, on integration between the limits $t = 0$ and $t = t$, gives

$$\ln \frac{(C_\infty - C)}{(C_\infty)} = -k_1' \frac{(C_0)}{(C_\infty)} t \quad (14)$$

TABLE II: Comparison of Second-Order Rate Constants for Deuterium and Tritium Exchange.^a

	Second-Order k_1		Second-Order k_2		T Exchange/D Exchange	
	D Exchange ($k_1 \times 10^6$)	T Exchange ($k_1 \times 10^6$)	D Exchange ($k_2 \times 10^6$)	T Exchange ($k_2 \times 10^6$)	(k_1)	(k_2)
1	3.0	1.4	9.3	8.1	0.47	0.87
2	4.4	1.9	13.7	11.2	0.43	0.82
3	5.5	2.5	17.2	14.9	0.45	0.87

^a The values in this table were calculated from the k_{exp} values in Table I.

The concentrations of the different isotopic species are expressed in units of per cent so that in these experiments $C_0 = 100$. In practice, since (C_∞) is a constant, we plot $\log (C_\infty - C)$ vs. time to give a rate constant, k_{exp} . This rate constant, k_{exp} , is equal to $k_1' + k_2'$. From eq 12, k_1' can be evaluated from k_{exp} by the relationship

$$k_1' = k_{exp} \frac{(C_\infty)}{(C_0)} \quad (15)$$

From eq 12 it can be seen that k_2' can also be evaluated from k_{exp} by the relationship

$$k_2' = k_1' \frac{(C_0)}{(C_\infty)} - k_1' = k_{exp} - k_1' \quad (16)$$

Knowing the values of k_1' and k_2' and the concentrations of H_2O and HTO (and HDO in the deuterium exchange), the values of the individual second-order rate constants, k_1 and k_2 , can be evaluated according to eq 8 and 9. To compare the deuterium and tritium exchanges, the tritium values in units of millivolts were converted to atom per cent values from the determined millivolt reading of the hydrogen produced from a known concentration of HTO , i.e., 1 or 2 mcuries/ml, and from the known half-life of tritium. For the tritium experiment, (C_∞) is 6680 mv ($= 1.030 \times 10^{-5}\%$ HT) and 1 mcurie/ml of HTO gave hydrogen gas with an activity of 40,668 mv ($= 6.18 \times 10^{-5}\%$ HTO). For the deuterium experiment, where the HDO concentration is 18%, (C_∞) is 6.6%.

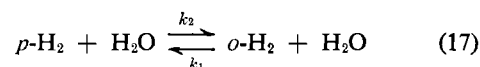
The calculated values for k_1 and k_2 are given in Table II. The K_{eq} for reaction 4a can be evaluated from a knowledge of k_2 and k_1 since $K_{eq} = k_1/k_2$. For the T exchange, the K_{eq} at 25° is 0.167, which is the value determined experimentally (see above). For the D exchange, the K_{eq} at 25° is 0.318, which is also the experimentally determined value (Krasna and Rittenberg, 1954b). The value of k_2 is approximately the same (85%) for the T exchange as it is for the D exchange, whereas k_1 for the D exchange is greater by a factor of 2.2 than k_1 for the T exchange. Thus, the reaction in which the isotope effect manifests itself is not in the splitting of the hydrogen molecule (HT compared with HD) but, rather, in the reaction of the proton of water

(T^+ compared with D^+) with the activated hydrogen.

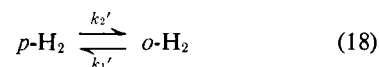
Discussion

Analysis of the kinetics of the hydrogenase-catalyzed exchange reaction between hydrogen gas and deuterated or tritiated water has shown that there is no difference between the rate of cleavage of HT compared with HD, whereas the rate of D^+ reacting with activated H_2 is twice the rate of T^+ reacting with H_2 . Here we have determined the isotope effect between D and T, and it would be of interest to compare the isotope effect between H and D. Experimentally this can be done by a comparison of the deuterium exchange with the parahydrogen conversion in H_2O . We have previously measured the over-all pseudo-first-order kinetics of these two reactions (Krasna and Rittenberg, 1954b) and found that the rate constant for the parahydrogen conversion is about three times that of the deuterium exchange. A comparison of the individual second-order rate constants, k_1 and k_2 , follows.

Since it has been shown (Krasna and Rittenberg, 1954b) that the hydrogenase-catalyzed parahydrogen conversion takes place by interaction with H_2O , the reaction can be represented in second-order form as



Since the H_2O concentration remains constant in this reaction, its concentration can be included in the second-order rate constant to give a pseudo-first-order reaction



where

$$k_1' = k_1(H_2O) \quad (19)$$

$$k_2' = k_2(H_2O) \quad (20)$$

If we let C = concentration of $p\text{-}H_2$ at any time t ,

TABLE III: Comparison of Deuterium Exchange and *p*-H₂ Conversion.

D Exchange			<i>p</i> -H ₂ Conversion			D Exchange/ <i>p</i> -H ₂ Conversion	
($k_{\text{exp}} \times 10^3$)	($k_1 \times 10^6$)	($k_2 \times 10^6$)	($k_{\text{exp}} \times 10^3$)	($k_1 \times 10^6$)	($k_2 \times 10^6$)	(k_1)	(k_2)
0.40	1.5	4.6	1.2	3.0	9.0	0.50	0.51
0.85	3.1	9.8	2.4	6.0	18.0	0.52	0.54
1.30	4.8	14.9	3.6	9.0	27.0	0.53	0.55

C_{∞} = concentration of *p*-H₂ at equilibrium, and C_0 be the total concentration of both *o*- and *p*-H₂, then the rate equation 14 will apply to the *p*-H₂-conversion reaction and the rate constants can be evaluated from eq 15, 16, 19, and 20. The concentrations of para- and orthohydrogen are expressed in units of per cent so (C_0) = 100, (H_2O) = 100, and (C_{∞}) = 25 (this is the experimental and theoretical value).

In Table III are given the values for k_{exp} , k_1 , and k_2 for the deuterium exchange and parahydrogen conversion at different concentrations of *Proteus* cells. The conversion and exchange in each line were done at identical cell concentrations. It is clear that for the conversion reaction both k_1 and k_2 are about two times greater than k_1 and k_2 for the deuterium exchange. The fact that k_1 is greater is not surprising, since k_1 for D exchange is greater than k_1 for T exchange, and one would also expect an isotope effect between H and D. However, in view of the fact that k_2 was the same for D exchange and T exchange, it is surprising that k_2 for conversion is greater than k_2 for D exchange. This result would suggest that there is an isotope effect in the splitting of HH compared to HD, but no effect in the splitting of HD compared to HT.

Acknowledgments

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