KINETIC DEUTERIUM ISOTOPE EFFECTS IN ENZYMIC FORMATE OXIDATION

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INTRODUCTION

Formate, one of the products of one-carbon-fragment metabolism, is found in small quantities in almost all living matter. Enzyme systems which oxidize formate or formic acid are present in mammalian cells, in plant tissues, and in bacteria. Although these enzymes have the same substrate, they are different in their composition and mechanism of action. In every instance, however, the hydrogen atom must be removed from the carbon atom in order to yield CO_2 or HCO_3^- as a reaction product. It is known that in formate oxidation by alkaline permanganate, there is a considerable deuterium isotope effect[1, 2] ranging from 6 to 10. Therefore, it is of interest to compare the activity of these three enzymes towards ordinary formate ($^{14}HCOO^-$) and heavy formate ($^{14}DCOO^-$). For this purpose, ordinary ^{14}C-formate and fully deuterated ^{14}C-formate, doubly-labelled *intra*molecularly was used, in order to exclude any ^{12}C–^{14}C-isotope effect. The deuterated formate was prepared by hydrolysis of ^{14}C-cyanide in heavy water, as described previously[3]. Formate oxidation in presence of the following enzyme systems has been tested: (*i*) formicodehydrogenase from *Phaseolus*; (*ii*) catalase from beef liver and (*iii*) formicodehydrogenase from *E. coli*. As will be demonstrated in this paper there is a considerable deuterium isotope effect in the plant enzyme. There may be an isotope effect in the catalase system, depending on experimental conditions. No isotope effect can be observed using the enzyme system of bacterial origin. Biological aspects and implications have already been discussed elsewhere[4] and in this comparative study it is the enzyme kinetic findings which will be presented and discussed.

FORMATE OXIDATION BY PLANT FORMICODEHYDROGENASE, ISOLATED FROM *PHASEOLUS MULTIFLORUS* L.

The activity of this enzyme has been measured either (*a*) manometrically or (*b*) photometrically. In the former method at least three reaction steps *A*, *B* and *C* are involved, whereas in the latter only the first step *A*, is followed.

$$A: \quad NAD^+ + HCOO^- \rightarrow NAD{-}H + CO_2$$
$$B: \quad NAD{-}H + R^+ \rightarrow NAD^+ + R{-}H$$
$$C: \quad R{-}H + \tfrac{1}{2}O_2 + H^+ \rightarrow R^+ + H_2O$$

(NAD = Nicotinamide–adenine–dinucleotide)
(R = Redox-substance, *e.g.* methylene blue)

Details of the methods used have been described previously[5]. As a measure of enzyme activity (*a*) oxygen consumption in presence of excess formate (0·06 M), NAD (0·05 per cent O) and methylene blue (0·05 per cent) or (*b*) NAD-reduction in presence of excess formate (0·05 M) and NAD (0·04 per cent) have been taken. In both instances D-formate is oxidized at a considerably lower rate, *e.g.*, at 27° the ratio of the initial reaction velocities amounts to $k_H/k_D = 2{\cdot}5$. From the enzymological point of view there are two possibilities, which might explain this difference in reaction rate: Either D-formate has a lower affinity than ordinary formate for the active site of the enzyme molecule ($K_{s(H)} < K_{s(D)}$), or the enzyme–substrate complex is formed or decomposed at a lower rate ($V_H > V_D$). In order to decide whether this is due to a difference in affinity or in maximal velocity three types of experiments have been performed:

Determination of the substrate constant (K_s) of formicodehydrogenase for $HCOO^-$ and for $DCOO^-$

For this purpose the initial reaction rates were measured at various substrate concentrations (HCOONa or DCOONa) and the corresponding substrate constants determined graphically by means of a Lineweaver–Burk plot. Based on 5 independent determinations done at 27° using the photometric technique, the following mean figures have been obtained $K_{s(H)} = 2{\cdot}7 \times 10^{-3}$ M (range $2{\cdot}6 - 2{\cdot}8 \times 10^{-3}$ M); $K_{s(D)} = 3{\cdot}0 \times 10^{-3}$ M (range $2{\cdot}8 - 3{\cdot}2 \times 10^{-3}$ M). Thus, there is only a small difference in respect to affinity, corresponding to a ratio of about $K_{s(D)}/K_{s(H)} = 1{\cdot}1$. Whereas the absolute values depend on temperature (*e.g.* they are about 20 per cent smaller at 17°), the ratio remains the same.

Oxidation of substrate mixtures (HCOONa + DCOONa)

Provided that the enzyme is saturated with substrate, there is a linear relationship between initial reaction rate and composition of the mixed substrate. As can be seen from *Figure 1*, the velocity is reduced to the same extent as the proportion of admixed deutero-formate is increased. This finding indicates that there is no particular preference for either substrate, and, therefore, confirms the above observation, that the difference in affinity between enzyme and substrate can be ruled out as a significant factor responsible for the manifestation of this marked isotope effect.

Variation of temperature

Measurements of initial reaction rates at various temperatures (range 14–37°) indicate that the dehydrogenation of ordinary formate is slightly less temperature-dependent than the analogous reaction with deuterated formate. Whereas, *e.g.*, at 16° the ratio of the reaction rates amounts to $k_H/k_D = 3{\cdot}0$, it steadily decreases the higher the temperature (at 37°, $k_H/k_D = 2{\cdot}0$). This difference in temperature dependence indicates that the activation energy required for enzymatic oxidation of deutero-formate is higher than that required for ordinary formate. The evaluation of the data in *Figure 2* indicates that there is a difference of about 3000 cal/mole

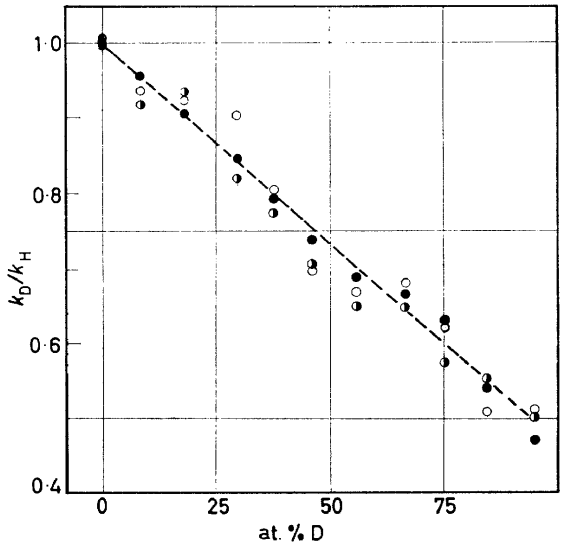

Figure 1. Formate oxidation by plant-formicodehydrogenase from *Phaseolus*: Correlation between relative reaction rate and percentage of deuterated formate in the substrate mixture; formate concentration kept constant at 0·06 M; the various symbols refer to three different Warburg experiments; the ratios k_D/k_H were calculated from the O_2-consumption figures (—O_2/30 min) (data from ref. 5)

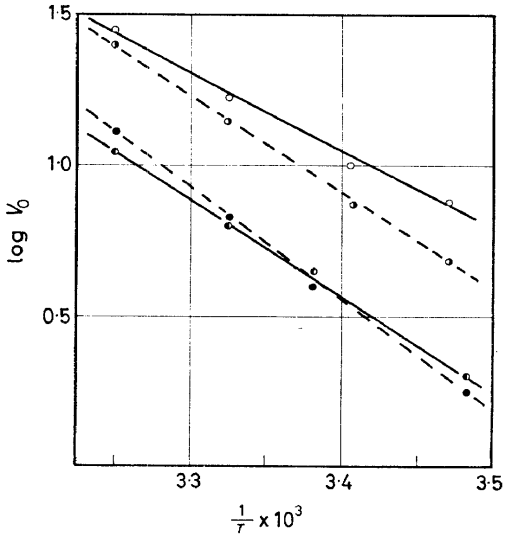

Figure 2. Formate oxidation by plant-formicodehydrogenase from *Phaseolus*: Reaction rates as a function of temperature; spectrophotometric technique from ref. 5

—○— substrate $HCOO^-$ } Solvent H_2O
—◐— substrate $DCOO^-$ }
- -◑- - substrate $HCOO^-$ } Solvent D_2O
- -●- - substrate $DCOO^-$ }

($E_H = 11{,}900 \pm 150$ cal/mole; $E_D = 14{,}940 \pm 170$ cal/mole). In addition, the same comparison has been made using D_2O as a solvent. At 27° replacement of H_2O by D_2O results in a 25 per cent decrease of formicodehydrogenase activity towards HCOONa. At this temperature, however, no such solvent isotope effect (D_2O/H_2O) can be observed in the oxidation of deutero-formate. As can be seen from *Figure 2* the regression lines for the oxidation of $DCOO^-$ in H_2O and D_2O have different slopes, but cross at this temperature. Therefore, deutero-formate, at temperatures below 27°, is oxidized more slowly in heavy water, but above 27° is oxidized even faster in D_2O than in ordinary water. The activation energy figures calculated from the data for D_2O as a solvent (*Figure 2*) are slightly higher than those valid for the aqueous system ($E_H = 14{,}330 \pm 260$ cal/mole; $E_D = 16{,}010 \pm 120$ cal/mole). The difference in activation energy between $HCOO^-$ and $DCOO^-$ oxidation is smaller in D_2O (1700 cal/mole) than in H_2O (3000 cal/mole).

The fact that both O_2-consumption and NAD-reduction exert the same isotope effect in plant-formicodehydrogenase activity suggests that the over-all reaction rate is limited by step *A*. This step consists essentially in the transfer of hydrogen (or deuterium) from the carbon atom of the substrate to the NAD-coenzyme. No isotope effect can be observed in step *B*, *i.e.*, when NAD–H is reoxidized in presence of a suitable H-acceptor such as methylene blue. This finding presents no objection to the above conclusion, because—as shown by Vennesland *et al.*[6]—the hydrogen atom transferred in step *B* is not identical with that one taken up in step *A*.

FORMATE OXIDATION BY CRYSTALLIZED BEEF LIVER CATALASE IN PRESENCE OF A H_2O_2-DONOR SYSTEM

In mammalian tissues, mainly in liver and kidney, formate is exclusively oxidized by catalase, acting as a peroxidase. For the measurement of peroxidatic catalase activity, ^{14}C-labelled formate represents a very convenient substrate, because of its sensitivity and its specificity. The coupled oxidation of $H^{14}COONa$ and $D^{14}COONa$ has been studied *in vitro* by means of a simple model, first described by Keilin and Hartree[7], consisting of notatin and glucose as a H_2O_2-generator, catalase and formate. The reaction sequence in this system consists of the following steps:

$$\begin{array}{lllll} A: & C_6H_{12}O_6 + O_2 + H_2O & \rightarrow C_6H_{12}O_7 & + H_2O_2 \\ B: & H_2O_2 + HCOOH & \rightarrow CO_2 & + 2H_2O \\ C: & H_2O_2 + H_2O_2 & \rightarrow 2H_2O & + O_2 \end{array}$$

Step *A* catalysed by notatin;
Step *B* and C catalysed by catalase.

Provided that H_2O_2 is produced at a sufficiently slow and steady rate, it is almost exclusively used for reaction *B*. Reaction *C*, therefore, takes place when no hydrogen donor, *e.g.*, formate is present or when H_2O_2 is formed in excess. For these experiments Warburg flasks with 2 side arms were used; the first containing 16·7 μmole of glucose and 40 μmole of ^{14}C-formate

in order to "start" the reaction and the second containing 0·2 ml 2N H_2SO_4 to be tipped in at the end of the incubation period. A buffered solution containing catalase and notatin in different proportions was placed directly in the main compartment of the flask. The base placed in the centre cup served as a trap for $^{14}CO_2$ produced by formate oxidation. The amount of $^{14}CO_2$ formed per unit of time, as well as excess oxygen consumption served as a measure of coupled formate oxidation by catalase. Further details concerning the experimental procedure have been published previously[8].

Isotope-effect in peroxidatic formate oxidation

If H_2O_2 is generated by glucoseoxidase (=notatin) at a moderate rate ($\sim$5 μmol ml^{-1} h^{-1}) and if only small amounts of catalase are present, ordinary formate ($H^{14}COONa$) and heavy formate ($D^{14}COONa$) are oxidized at different rates. Provided that the over-all reaction is exclusively limited by step *B*, the resulting ratio between the two initial reaction velocities equals $k_H/k_D = 1{\cdot}5$. Since the absolute amount of hydrogen donor, oxidized under these conditions, is relatively small, this difference hardly can be detected manometrically by means of measuring excess

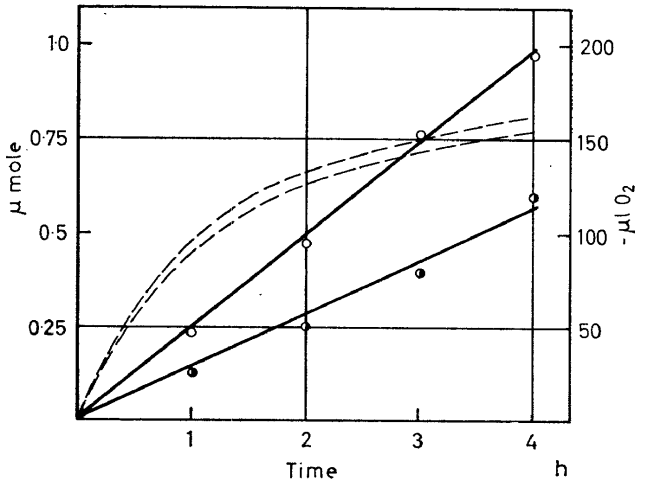

Figure 3. Oxidation of ^{14}C-formate in a model system containing excess notatin and traces of catalase; Ordinates: left side: μ moles $^{14}CO_2$/flask produced (○ = $H^{14}COONa$; ◑ = $D^{14}COONa$); right side: μl O_2/flask consumed in presence of HCOONa (upper curve – – –) and DCOONa (lower curve – – –) (data from ref. 8)

oxygen consumption. As shown in *Figure 3* the estimation of $^{14}CO_2$-formation is a much more sensitive indicator for small differences in coupled formate oxidation.

Variation of proportion between notatin and catalase concentration in the model system

In order to keep the rate of H_2O_2 production at a suitable level, the ratio was varied mainly by increasing or decreasing catalase concentration. If

large amounts of catalase are added the hydrogen peroxide produced is used to a much greater extent for coupled formate oxidation. However, no isotope effect can be observed under these conditions. This is most likely due to the fact that step *B*, which is catalysed by catalase, is no more limiting. If intermediate quantities of catalase are present in the system isotope effects of variable size occur. In *Figure 4* the ratios between the

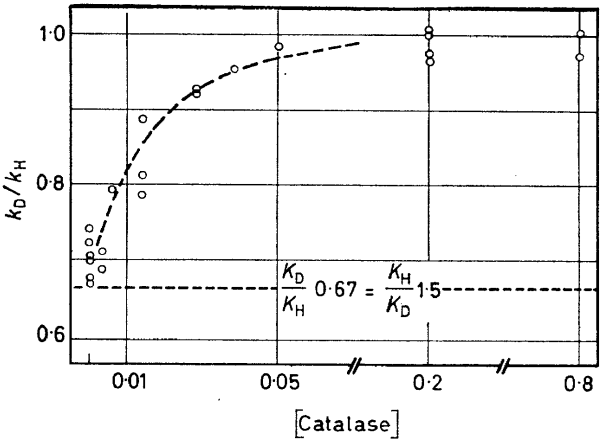

Figure 4. Oxidation of ^{14}C-formate in presence of notatin, glucose and catalase; effect of catalase concentration on the extent of isotope effect; Ordinate: ratio of reaction rates for $DCOO^-$ and $HCOO^-$; Abscissa: amount of crystallized liver catalase added, expressed in arbitrary units (ml of a 0·5% solution; activity of the preparation Kat. F. 36,000): notatin and glucose concentration are kept constant (data from ref. 8)

respective reaction rates (k_D/k_H) are plotted against catalase concentration. The resulting curve shows that there is a steady change between the two extreme situations. Experiments with a rather low catalase concentration are limited by the fact that all notatin preparations which have been available to us, are contaminated by small amounts of catalase. Therefore, experiments of that type have been accomplished by adding a suitable inhibitor to the model system, such as sodium azide or dichlorphenol. In so doing the absolute reaction rates for formate oxidation (HCOONa or DCOONa) are reduced still further. However, the presence of these inhibitors does not alter the isotope effect to a significant extent. Therefore, it seems allowable to accept this figure ($k_H/k_D = 1{\cdot}5$) as a value characterizing the isotope effect in step *B* of coupled formate oxidation, at least under the experimental conditions chosen.

The extreme situations in this two-enzyme system with respect to limiting factor and isotope effect are schematically drawn in *Table 1*. Finally, it may be added here, that in coupled peroxidatic formate oxidation, too, there is a linear relationship between reaction velocity and percentage of deuterated compound present in the substrate mixture. From this it may be concluded, that, as is the case in the plant enzyme, the isotope effect in peroxidatic catalase activity is due to a difference in turnover rather than a difference in affinity.

Table 1. Possible interpretation of presence of absence or an isotope effect in a multi-enzyme system (*e.g.* coupled oxidation of formate; system containing notatin, glucose, catalase and formate)

Case I: Catalase ≫ notatin: over-all reaction limited by notatin		
Glucose $\xrightarrow{\blacktriangledown\blacktriangle}$ H_2O_2 + Formate $\longrightarrow$ CO_2 $\uparrow$ (Notatin) $\qquad\qquad$ $\uparrow$ (Catalase)	$\frac{k_H}{k_D} = 1{\cdot}0$	There is *no* isotope effect!
Case II: Notatin ≫ catalase: over-all reaction limited by catalase		
Glucose $\xrightarrow{\uparrow\blacktriangledown\blacktriangle}$ H_2O_2 + Formate $\longrightarrow$ CO_2 $\uparrow$ (Notatin) $\qquad\qquad$ $\uparrow$ (Catalase)	$\frac{k_H}{k_D} = 1{\cdot}5$	There is an isotope effect

Applications to biological objects

The deuterium isotope effect, which can be observed in formate oxidation using tissue slices or intact animals, falls within the same range as in the model experiments mentioned above. In slices of guinea-pig liver, incubated in a suspending medium containing 0·025 M ^{14}C-formate the deuterated substrate is oxidized at a definitely, but variably lower rate (k_H/k_D = 1·1 — 1·3). Although a number of experimental conditions have been tested, the maximal ratio of 1·5 was never attained. *In vivo* loading experiments performed in rats have revealed a similar situation. If a standard dose of 0·5 meq/100 g body weight is given, the rate of $^{14}CO_2$ elimination is highest

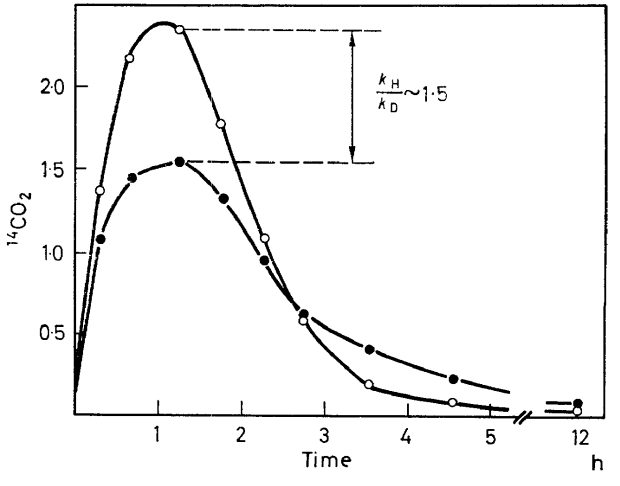

Figure 5. Oxidation of ^{14}C-formate *in vivo*; elimination of $^{14}CO_2$ in expired air after intraperitoneal application of a single dose of H^{14}COONa (= ○) or D^{14}COONa (= ●) to rats; average of 4 experiments, each; Dose: 0·5 meq/100 g body weight (data from ref. 9)

after intraperitoneal injection of ^{14}C-formate; it is distinctly lower after subcutaneous and even more so after peroral application. On the other hand, the isotope effect is largest in the first type of experiment; it is smaller or even negligible in the second and third case. As demonstrated in *Figure 5.* the rates of $^{14}CO_2$ elimination exert almost exactly the same proportion ($k_H/k_D = 1{\cdot}5$) as has been found to be the maximal value for $k_H : k_D$ in the model system. This is the case, at least, if the rates in the steady state period (30–90 min) are considered[9]. Based on the results obtained in the model experiments one may deduce from these findings, which one is the limiting step in formate oxidation *in vivo*. If this extrapolation is correct the presence of an isotope effect indicates that *in vivo*, as well as in isolated tissue, the conversion capacity in respect to formate oxidation is mainly limited by peroxidic catalase activity and not by the rate of H_2O_2 formation in the tissue. This conclusion is in full agreement with results obtained by other techniques, indicating that tissues, such as liver and kidney, exert an exceedingly high H_2O_2 formation capacity[10, 11].

Formate oxidation by bacterial formicodehydrogenase from *Escherichia coli*

In contrast to the plant enzyme, bacterial formicodehydrogenase is not a NAD-dependent enzyme, but hydrogen is transferred to a carrier of unknown structure, which is rather sensitive to oxygen. Formicodehydrogenase acts either as a single enzyme or co-operates with hydrogenase, thus forming a complex enzyme system called hydrogenylase. According to Gest[12] bacterial formicodehydrogenase reacts as follows:

$$HCOOH \rightarrow CO_2 + 2H^+ + 2e$$

The electrons are then taken up by a carrier and transferred along some respiratory chain. For the determination of enzyme activity either oxygen consumption is followed manometrically, during aerobic incubation (gas phase: 5 per cent O_2 and 95 per cent N_2) in Warburg vessels, or methylene blue reduction may be taken as an indicator under anaerobic conditions (Thunberg-Technique). As reported in a previous paper[13] there is no deuterium isotope effect at all in formate oxidation by *E. coli* preparations, whether oxygen consumption or methylene blue reduction is measured. Only after prolonged incubation (air as a gas phase!), when the reaction rate is decreasing considerably, is a small isotope effect in respect to oxygen consumption observed.

If complex hydrogenlyase activity is measured, a distinct isotope effect can be observed. When a suspension of *E. coli* is incubated anaerobically (gas phase N_2) and production of molecular hydrogen is taken as an indicator, $HCOO^-$ and $DCOO^-$ are metabolized at different rates. The ratio of the corresponding reaction velocities, as measured by hydrogen gas production, is $k_H/k_D = 1{\cdot}5$. An analysis of this isotope effect has revealed, that at least part of it is due to difference in affinity. Half maximal velocity is reached at a substrate concentration of 0·018 M in presence of HCOONa, and at 0·022 M in presence of DCOONa. If the total substrate concentration is kept constant at 0·04 M and the proportion HCOONa/DCOONa

is varied, a small but distinct deviation of the corresponding hydrogen production rates from linearity occurs[13]. In comparison to formicodehydrogenase activity the reaction rates for hydrogenlyase activity are relatively low. In either case, however, no definite statement about the mechanism or the rate-limiting factor can be made.

DISCUSSION

Comparison with isotope effect in non-enzymic-formate oxidation

As in non-enzymic oxidation of formate the three enzyme reactions which have been analysed, exert a certain deuterium isotope effect. This is the case, at least, if the very step, in which the bond between carbon and hydrogen is split, limits the reaction under investigation. Whereas the isotope effect in formate oxidation by alkaline permanganate as determined by Aebi *et al.*[2] is considerable (k_H/k_D = 6–10), those reported here for enzymatic oxidation are much smaller. A comparison can be made by means of the data summarized in *Table 2*. The figure given there for oxida-

Table 2. Comparison of various deuterium isotope effects in formate oxidation

Enzyme (experimental conditions)	*Substrate constants* (H = $HCOO^-$; D = $DCOO^-$)	*Isotope effect* (ratio k_H/k_D)	*Reference*
Oxidation by alkaline $KMnO_4$ (25°; pH ~ 14)	—	7·4	Wiberg and Stewart[1]
(20°; pH ~ 14)	—	6·6 (6–10)	Aebi, Buser and Lüthi[2]
Plant-formicodehydrogenase from *Phaseolus multiflorus* L. (27°; pH = 6·8)	(H) $2·7 \times 10^{-3}$ (D) $3·1 \times 10^{-3}$	2·5 (at 16° 3·0) (at 37° 2·0)	Aebi, Frei and Schwendimann[5]
Peroxidatic catalase activity (beef liver catalase) (27°; pH = 5·9)	($\sim 10^{-3}$)	1·5	Frei and Aebi[8]
Hydrogenlyase from *E. coli* (32°; pH = 6·8)	(H) $1·8 \times 10^{-2}$ (D) $2·2 \times 10^{-2}$	1·5	Aebi and Frei[13]
Formicodehydrogenase from *E. coli* (32°; pH = 6·8)	($\sim 10^{-5}$)	1·0	Aebi and Frei[13]
Theoretical "maximal" value (25°)	—	6·9	Wiberg[14]
Theoretical "minimal" value	—	$\sqrt{(2)} = 1·42$	

tion by alkaline permanganate closely meets the theoretical maximum value given by Wiberg[14]. According to his calculation a primary isotope effect, due to splitting a bond H–C or D–C, may reach a maximal value of k_H/k_D = 8·3 at 0° and 6·9 at 25°. The correctness of these figures has been experimentally verified by various authors, *e.g.*, for formate oxidation by

Wiberg and Stewart[1], or for the oxidation of 2-deuteropropanol by Westheimer and Nicolaides[15]. As has been pointed out by Wiberg[14] a deuterium isotope effect of that kind may become smaller the more the zero point energy of the bond is conserved in the activated state. Thus, the values obtained for enzymatic formate oxidation ($k_H/k_D = 1{\cdot}5$–$3{\cdot}0$) indicate a high degree of conservation of zero point energy in the transition state, presumably in the activated enzyme–substrate complex. Whereas the action of the plant-formicodehydrogenase represents an intermediate situation in respect to activation energy ($1{\cdot}2$–$1{\cdot}5 \times 10^3$ cal/mole) as well as to the extent of the isotope effect ($2{\cdot}0$–$3{\cdot}0$), the figures obtained for peroxidatic formate oxidation by catalase are close to the theoretical minimum. On one hand the activation energy in reactions brought about by catalase is believed to be extremely small[16], *i.e.* in the range of 1000 cal/mole. On the other hand the experimental value for a deuterium isotope effect in presence of this enzyme ($k_H/k_D = 1{\cdot}5$) is close to the theoretical minimum figure. This value is obtained, if the mass factor is considered exclusively ($k_H/k_D = \sqrt{(2)} = 1{\cdot}42$) in the formula given by Wiberg[14]. According to the present author this would mean that in peroxidatic formate oxidation bonding H–C (D–C) in the activated complex is as strong as that in the reactants. It may be of interest that there exists a parallelism, at least where the formal aspect is concerned, between enzymic and thermal activation. In either case, *i.e.*, at high temperature as well as in presence of a suitable enzyme system, the size of the resulting isotope effect is almost exclusively due to the mass factor. This "theoretical minimum value" of $1{\cdot}42$ is approximately attained in two different enzyme systems, splitting H (or D) from C in formate, *i.e.*, in peroxidatic catalase and in hydrogenlyase activity.

Comparison of isotope effects in formate oxidation with those in other enzyme systems

Only few dehydrogenating enzymes have been analysed so far. This is due, to a certain extent, to the fact that fully deuterated substrates have been available only in exceptional cases in the past. This situation has changed since Katz and his associates have succeeded in growing a variety of organisms, such as algae and bacteria in heavy water[17, 18]. Only three similar processes will be considered here as examples; *viz.* oxidation of succinate, glucose and glucose-6-phosphate. Deutero-succinate, first prepared and investigated by Erlenmeyer *et al.*[19] may be considered as the "classical" substrate. According to studies made more recently by Thorn[20] and Thomson and Klipfel[21] dehydrogenation of tetradeuterated succinate by heart-muscle preparation or kidney homogenate exerts an isotope effect of $k_H/k_D = 4{\cdot}5$–$5{\cdot}5$. In presence of excess substrate there is a linear relationship between deuterium content of the substrate and reaction velocity. According to Thorn the affinities for succinate and tetradeutero-succinate differ by a factor of $1{\cdot}4$–$1{\cdot}8$. Thus, this isotope effect is likely to be due to a difference in maximal velocity ($V_{max.}$) as well as, to some extent, a difference in affinity. Katz and his associates have compared the oxidation rates of glucose and fully deuterated glucose in presence of notatin[17]. From the curves an approximate figure for $k_H/k_D = 3{\cdot}2$ can be calculated. The

dehydrogenation of glucose-1-deutero-6-phosphate by glucose-6-phosphate-dehydrogenase of various origin has been studied by Rose[22]. All preparations tested (from yeast, *E. coli* and red cells) reveal a different situation in as much the deuterated substrate has a higher affinity than ordinary glucose-6-phosphate ($K_{S(H)}/K_{S(D)} = 1{\cdot}4$–$1{\cdot}9$). On the other hand, as usual, the maximal velocity, *i.e.* dehydrogenation rate at $[S] \to \infty$ is slower for the deuterated substrate, the calculated ratios ranging from $k_H/k_D = 2{\cdot}0$ to $2{\cdot}7$.

These few examples demonstrate that it is very difficult to make any generalizing statement or prediction about the extent or the sense of changes in affinity or maximal reaction velocity, when H is replaced by D in a substrate of dehydrogenating enzymes. At the present moment it seems, that, according to the structure of substrate and nature of the enzyme, either alteration ($K_{S(H)} \neq K_{S(D)}$ or $V_{max.(H)} \neq V_{max.(D)}$) in varying proportion may contribute to a deuterium isotope effect. It seems justified, however, to assume that formate, as a relatively small molecule containing one single H—C bond only, offers a situation which is least complicated by interactions due to other groups in the substrate molecule.

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