STUDIES ON *AEROBACILLUS POLYMYXA*

V. THE SIGNIFICANCE OF THE MICHAELIS CONSTANTS OF THE HYDROGENLYASE SYSTEMS AND RELATED ENZYMES

By W. G. CREWTHER*

[*Manuscript received September* 23, 1952]

Summary

Washed-cell suspensions of *Aerobacillus polymyxa* have been used to determine the Michaelis constants of the enzyme systems producing molecular hydrogen from formate, glucose, and pyruvate and of the dehydrogenases acting on these substrates. For each substrate the K_m value of the dehydrogenase was considerably smaller than for the analogous hydrogenlyase.

A theoretical examination of the significance of the K_m values of a model dehydrogenase system showed that different values might be expected according to the concentration and nature of the hydrogen acceptor used.

It was shown experimentally that substitution of rosinduline, potassium indigo trisulphonate, or phenol indophenol for methylene blue in the Thunberg technique resulted in a wide range of K_m values for the formic dehydrogenase of *Escherichia coli*. There was also a significant variation in the results obtained with different concentrations of methylene blue.

It has been concluded that discrepancies in the K_m values of dehydrogenases and the corresponding hydrogenlyases of bacteria cannot be used as evidence of the existence of hydrogenlyases as enzymes distinct from the dehydrogenases and hydrogenase of bacteria.

I. INTRODUCTION

Stephenson and Stickland (1932) have postulated the existence in certain bacteria of enzymes, the hydrogenlyases, which catalyse the production of molecular hydrogen from substrates such as formate and glucose. Their experimental evidence supported the hypothesis that the hydrogenlyases are distinct from the corresponding dehydrogenases and hydrogenase. The experimental data adduced by these authors included the observation that the Michaelis constants of the formic dehydrogenase and of the formic hydrogenlyase of *Escherichia coli* were of a different order of magnitude, the former being considerably smaller than the latter. Ordal and Halvorson (1939) suggest that, as the Michaelis constant evaluates the dissociation constant of the enzyme-substrate complex, this observation supports the hypothesis of a separate hydrogenlyase system though they consider their own results to favour the opposite view.

In a previous paper of this series (Crewther 1953) it has been shown that, under suitable conditions, washed suspensions of *Aerobacillus polymyxa*

* Biochemistry Unit, Wool Textile Research Laboratory, C.S.I.R.O., Melbourne.

produce hydrogen from formate, glucose, and pyruvate. The results obtained suggested that hydrogen may arise from formate and glucose as a result of the consecutive action of the specific dehydrogenase and hydrogenase, though no conclusive evidence was presented.

In developing the expression

$$r = \frac{[S] \,.\, [E] \,.\, k}{[S] \quad + \quad K_m}$$

$$= \frac{[S] \,.\, R}{[S] \quad + \quad K_m} \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

for the rate of an enzyme catalysed reaction (r being the rate of the reaction, [S] and [E] the concentrations of substrate and enzyme respectively, R the maximum reaction rate, K_m the dissociation constant of the enzyme substrate complex, and k another constant), Michaelis and Menten (1913) assumed that an equilibrium existed between substrate, free enzyme, and the enzyme-substrate complex. On the assumption that a steady state is set up between enzyme, substrate, and enzyme-substrate complex, Briggs and Haldane (1925) have developed a similar equation for the rate of an enzymic reaction of the form

$$r = \frac{[S] \,.\, R}{[S] \quad + \quad \dfrac{k'_1 + k_2}{k_1}}, \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

in which k_1, k'_1, and k_2 are the rate constants for the reactions

$$\begin{aligned} S + E &\longrightarrow ES \qquad (k_1) \\ ES &\longrightarrow E + S \qquad (k'_1) \\ ES &\longrightarrow E + A + B \quad (k_2). \end{aligned}$$

Consequently the value obtained by determining the concentration of substrate giving half the maximum overall reaction rate will be $(k'_1 + k_2)/k_1$ and the error incurred in assuming that $K_m = k'_1/k_1$ is greatest when the rate of decomposition of the complex is great relative to its rate of dissociation into enzyme and substrate.

Application of the theory of reaction rate kinetics to a system consisting of a specific dehydrogenase catalysing the transfer of hydrogen or electrons to a carrier, the reduced form of which serves as substrate for the enzyme, hydrogenase, provides an expression in the form of a quadratic in "r", the steady state reaction rate. This is due to the fact that the reduced carrier, which is a product of the first reaction, is the substrate of the second; consequently the reactions of this intermediate cannot be neglected in the way Michaelis and Menten neglect the effects of the products of the reaction. The value of the substrate concentration giving half maximum activity would have little meaning in such a system. This therefore constitutes a theoretical reason for questioning the validity of a comparison of the K_m values of the hydrogenlyase systems and dehydrogenases of bacteria.

In applying the theory to the Thunberg technique the following simple reactions are postulated:

$$\begin{aligned} E + SH_2 &\rightleftharpoons ESH_2 \\ ESH_2 + D &\rightleftharpoons ES + DH_2 \\ ES &\rightleftharpoons E + S, \end{aligned}$$

and on attainment of a steady state

$$\begin{aligned} r &= k_1 [E] [SH_2] - k'_1 [ESH_2] \\ r &= k_2 [ESH_2] [D] - k'_2 [ES] [DH_2] \\ r &= k_3 [ES] - k'_3 [E] [S] \\ [E_T] &= [E] + [ESH_2] + [ES]. \end{aligned}$$

Eliminating $[ESH_2]$, $[ES]$, and $[E]$, the expression for r becomes

$$\frac{k'_1 k_3 [E_T](k_1 k_2 k_3 [D][SH_2] - k'_1 k'_2 k'_3 [S][DH_2])}{(k_2 k_3 [D] + k'_1 k_3 + k'_1 k'_2 [DH_2])(k'_1 k_3 + k_1 k_3 [SH_2] + k'_1 k'_3 [S]) - (k_3 - k'_1)(k_1 k_2 k_3 [D][SH_2] - k'_1 k'_2 k'_3 [S][DH_2])}.$$

If $k'_2[DH_2]$ and $k'_3[S]$ are comparatively insignificant,

$$r = \frac{k_1 k_2 k_3 [E_T][D][SH_2]}{[SH_2]\,(k_1 k_3 + k_1 k_2 [D]) + k'_1 k_3 + k_2 k_3 [D]}$$

and when $r = \tfrac{1}{2} \cdot \dfrac{k_2 k_3 [E_T][D]}{k_3 + k_2 [D]}$, $[SH_2] = \dfrac{k'_1 k_3 + k_2 k_3 [D]}{k_1 k_3 + k_1 k_2 [D]}$,

it follows that K_m would vary with [D]. As [D] increased, K_m would approach k_3/k_1 and as [D] approached zero K_m would approach k'_1/k_1, the dissociation constant of the enzyme-substrate complex. At high concentrations of the hydrogen acceptor, D, K_m would be practically independent of its concentration and of its chemical structure. However, the concentration at which this desirable condition is attained would depend on the relative magnitudes of k_2 and k'_1 and of k_2 and k_3, i.e. it would depend on the chemical nature of the hydrogen acceptor. At low concentrations of D both its concentration and chemical nature — E_0 value, pK values, and steric properties — would be expected to affect the value of K_m obtained.

This paper therefore compares the K_m values of the hydrogenlyase and dehydrogenase systems of *A. polymyxa* and *E. coli*, and demonstrates the influence of the concentration and nature of the hydrogen acceptor used in the Thunberg determination of dehydrogenase activity on the values of K_m obtained for these enzyme systems.

II. Experimental

Washed-cell suspensions of *A. polymyxa* strain GO(7)) were prepared and tested as previously described (Crewther 1953). Media for growing *A. polymyxa* contained 0.2 per cent. $FeSO_4.7H_2O$ unless otherwise stated. Cells of *E. coli* (strain 557 R) grown on 500 ml. basal medium containing no yeast extract (Crewther 1953) were washed and suspended in 200 ml. buffer.

In determining the effect of substrate concentration on the rate of hydrogen production it was observed that the rate of evolution was practically constant during the period of the experiments. Consequently it was unnecessary to plot

hydrogen production against time to obtain the initial reaction rate. Curves relating formate concentration to the rate of hydrogen production are shown in Figure 1, and it will be observed that significant differences occur from one experiment to another. The chief difference appears to lie in the concentration of formate at which inhibition occurs. Similar curves for the production of

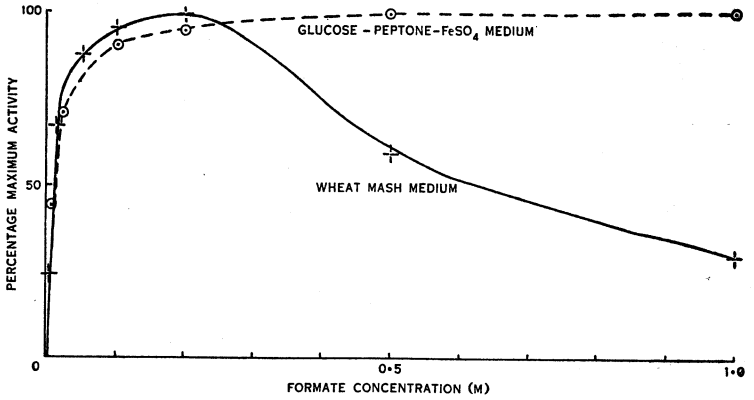

Fig. 1.—Relationship between substrate concentration and activity of the formic hydrogenlyase system of *A. polymyxa* grown on wheat mash and on glucose-peptone-ferrous sulphate medium.

hydrogen from glucose and pyruvate, and for the corresponding dehydrogenases, are plotted in Figures 2 and 3. The curves for pyruvic hydrogenlyase and dehydrogenase are seen to be atypical and resemble that obtained for pyruvic

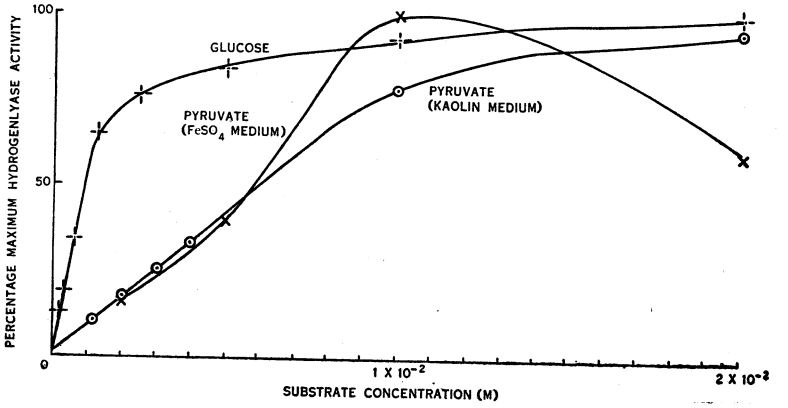

Fig. 2.—Relationship between substrate concentration and activity of glucose hydrogenlyase and pyruvic hydrogenlyase of *A. polymyxa*. Experiments with pyruvic hydrogenlyase were done on cells grown on glucose peptone medium containing ferrous sulphate and on similar medium containing 1 per cent. kaolin but only 0.005 per cent. $FeSO_4.7H_2O$.

hydrogenlyase of *Clostridium acetobutylicum* by Davies (1942). It was found that in some cases normal curves were obtained (Fig. 2) when the cells of *A. polymyxa* were grown on a medium in which $FeSO_4$ was replaced by 1 per

cent. kaolin (Crewther 1953). The values of K_m obtained from the atypical curves of Figures 2 and 3 will have doubtful significance but are included for comparison.

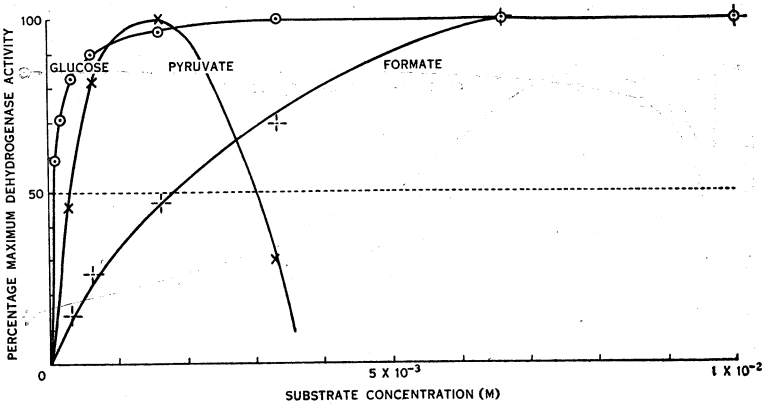

Fig. 3.—Relationship between substrate concentration and the activity of formic, glucose, and pyruvic dehydrogenase systems of *A. polymyxa*. A final concentration of 0.02 per cent. methylene blue was used in the dehydrogenase estimations.

The K_m values obtained in this way are summarized in Table 1, the constants for the dehydrogenases being considerably smaller in each case than those of the corresponding hydrogenlyase. Stickland's observation (1929) that the K_m value of the formic dehydrogenase of *E. coli* is less than that of the hydrogenlyase has therefore been confirmed with *A. polymyxa*.

TABLE 1

K_m VALUES OF THE DEHYDROGENASES AND HYDROGENLYASES OF *A. POLYMYXA*

Substrate	Michaelis Constant (M)	
	Dehydrogenase	Hydrogenlyase
Formate	$1{\cdot}8\times10^{-3}$	$1{\cdot}0\times10^{-2}$
Glucose	$6{\cdot}0\times10^{-5}$	$9{\cdot}0\times10^{-4}$
Pyruvate	$3{\cdot}0\times10^{-4}$	$6{\cdot}0\times10^{-3}$

The theoretical investigation of the significance of the K_m values obtained by the Thunberg technique indicated that the value obtained would depend on the concentration of the dyestuff used as hydrogen acceptor, and on its chemical structure. In order to test this experimentally, *E. coli* was used because of the greater stability of the hydrogenlyase systems of this organism, and to eliminate the need for a protecting sludge in the cell suspensions. In each

case reduction of nine-tenths of the original amount of dye present was used as an end-point. The curves relating substrate concentration and the rate of reduction of methylene blue, using 0.02, 0.002, and 0.001 per cent. methylene blue in the final mixtures are shown in Figure 4. The K_m values obtained from these curves were 1.9×10^{-3}, 1.1×10^{-3}, and 1.9×10^{-4}M respectively. In a similar manner, considerable differences were obtained in the curves when using final concentrations of 0.5×10^{-5}M rosinduline, potassium indigo trisulphonate, methylene blue, and phenol indophenol, with E'_0 values at pH 7.0 of -0.254, -0.083, $+0.011$, and $+0.227$ V. respectively (Fig. 5). The K_m values obtained in this way for formic dehydrogenase of *E. coli* varied from 8×10^{-5} to 5×10^{-3}M.

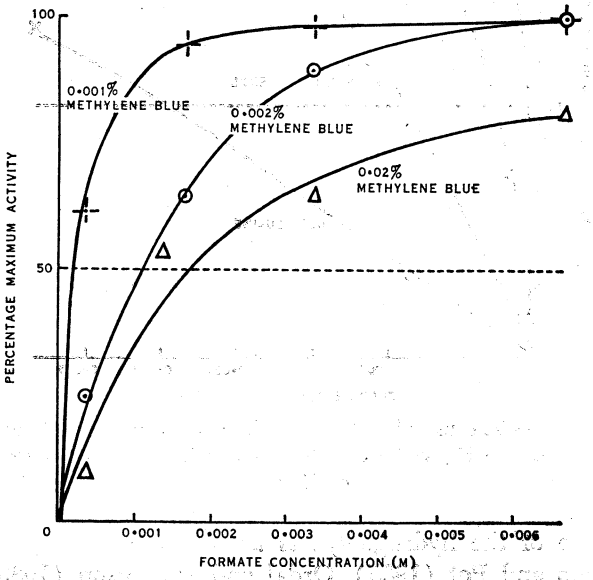

Fig. 4.—The use of different concentrations of methylene blue as hydrogen acceptor in the determination of the K_m value of the formic dehydrogenase system of *E. coli*. The final concentrations of methylene blue are indicated.

III. Discussion

As predicted by a theoretical examination of the reactions taking place in the estimation of dehydrogenase activity by the Thunberg technique, the experimental data presented in this paper show clearly that the K_m values obtained for the formic dehydrogenase system of *E. coli* are dependent on the nature and the concentration of the hydrogen acceptor used in estimating dehydrogenase activity. The theoretical examination of the significance of the K_m values of the hydrogenlyase systems of bacteria has shown that, if hydrogen is produced as the result of the consecutive action of a specific dehydrogenase linked to hydrogenase by a suitable carrier, the equation evaluating the rate of the reaction as a function of substrate concentration would not have the form of the classical Michaelis-Menten equation. Consequently the experimental values

for the Michaelis constant would not bear a simple relationship to the dissociation constant of the enzyme-substrate complex. For these reasons the differences in K_m value of the hydrogenlyase systems and the corresponding dehydrogenase systems do not conflict with the view that gaseous hydrogen arises as the result of the consecutive action of a dehydrogenase and the enzyme hydrogenase.

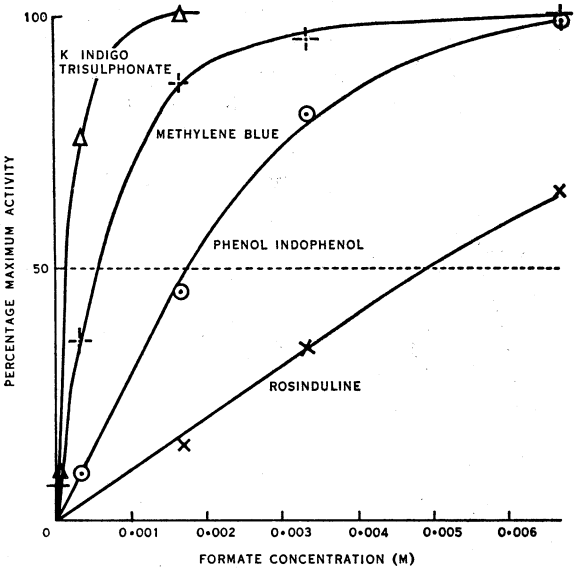

Fig. 5.—The use of different hydrogen acceptors in determining the K_m value of the formic dehydrogenase system of *E. coli*.

The existence of the hydrogenlyases as separate enzymes has been questioned by Tasman and Pot (1935), Ordal and Halvorson (1939), and Waring and Werkman (1944), but the experimental facts on which the concept of the hydrogenlyases was originally based (Stephenson and Stickland 1932) have not been satisfactorily explained by another hypothesis. The present paper and the preceding paper of this series (Crewther 1953) suggest that further investigation of the problem is necessary.

IV. References

Briggs, A. E., and Haldane, J. B. S. (1925).—*Biochem. J.* **19**: 338.
Crewther, W. G. (1953).—*Aust. J. Biol. Sci.* **6** (2): 190.
Michaelis, L., and Menten, M. L. (1913).—*Biochem. Z.* **49**: 333.
Ordal, E. J., and Halvorson, H. O. (1939).—*J. Bact.* **38**: 199.
Davies, R. (1942).—*Biochem. J.* **36**: 582.
Stephenson, M., and Stickland, L. H. (1932).—*Biochem. J.* **29**: 1749.
Tasman, A., and Pot, A. W. (1935).—*Biochem. J.* **29**: 1749.
Waring, W. S., and Werkman, C. H. (1944).—*Arch. Biochem.* **4**: 75.