tion, at moderate temperatures. On the other hand, Nylon, in which the R-group is only a hydrogen atom, sorbed the stoichiometric amount of BF_3 to form a complex which did not break down on desorbing at temperatures up to 39°.

The evidence presented thus indicates that the strong, "permanent" binding of BF_3 by proteins is not stoichiometric in nature. On the other hand the fact that the total BF_3 sorption can be experimentally separated into chemically (*i.e.*, permanent) and physically sorbed parts does point to a specific, chemical interaction for this permanently bound portion despite the absence of stoichiometry. The reason for the non-stoichiometric addition of BF_3 may be at least threefold. One is the dispersity of particle size of the solid protein samples used; the second is the interaction between sorbed groups and the third is lack of true equilibrium in the system.

The first explanation cannot be completely ruled out by the results of the present investigation since no systematic studies were made of the effects of particle size on BF_3 sorption. However, the fact that different samples of protein did give quite reproducible results is an indication that this may not be too important an effect.[33] In addition the results of Benson and Zwanzig[34] on H_2O sorption indicates that in a comparable system, particle dispersity is an unimportant factor.

The second and third explanations cannot be completely disentangled. The high concentrations of permanently bound BF_3 (*i.e.*, about 10 *M* per volume of dry protein) leads to an average distance of separation of about 10 Å. between binding sites. In view of the diverse nature of the functional groups present in the protein and the relatively large size of the BF_3 molecule it is not at all unreasonable to expect a "smearing" out of the differences in binding energies and in chemical potential of the different sites leading to a behavior resembling that of solid solution. Such behavior would still be compatible with the observations of Seehof and Benson,[11] that in back-titration with NH_3, stoichiometric relations could be found between very strongly binding groups and the amounts of BF_3 not back-titrated by NH_3. If these back-titrations are not all fortuitous, they could be interpreted as arising from "energy bands" in the solid protein which could appear as non-stoichiometric binding from the rigorous test of a phase diagram though still stoichiometric from the point of view of NH_3:BF_3 formation if the "gaps" between the different sites were sufficiently large. For a more rigorous test of this model, it would be necessary to study the effects of temperature on the back titrations.

LOS ANGELES, CALIF.

(33) Even though such samples usually came from the same "batch," it is unlikely that the particle dispersity was the same since the larger particles usually accumulate at the bottom of the bottle.

(34) S. W. Benson, D. A. Ellis and R. W. Zwanzig, THIS JOURNAL, **72**, 2102 (1950).

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

On the Mechanism of Dehydrogenation of Fatty Acyl Derivatives of Coenzyme A. IV. Kinetic Studies

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RECEIVED JANUARY 25, 1956

The kinetics and the equilibrium of the enzymatic α,β-oxidation of fatty acyl derivatives of CoA were investigated. With indophenol as acceptor the following three step mechanism of reaction appears likely: electrons are passed from substrate to a primary dehydrogenase (flavoprotein), thence to a specific electron transferring enzyme (flavoprotein) and finally to the dye. The interaction of the two enzymes concerned with their respective substrates, *i.e.*, fatty acyl CoA and reduced dehydrogenase, follows Michaelis–Menten behavior. The three primary dehydrogenases differ markedly in their ability to react with phenazine–cytochrome *c* as acceptor system as compared to flavoprotein–indophenol. With pyocyanine as acceptor a measurable equilibrium was established, and the oxidation–reduction potential E_0' of the system butyryl CoA–crotonyl CoA was determined to be −0.015 v. It is emphasized that oxidative enzyme systems, the components of which have tightly bound prosthetic groups, have unique kinetic properties. Traditional Michaelis constants and turnover numbers of such enzymes cannot be obtained when over-all reactions are measured. The observed constants give the kinetic characteristics of the total substrate–enzyme–acceptor system.

During the last two years four flavoproteins have been shown to participate in the first oxidative step of the fatty acid cycle as it occurs in pig liver mitochondria.[2–5] The evidence indicates that FAD is the prosthetic group in at least three of these enzymes.[6] Three of these are primary dehydrogenases, differing in their substrate specificity and other characteristics. They have been named butyryl dehydrogenase,[5,7] general fatty acyl dedrogenase[2] and palmityl dehydrogenase,[4] abbreviated G, Y and Y', the letters indicating their green and yellow colors, respectively.

The fourth of these flavoproteins functions in the

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(2) F. L. Crane, S. Mii, J. G. Hauge, D. E. Green and H. Beinert, *J. Biol. Chem.*, **218**, 701 (1956).

(3) F. L. Crane and H. Beinert, *ibid.*, **218**, 717 (1956).

(4) J. G. Hauge, F. L. Crane and H. Beinert, *ibid.*, **219**, 727(1956).

(5) F. L. Crane, J. G. Hauge and H. Beinert, *Biochim. Biophys. Acta*, **17**, 293 (1955).

(6) The following abbreviations will be used: CoA, coenzyme A; C_n, acyl CoA with *n* carbon atoms in the acyl moiety; FAD, flavin-adenine dinucleotide; indophenol, 2,6-dichlorophenolindophenol; OD, optical density; Δ_{OD}, change of OD; phenazine, phenazine methosulfate; Tris, tris-(hydroxymethyl)-aminomethane; G, Y, Y' and ETF are defined in the text; E_p, primary dehydrogenase (G, Y, Y'); GH_2, YH_2, etc., are the reduced forms of G, Y, etc.; $C_4 - E_p$, C_4 used with E_p.

(7) D. E. Green, S. Mii and H. R. Mahler, *J. Biol. Chem.*, **206**, 1 (1954).

oxidation of GH_2, YH_2 and $Y'H_2$, the reduced forms of the primary dehydrogenases.[3] In turn this enzyme, which has been given the name electron transferring flavoprotein or ETF, can be reoxidized by conventional dyes and under certain circumstances by cytochrome *c*. Recently it has been shown that a particulate enzyme complex that is able to catalyze oxidation by O_2 of succinate and reduced diphosphopyridine nucleotide will also accept electrons from reduced ETF.[8]

This action of two flavoproteins in sequence is in itself unique. The fact that FAD does not become appreciably dissociated from the enzymes at the *p*H and ionic strength at which they act presents a situation of considerable interest from a kinetic and mechanistic point of view. Thus, one flavoprotein after catalyzing the primary dehydrogenation becomes in its reduced form the substrate on which the next flavoprotein acts.

The present work was undertaken in an attempt to throw further light on the mechanism of these steps of fatty acid oxidation, and upon the kinetics which apply in general when oxidative enzymes having tightly bound prosthetic groups interact in solution.

The experimental approach was limited by the fact that only over-all reaction rates could be measured. The experiments were designed to establish how this over-all rate depends on the concentrations of the several components of the system.

Experimental

Materials and Methods.—Acyl derivatives of CoA were prepared enzymatically by the use of the appropriate activating enzymes.[9,10] Their concentrations were assayed enzymatically in the indophenol–phosphate buffer system described below. An excess of ETF and the proper dehydrogenase was first added to the cuvette. Subsequent addition of acyl CoA then caused a reduction of indophenol in an amount equal to the amount of the substrate added.

G, Y, Y′ and ETF were purified from pig liver as described in the previous papers of this series.[2-6] The concentrations of the enzymes were evaluated in terms of their FAD content determined as described previously.[2] The likely assumption was made that ETF has one and Y and Y′ two molecules of FAD per molecule of protein.[2-4] No data are available on the molecular weight of G from pig. A preparation of G that was purified twice by electrophoresis on a starch column had a riboflavin content of 0.43%. This content of flavin corresponds to a minimum molecular weight of 87,000. If G from pig is assumed to have a molecular weight in the same range as the beef enzyme,[7] it follows that each molecule has two molecules of flavin. The evaluation of the concentration of G was based on these assumptions. The preparations of dehydrogenases and ETF used were from 30 to 95% pure. The dehydrogenases were, however, essentially free of ETF, and the ETF free of G, Y and Y′. Indophenol and cytochrome *c* were commercial products. Phenazine, prepared by the method of Dickens and McIlwain,[11] was kindly supplied by Dr. T. P. Singer. The pyocyanine was obtained by recrystallization of a commercial preparation from petroleum ether–chloroform. The molar extinction coefficient at 700 mμ and *p*H 7 was found to be 3.4×10^6 cm.²/mole on a gravimetric basis.

Rate Measurements.—The spectrophotometric experiments, when not otherwise stated, were carried out in 0.02 *M* phosphate buffer at *p*H 7.2 and 30° in a final volume of 0.5 ml. Initial rates were evaluated from the recorded OD changes in the Beckman DUR spectrophotometer. Indophenol was used in most of the experiments as the final electron acceptor, at a level of 4×10^{-5} *M*. The optical density was recorded at 600 mμ where the extinction coefficient is 16.1×10^6 cm.²/mole.[12] In some experiments phenazine was used in conjunction with cytochrome *c*, instead of ETF–indophenol, and the increase in OD at 550 mμ was measured. The difference in molar extinction coefficient between the reduced and the oxidized states was taken to be 19.7×10^6 cm.²/mole.[13] The concentration of cytochrome *c* in these experiments was 1 mg. per ml. The rate of reduction was the same when the concentration of cytochrome *c* was varied between 0.5 to 2.0 mg. per ml. Although phenazine is autoöxidizable, oxygen here apparently played an insignificant role compared to cytochrome *c* in the reoxidation of reduced phenazine. Phenazine was used at different concentrations to be given below.

The reaction was started by addition of either ETF or acyl CoA. These two procedures lead to identical rates. In all cases except for the green enzyme the rate was the same whether or not the dehydrogenase was added last. A 25% reduction in the rate was found when G was the last compound to be added. The reason for this behavior was not investigated.

Dialysis of ETF and G together against 0.20 *M* Tris at *p*H 7.5 was carried out in order to determine if a complex of G and ETF would slowly form that would show increased catalytic activity. The codialysis was carried out with protein concentrations of 4 and 0.4 mg./ml. In neither case was a higher rate observed by this procedure than when the dialyzed components were added together at the time of the assay.

Results

Requirement for the Acceptors ETF and Phenazine.—In the study of the kinetic constants of the primary dehydrogenation step for the three enzymes and various substrates and inhibitors, ETF and indophenol were present in "excess." The rate was found to be zero order with respect to indophenol at the standard concentration of 4.0×10^{-5} *M*. The concentration of ETF required to give half maximal rate, K_{ETF}, was evaluated from double reciprocal plots.[14] The value of K_{ETF} depends on which dehydrogenase is under test. The maximal velocities and the K_{ETF} values are recorded in Table I. The rates were kept in the normal assay range 0.02 to 0.04 OD unit per minute.

TABLE I

DEPENDENCE OF RATE ON ACCEPTOR CONCENTRATION[a]

Enzyme–substrate	ETF $K_{ETF} \times 10^7$, *M*	ETF $V_{max.}$, min.$^{-1}$	Phenazine $K_{phen.} \times 10^4$, *M*	Phenazine $V_{max.}$, min.$^{-1}$
C_4–G	11.3	333	0.9	530
C_4–Y	4.5	210		
C_8–Y	4.9	834		
C_{16}–Y	3.9	190	10	122
C_8–Y′	6.3	370		
C_{16}–Y′	6.3	345	33	67

[a] The concentrations of C_4, C_8 and C_{16} were, respectively, 9.6×10^{-5} *M*, 1.3×10^{-5} *M* and 3.2×10^{-5} *M*. The dehydrogenase was present in concentrations giving rates in the range 0.020 to 0.040 OD unit/min. The concentrations of ETF and phenazine were varied within a 10-fold concentration range that included K_{ETF} and K_{phen} respectively. Other conditions were as described under Experimental.

In the two last columns of Table I are given corresponding data for phenazine–cytochrome *c* as the

(8) H. Beinert and F. L. Crane, in W. D. McElroy and B. Glass, "Inorganic Nitrogen Metabolism," Johns Hopkins Press, Baltimore, Maryland, 1956, p. 601.

(9) H. R. Mahler, S. J. Wakil and R. M. Bock, *J. Biol. Chem.*, **204**, 453 (1953).

(10) A. Kornberg and W. E. Pricer, Jr., *ibid.*, **204**, 329 (1953).

(11) F. Dickens and H. McIlwain, *Biochem. J.*, **32**, 1615 (1938).

(12) This value was obtained by Dr. F. L. Crane by titration with ferrous ammonium sulfate. The latter was standardized against potassium dichromate, the concentration of which was established spectrophotometrically.

(13) G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.*, **194**, 513 (1952).

(14) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

final electron acceptor instead of ETF–indophenol. A wide variation is noted in the range of concentration at which phenazine can serve as efficient electron acceptor for the different dehydrogenases. Such a variation applies not only to the acceptor concentration required to give half maximal rate, but also to the magnitude of this maximal rate. Only for the green enzyme is a higher turnover possible with phenazine than with ETF, and this at a comparatively low phenazine concentration.

Apparent Michaelis Constants and Turnover Numbers for the Dehydrogenase Step.—Apparent Michaelis constants K_s of the order 1 to 3 $\times$ 10^{-6} M were found in all cases except one (Table II).

TABLE II

KINETIC CONSTANTS FOR THE DEHYDROGENASE STEP[a]

Enzyme–substrate	ETF $K_s \times 10^6$, M	ETF $K_s/(SH_2)$	ETF TN_s, min.$^{-1}$	Phenazine $K_s \times 10^6$, M	Phenazine $K_s/(SH_2)$	Phenazine TN_s, min.$^{-1}$
C_4–G	2.2	0.04	350	2.6	0.03	550
C_4–Y	40	.04	300			
C_8–Y	0.9	.07	900			
C_{16}–Y	3.4	.11	212	4.0	.08	131
C_8–Y′	1.2	.09	405			
C_{16}–Y′	1.6	.05	365	1.6[b]	.05	70

[a] Conditions were as given for Table I, with the exception that the substrate concentrations were varied within a 10 to 20-fold concentration range that included K_s, and the acceptors added at concentrations of 1.3 to 3 $\times$ K_{ETF}, 3 to 10 $\times$ $K_{phen.}$ Columns 3 and 6 give the $K_s/(SH_2)$ ratios that obtained in Table I, here used to compute TN_s, the turnover number for excess substrate and acceptor, by help of the relationship $TN_s = V_{max.}\ (K_s/(SH_2) + 1)$. [b] Assumed equal to K_s for ETF as acceptor.

For the Y enzyme a 10 times higher concentration of C_4 was needed to attain half maximal velocity. For C_{16} oxidation by Y or Y′ a progressive decrease in rate was observed when the C_{16} concentration was made higher than 3 $\times$ 10^{-5} M, indicating inhibition by substrate.

These data were obtained at the time when the variable requirement for ETF was not realized. The constants were thus measured at a variable $K_{ETF}/(ETF)$ ratio (Table II), although the ETF concentration was constant, 1.4 $\times$ 10^{-6} M. For the bulk of the experiments it was not economical to employ ETF concentrations much higher than this value. The effect on K_s of raising the ETF level is, however, small in this region. The change in K_s for C_4–G when $K_{ETF}/(ETF)$ was brought from 0.8 to 0.3 was within experimental error. When $K_{ETF}/(ETF)$ was changed from 6.5 to 1.3 for C_4–Y, K_s was increased 20%.

Product Inhibition.—Oxidation of C_4 by Y is inhibited by crotonyl CoA, which is the probable product of the oxidation[2] (Fig. 1). Assuming the occurrence of competitive inhibition, K_I may be computed knowing the concentration of inhibitor I giving 50% inhibition $[(I)_{0.5}]$ in the manner

$$(I)_{0.5} = K_I[1 + (SH_2)/K_s] \qquad (1)$$

This relationship is based on equation 10 below, and gives a K_I for C_4–Y of 1.4 $\times$ 10^{-5} M. For C_4–G the value for crotonyl CoA inhibition similarly obtained was 3.6 $\times$ 10^{-7} M. The product inhibition in the C_{16}–Y case could only be measured indirectly. A value of 1.5 $\times$ 10^{-6} M, or about one-half the K_s value, was obtained based on the following type of experiment: when a small amount of substrate is added, and the reaction allowed to proceed to completion in a short time, then the rate which is obtained on addition of another portion of substrate is reduced in magnitude mainly because of the presence of the product. The approximate validity of this method was verified in the C_4–Y and C_4–G systems.

Assuming that the oxidations of C_4 and C_{16} take place at the same site on the enzyme, a relatively strong inhibition of C_4 oxidation by α,β-unsaturated C_{16} would be expected, since the K_s and K_I, as mentioned above, are 4.0 $\times$ 10^{-5} M and 1.5 $\times$ 10^{-6} M, respectively. When experiments were performed where Y was allowed to oxidize a small amount of C_{16} before the addition of C_4, an inhibition of the C_4 oxidation was revealed that was considerably stronger than expected. The inhibition was competitive in nature, with a K_I of 1 $\times$ 10^{-7} M. The reason for this extra inhibition beyond what was observed as product inhibition for C_{16}–Y remains obscure. A K_I of the same order of magnitude was found when C_8 was used instead of C_{16}. When potassium palmitate was tested, a K_I of the order of 10^{-3} to 10^{-4} M was indicated.

C_4 oxidation by G is not inhibited by α,β-unsaturated C_8 or C_{16}. The results of an experiment showing these relationships are given in Fig. 2. When, in the presence of Y and ETF, C_4 is added, a rapid reduction of indophenol takes place. This reaction nearly ceases as a result of the addition of a small amount of C_{16}. The reaction resumes when G is added.

On the Oxidation Potential of the Couple Butyryl–Crotonyl CoA.—Although claims have been made that a measurable equilibrium is established when indophenol enzymatically oxidizes butyryl CoA[7] or the corresponding pantetheine derivative,[15] various lines of evidence obtained during the present work showed that the reaction went practically to completion. A measurable equilibrium was consequently searched for with other dyes. Pyocyanine was found to have a suitable potential. The measurements were carried out anaerobically, and the absence of hydrase in the dehydrogenase preparation was ascertained (Table III). From the equilibrium constant observed,

TABLE III

EQUILIBRIUM CONSTANT[a]

Exp.	AH_2	A	SH_2	S	K[b]
1	0.027	0.063	0.064	0.027	0.18
2	.029	.061	.062	.029	.22
3	.029	.061	.062	.029	.22
4[c]	.050	.040	.041	.010	.30

[a] The reactions were carried out in a total vol. of 1 ml. with initially 0.090 μmole pyocyanine (A) present and 0.091 μmole C_4 (SH_2). Columns 1–4 give the amounts in μmoles of reduced pyocyanine, pyocyanine, butyryl–CoA and crotonyl–CoA after completion of the reaction caused by addition of 0.1 mg. G (no ETF). [b] $K = (AH_2)(S)/(A)(SH_2)$. [c] This experiment was carried out in the presence of hydrase (kindly given by Dr. S. Wakil). The concentration of S was computed based on the equilibrium constant for the hydrase reaction reported by J. R. Stern and A. del Campillo, *J. Biol. Chem.*, 218, 985 (1956).

(15) J. R. Stern, THIS JOURNAL, 77, 5194 (1955).

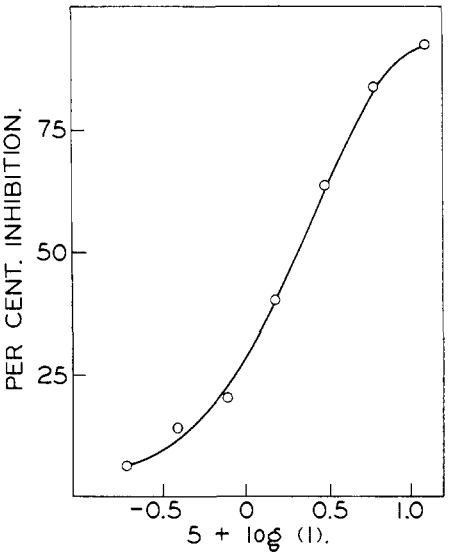

Fig. 1.—Inhibition of C_4 oxidation by crotonyl CoA. Twenty γ Y and 50 γ ETF were used together with 0.96×10^{-2} μmole C_4. The rate of the reaction was tested in the presence of variable amounts of crotonyl CoA. (I) is molarity of the latter. Other conditions as described under Experimental.

and the known E_0' of -0.034 volt for pyocyanine,[16] a E_0' of -0.015 v. was computed for butyryl CoA–crotonyl CoA (pH 7.0, 30°). This value is close to the value -0.025 v. computed by Kalckar for the butyrate–crotonate couple.[17]

Assay of Acyl CoA.—Since the reaction with indophenol goes to completion, this system furnishes a convenient and rapid assay system for the acyl derivatives of CoA, as pointed out above. The small autoöxidation of reduced indophenol that takes place can be corrected for easily. When comparison was made of the assay values for C_4 obtained by this method and the values obtained from hydroxamic acid formation[18] or SH-liberation on hydrolysis as measured by the nitroprusside test,[19] the agreement was good. The agreement was likewise good for C_{16}, where the enzymatic assay was compared to hydroxamic acid formation[10] and absorption at 260 mμ.[20]

Kinetic Constants for the Dehydrogenase–ETF Interaction.—Experiments were designed to obtain data pertaining to the action of ETF on E_pH_2 as substrate. It was not feasible to add E_pH_2 as such as substrate for the ETF reaction. Instead E_p was used under conditions where in the steady state a maximal amount of E_p would be present as E_pH_2. The concentration of ETF was adjusted so that even for the lowest level of E_p more than a 10-fold increase in the rate was obtained when ETF was subsequently added in excess. Preliminary studies under these conditions indicated that regular Michaelis–Menten kinetics[21] were followed.[3]

(16) H. A. Lardy, "Respiratory Enzymes," Burgess Publishing Co., Minneapolis, Minn., 1950, p. 80.
(17) H. M. Kalckar, *Chem. Revs.*, **28**, 711 (1941).
(18) F. Lipman and L. C. Tuttle, *J. Biol. Chem.*, **161**, 415 (1945).
(19) R. R. Grunert and P. H. Phillips, *Arch. Biochem.*, **30**, 217 (1951).
(20) The comparisons were carried out by Dr. Helmut Beinert.
(21) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

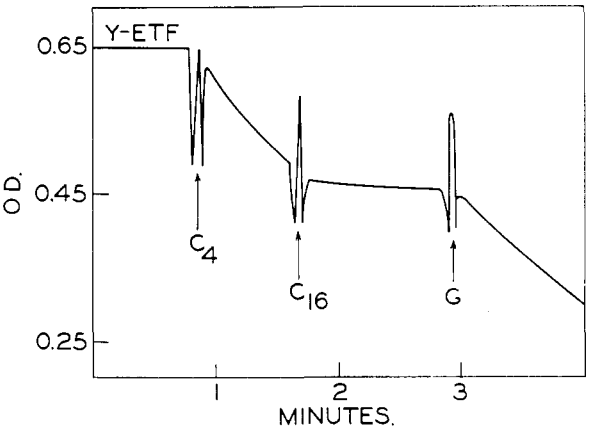

Fig. 2.—Effect of α,β-unsaturated C_{16} on C_4 oxidation by Y and G. The figure shows the OD change as recorded in a Beckman DK; 250 γ ETF and 44 γ Y was initially present in the standard test system. At the first arrow 2.9×10^{-2} μmoles C_4 was added, at the second arrow 6.6×10^{-4} μmoles C_{16}, and at the last arrow 42 γ G.

This was confirmed in more complete experiments (Fig. 3). ETF was limiting in these experiments to the degree that the ratio of total concentrations of E_p to ETF was approximately 1000-fold higher than in the experiments using ETF in excess, in spite of the fact that the over-all rates were similar. The values for V_{max} and K_s' are given in the first two columns of Table IV (Fig. 3).

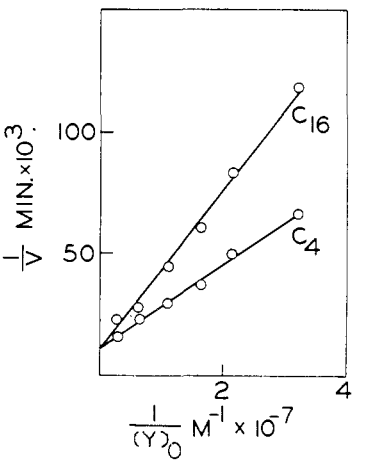

Fig. 3.—YH_2 as substrate for ETF; 3.4×10^{-5} μmole of ETF was tested with 4.8×10^{-2} μmole C_4 or 1.6×10^{-2} μmoles C_{16} in the presence of variable amounts of Y. Other conditions as described under Experimental.

It was found that under these circumstances of low ETF concentration the rate was not zero order with respect to indophenol. The concentrations of indophenol that gave half maximum velocity are given in the third column. These values are largely independent of the nature of E_pH_2, as would be expected. Using these data to correct the V_{max}' observed to the velocity for saturation both with respect to E_pH_2 and indophenol, the TN_s' values of the fourth column are obtained. The TN_s' values are thus of the same order of magnitude as the TN_s

TABLE IV

KINETIC CONSTANTS FOR THE DEHYDROGENASE–ETF INTERACTION[a]

Enzyme–substrate	V'_{max}, min.$^{-1}$	$K_s' \times 10^7$, M	$K_s'' \times 10^5$, M	TN_s', min.$^{-1}$
C_4–G	84	3.7	7.8	260
C_4–Y	91	1.6	7.8[b]	280
C_{16}–Y	91	3.0	7.8	280
C_8–Y′	91	2.2	7.8[b]	280
C_{16}–Y′	100	4.5	7.3	295

[a] 2.4×10^{-5} μmole ETF used for G and Y′, 3.4×10^{-5} μmole ETF used for Y for the establishment of V'_{max} and K'_s. The dehydrogenase concentrations were varied within a 10-fold range of concentrations that included the K'_s. The amounts of C_4, C_8 and C_{16} used were, respectively, 4.8×10^{-2}, 0.67×10^{-2} and 1.6×10^{-2} μmole. V'_{max} was obtained with the standard indophenol concentration of 4×10^{-5} M. The dependence of the rate on indophenol concentration was then tested to obtain K_s'' and TN_s'. Other conditions as described under Experimental. [b] These values were assumed equal to 7.8×10^{-5} M.

values, and the K_s' constants indicate a very high affinity of ETF for the reduced dehydrogenases.

It is noted in Fig. 3 and Table IV that different values were found for K_s' for YH_2 as substrate depending on whether C_4 or C_{16} was the reducing agent. Likewise, when C_8 and C_{16} were used with Y′, the K_s' values were not the same. The turnover numbers TN_s' appear, however, to be little affected by the nature of the reducing substrate.

Dependence of Rates upon *p*H and Buffer.—A study of the effect of *p*H on maximal velocities and Michaelis constants was not undertaken. Rather, information was sought on the position of the *p*H optimum for two cases only, *viz.*, when ETF is in excess and when ETF is limiting. As is shown in Table V, the optimal *p*H varies from 7.6 to 8.4 for high ETF concentration, whereas the *p*H peak is shifted to 6.8 or 6.9 in all cases when ETF is low. When the combination phenazine–cytochrome *c* was used as the electron acceptor system, data close to those for ETF excess were observed or indicated. It was not possible to go beyond *p*H 8 with this system because of the reduction of cytochrome *c* by oxidized phenazine.

TABLE V

*p*H OPTIMA[a]

Enzyme–substrate	ETF excess	ETF limiting	Phenazine excess
C_4–G	7.6	6.9	7.5
C_4–Y	8.0	6.8	8
C_{16}–Y′	8.4	6.8	8

[a] The rates with ETF and indophenol were recorded from *p*H 6.25 to 9.75, taking the indophenol–leucoindophenol equilibrium into account. The buffers used were phosphate, Tris and glycine. All buffer concentrations were 0.02 M. For ETF in excess, 200 γ ETF and 4 γ Y were used, whereas for ETF limiting 12 γ ETF and 40 γ Y. 100 γ phenazine were used with G, 300 γ with Y and Y′. C_{16} and C_4 were added in amounts of 1.6×10^{-2} and 3.3×10^{-2} μmole, respectively. Other conditions as described under Experimental.

The rate was found higher at *p*H 8 when phosphate was replaced by Tris or glycine as buffer for C_4–Y. This effect was less pronounced for C_{16}–Y, and not noticeable at all with C_4–G.

Mechanism of the Reaction.—The results given above show that the rate increases toward a maximum when either substrate concentration (SH_2), the acceptor concentration (A), the total concentration of dehydrogenase $(E_p)_0$, or the total concentration of ETF $(E_t)_0$ is increased while the others are held constant.[22] If $(E_p)_0$ and $(E_t)_0$ are increased simultaneously, a saturation behavior is, however, not observed. These observations require an empirical rate law of the form

$$V = \frac{k(E_t)_0(E_p)_0}{1 + K_a/(SH_2) + K_b/(A) + K_c(E_t)_0 + K_d(E_p)_0} \quad (2)$$

From the kinetic data it is possible to calculate the five constants of this expression for the various dehydrogenase–acyl CoA combinations. It is more convenient, however, first to find a reaction mechanism that satisfies this rate law, and then relate the observed kinetic data directly to the constants of the mechanism. The following is such a mechanism (mechanism I)

$$SH_2 + E_p \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SH_2E_p \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_pH_2 + S \quad (3)$$

$$E_pH_2 + E_t \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E_pH_2E_t \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} E_p + E_tH_2 \quad (4)$$

$$E_tH_2 + A \underset{k_2''}{\overset{k_1''}{\rightleftharpoons}} E_tH_2A \underset{k_4''}{\overset{k_3''}{\rightleftharpoons}} E_t + AH_2 \quad (5)$$

This reaction sequence is supported by additional evidence[2,3]: E_p is reduced by SH_2 in the absence of ETF, and ETF in substrate amounts is reduced by SH_2 in the presence of catalytic amounts of E_p.

In carrying out the steady-state treatment[23] for this sequence of reactions, which in the present formulation involves seven intermediates whose concentrations are to remain constant, it is assumed that SH_2 and A are present in high enough concentrations to make the difference between their free and total concentrations negligible. It is assumed also that the reversal of the steps (3–5) gives a negligible contribution to the initial steady-state rate.[24] Under these conditions, the resulting expression that relates the velocity V of the over-all reaction to the four variables (SH_2), (A), $(E_p)_0$ and $(E_t)_0$ can be shown to be

$$[(E_p)_0 - V(K/(SH_2)k_3 + 1/k_3 + 1/k_3')] \times [(E_t)_0 - V(K''/(A)k_3'' + 1/k_3'' + 1/k_3')] = VK'/k_3' \quad (6)$$

where $K = (k_2 + k_3)/k_1$, $K' = (k_2' + k_3')/k_1'$ and $K'' = (k_2'' + k_3'')/k_1''$. Since the expression is quadratic, it is not advantageous to render it explicit in V. This expression fulfills the requirements stated above that were the basis for expression (2). Thus when $(E_t)_0$ is very large, we obtain

$$V = \frac{(E_p)_0k_3}{K/(SH_2) + 1 + k_3/k_3'} = \frac{V_{max}}{K_s/(SH_2) + 1} \quad (7)$$

The expression for the maximal rate with (SH_2) as

(22) $(E_t)_0$ and $(E_p)_0$ are total molar concentrations of enzymes, not active sites or active forms. It should therefore be borne in mind that the data obtained apply to a certain set of experimental conditions (*cf.* Experimental).

(23) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

(24) Such an assumption may not always be justified. Indeed, in the experiments where ETF was rate limiting, a condition was intentionally sought where step (3) would be practically in an equilibrium condition. This leads to a modified rate expression (11) given below.

the variable is quadratic unless $(E_t)_0$ at the same time is assumed to be very large

$$V = \frac{(E_p)_0 k_3}{k_3/k_3' + 1} = TN_s(E_p)_0 \quad (8)$$

If an acceptor dye A′ were used instead of E_t, the rate expression is derived from (6) by setting $(E_t) = (E_t)_0 = (A')$

$$V = \frac{(E_p)_0 k_3}{K/(SH_2) + K'k_3/(A')k_3' + 1 + k_3/k_3'} \quad (9)$$

An equivalent expression was derived by Alberty as a special case in his treatment of various mechanisms for the reaction $A + B \rightleftarrows C + D$.[25] When a competitive inhibitor is present which establishes the equilibrium $(E_p)(I)/(E_pI) = K_I$, an additional term must be added in the denominator of (9). This term is $K(I)/K_I(SH_2)$. For (A′) very large, it follows then that

$$V_{max}/V = 1 + K_s/(SH_2) + K_s(I)/K_I(SH_2) \quad (10)$$

This relationship is the same as that developed by Lineweaver and Burk,[14] except for the definition of K_s that is given implicitly in equation 7 above, and explicitly in (12).

If we for the condition of ETF being present in limiting concentration take the equilibrium concentration of E_pH_2 as the concentration of the substrate for ETF (*cf.* ref.), the rate equation will be analogous to (9)

$$V = \frac{(E_t)_0 k_3'}{K'/(E_pH_2)_{eq.} + K''k_3'/(A)k_3'' + 1 + k_3'/k_3''} \quad (11)$$

Another mechanism (mechanism II) which in a qualitative way also is seen to lead to the rate-law given in equation 2 is

$$E_p + E_t \rightleftarrows E_pE_t \quad (a)$$

$$SH_2 + E_pE_t \rightleftarrows SH_2E_pE_t \rightleftarrows S + E_pE_tH_2 \quad (b)$$

$$E_pE_tH_2 + A \rightleftarrows AE_pE_tH_2 \rightleftarrows AH_2 + E_pE_t \quad (c)$$

The spectral studies cited above would favor mechanism I.[2,3] There is in addition some kinetic evidence that tends to exclude II in favor of I. This evidence comes partly from a comparison of the rates obtained when ETF was limiting and E_p present in saturating amounts with those which apply when ETF was present in excess (Tables II, IV). V_{max} values of 91 and 405 min.$^{-1}$, respectively, were, for example, found in the C_8–Y′–E_t–A system. The same V_{max} should have been reached in the two cases if $E_p + E_t \rightleftarrows E_pE_t$ were the first step, SH_2 reacting only with E_pE_t. Furthermore, the reaction is practically zero order with respect to (A) over a large range of (A) when ETF is present in excess. This is not so for the same range of (A) when ETF is present in limiting concentrations. Such behavior is expected in mechanism I but not in II.

Mechanism I (eq. 3–5) may be modified in some details and still correspond to the observations. The substrate in the second step might for instance be SE_pH_2 rather than E_pH_2. The constant k_3 would then be the rate of formation of SE_pH_2 from SH_2E_p.

Evaluation of the Experimental Kinetic Constants.—Equation 8 bears out an important point that is characteristic for the kind of enzyme system studied. The maximal velocity $TN_s \times (E_p)_0$ obtained for saturation with both substrate and acceptor is not equal to the turnover number k_3 times the total enzyme concentration. TN_s is less than k_3 by a factor of $(k_3/k_3' + 1)$. This is understandable when we realize that increasing the ETF concentration has two opposite effects on the total rate. The reaction rate will be increased because there is more enzyme available to oxidize reduced dehydrogenase. However, increasing (ETF) also leads to a decrease in the steady-state concentration of E_pH_2, the substrate for ETF. These two effects balance out before the maximal rate of the primary dehydrogenation has been reached, unless $k_3/k_3' \ll 1$. As seen in eq. 9, the factor $(1 + k_3/k_3')$ is involved in the same manner when an acceptor dye A′ is used to reoxidize the reduced dehydrogenase. The rate will here increase with increasing dye concentration until E_pH_2 becomes saturated.

It is furthermore seen that the traditional Michaelis constant[21,23] $K = (k_2 + k_3)/k_1$ is not observable. What is determined on varying (SH_2) in the presence of excess ETF is $K_s = K \times k_3'/(k_3 + k_3')$. The k_3/k_3' ratio is unknown and unavailable experimentally with the present technique which only measures the over-all reaction.

The same problem presents itself in the evaluation of the data for the flavoprotein interaction (Table IV). In addition, we do not know to what extent the dehydrogenase is present in the form of E_pH_2. Even though (E_p) may be insignificant, the concentrations of the enzyme–substrate complexes are not necessarily also small. The values of $(E_p)_0$ that give half maximal velocity are in consequence to be regarded as the upper limit for the concentration of E_pH_2.

Discussion

Of the two basic mechanisms I and II that were proposed, the present evidence favors the first. Although mechanism I gives a satisfactory framework for the observed data, and the data were interpreted in terms of this scheme, it is not excluded that some other satisfactory mechanism could be devised.

A notable characteristic of the primary dehydrogenation step is the marked product inhibition observed. K_I for crotonyl CoA inhibition in C_4 oxidation by G was found to be $^1/_6$ of the observed K_s for this reaction. Similar inhibitions were noted for C_4–Y and C_{16}–Y. Since crotonyl CoA most likely is the product of C_4 oxidation, the role of these enzymes in the reductive direction is thus indicated.

The velocity of interaction of the two flavoproteins is remarkably high considering the complexity of the reacting species. The observed turnover numbers of about 280 min.$^{-1}$ are minimal values that may be several fold larger, depending on the relative values of k_3' and k_3''. The apparent Michaelis constants are of the magnitude 3×10^{-7} M, indicating a high affinity of ETF for E_pH_2. It would, in fact, hardly have been feasible to determine these Michaelis constants by measuring indophenol reduction if it were not for the fact that the substrate E_pH_2 was generated in the cuvette as it was utilized, and kept at a constant level.

(25) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953).

The dependence of the apparent Michaelis constants for the flavoprotein interaction on the nature of the fatty acyl CoA may reflect differences in the stability constants of the enzyme–substrate complexes. For the same total amount of dehydrogenase and an excess of substrate, differing relative concentrations of SH_2E_p and E_pH_2 can be expected. If the variation in the K_s' reflects this variable E_pH_2 concentration, a maximal velocity should be obtained that does not depend on the nature of the reducing substrate. The data of Table IV support this thesis.

The variables that depend on *p*H and in turn determine the rate are numerous. At the peak of the rate *vs.* *p*H curve for ETF in excess, the optimal relative concentrations of the active species of E_pH_2, E_p, ETF and SH_2 will be present. Since no change in the optimum could be observed when phenazine–cytochrome *c* was substituted for ETF–indophenol, E_p, E_pH_2 and SH_2 are the most likely determining components. These *p*H optima were found to be dependent on the nature of E_p, ranging from *p*H 7.6 to 8.4. For ETF being limiting, only one *p*H optimum was observed, at *p*H 6.8 to 6.9. This may thus reflect that dissociations involving ETF influence the process of reduction by E_pH_2 and reoxidation by indophenol.

The application of these data in more than a qualitative way to the *in vivo* situation would require knowledge of the actual concentrations of the substrates and enzymes. Furthermore, since these mitochondrial flavoproteins are part of a heterogeneous complex of enzymes, their relative localizations will be of even greater influence than the actual concentrations in determining the rate picture. A close *in vivo* association of the flavoproteins studied is indicated by the great difficulty encountered in the process of their mutual separation.[2,3,5]

Some properties of general nature concerning sequences of oxidation–reductions such as described in eq. 3–5 were brought to attention by the present work. The assay of the activity of such enzymes has certain complications. Intuitively it is felt that when substrate and acceptor systems are present in excess, the steady-state rate will directly measure the activity of the enzyme assayed. This is true in the case of a sequence of enzymatic reactions such as

$$S \xrightarrow{E_1} P_1 \xrightarrow{E_2} P_2 \xrightarrow{E_3} P_3$$

where the enzymes are not dependent on each other for their action. For a sequence of oxidation–reduction reactions catalyzed by enzymes having tightly bound prosthetic groups the situation is, however, a different one. As pointed out above, the turnover numbers observed in the presence of excess substrate and acceptor differ from the real values by a factor that depends on the relative magnitude of the k_3 and k_3' constants. It is a consequence of this situation that a series of different turnover numbers for one and the same substrate and enzyme is to be expected when a series of electron acceptors is tested. This is illustrated in Table II above, and also, for example, by the data of Mahler, *et al.*, for a number of flavoproteins where various electron acceptors were compared.[26] Superimposed on this effect on k_3' caused by the change of acceptor may be a variable inhibition or activation of the enzyme. It is moreover to be expected that for a series of acceptors, a series of differing apparent Michaelis constants K_s will be observed for the same substrate. These constants will all be lower than the traditional $(k_2 + k_3)/k_1$ value by the undetermined factor $(1 + k_3/k_3')$, assuming that possible inhibitory or activating influence by the acceptor has been taken into account.

Although K and k_3 remain unknown, differing from the experimental K_s and TN_s by factors that cannot be determined by a method of measuring multistep reactions, K_s and TN_s are valid kinetic constants in their own right. Their definition in terms of the rate constants of the mechanism is

$$TN_s = \frac{k_3k_3'}{k_3 + k_3'}, \text{ and } K_s = \frac{(k_2 + k_3)k_3'}{(k_2 + k_3')k_1} \qquad (12)$$

These may be the constants that are often obtained for enzymes with tightly bound prosthetic groups. They depend on the acceptor as much as on the substrate, and should consequently be understood as kinetic characteristics of the total substrate–enzyme–acceptor system.

Acknowledgments.—The author wishes to thank Dr. D. E. Green for his interest in this work. The author is also indebted to Dr. H. Beinert and Dr. F. L. Crane for their encouragement and advice, and to Dr. R. A. Alberty of this University for many stimulating discussions. The skilled assistance of Mrs. Mildred Van De Bogart is gratefully acknowledged. The investigation was supported by a research grant from the National Science Foundation to Dr. H. Beinert. Oscar Mayer and Co. generously provided the tissues used in this work.

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(26) H. R. Mahler, A. S. Fairhurst and B. Mackler, THIS JOURNAL, **77**, 1514 (1955).