Kinetics of Nicotinamide Nucleotide Transhydrogenation by Human Placenta

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1. Measurements of kinetic constants for a purified preparation of human-placenta oestradiol dehydrogenase have been made. 2. These constants have been compared with similar measurements made on crude ammonium sulphate precipitates of human-placenta homogenates. The comparison indicates that nearly half of the observed nicotinamide nucleotide-transhydrogenation activity in the crude preparations is due to a specific oestrogen-dependent transhydrogenase. 3. The remainder of the observed activity results from a substrate-mediated transhydrogenation catalysed by the oestradiol-dehydrogenase activities present in the preparation.

Evidence has been presented for the existence in human term placenta of a specific oestrogen-dependent nicotinamide nucleotide transhydrogenase (Hagerman & Villee, 1959). This enzyme (a transhydrogenase) catalyses the transfer of hydrogen from one nicotinamide nucleotide to another without simultaneous oxidation or reduction of any dissociable non-nicotinamide nucleotide reactant. It has also been demonstrated that highly purified oestradiol-17 β dehydrogenase from human term placenta can catalyse a substrate-mediated transhydrogenation reaction (Jarabak, Adams, Williams-Ashman & Talalay, 1962). In this case an enzyme with a primary dehydrogenase function catalyses transhydrogenation by a reversible oxidation and reduction of its primary substrate by using different nicotinamide nucleotides in the forward and reverse directions.

An oestrogen-dependent nicotinamide nucleotide transhydrogenase has been isolated from human myometrium, and this enzyme was conclusively demonstrated to possess no 17 β -hydroxy steroid-dehydrogenase activity (Abe, Hagerman & Villee, 1964). It has not been possible to prepare in bulk a similar enzyme from human placenta, although small amounts of such an enzyme have been obtained (Hagerman & Villee, 1959). The kinetic analysis described below was undertaken to improve the method of assay of these transhydrogenating systems in the hope that more definitive assays would assist in the preparation of the enzyme.

THEORY

An analytic solution for the general case of cyclic reaction systems was obtained by Hearon (1948), who showed that such systems, under certain specific restrictions, follow Michaelis-Menten kinetics. If one or two dehydrogenases with appropriate substrate specificity individually follow Michaelis-Menten kinetics, it can readily be shown either from first principles or from algebraic manipulation of a pair of Michaelis-Menten functions that the velocity of a dehydrogenase-catalysed substrate-mediated transhydrogenation as a function of substrate concentration follows an equation of the form:

$$v = \frac{Vs}{s + K}$$

where

$$V = \frac{V_1 V_2}{V_1 + V_2}$$

and

$$K = \frac{K_2 V_1 + K_1 V_2}{V_1 + V_2}$$

in which the subscripts identify the kinetic constants of the dehydrogenase activities. That is, the maximal velocity of substrate-mediated transhydrogenation, V , is a simple function of the reciprocals of the maximal velocities, V_1 and V_2 , of the individual dehydrogenation reactions, whereas the apparent Michaelis-Menten constant of the transhydrogenation system, K , is equal to the velocity-weighted mean of the Michaelis constants, K_1 and K_2 , of the two dehydrogenase reactions. For a transhydrogenation catalysed by a single dehydrogenase, the cited result is exact; for a transhydrogenation catalysed by two dehydrogenases, the exact equation contains quadratic and cubic terms that can be neglected for all values of the constants

Table 1. *Comparison of experimentally observed and calculated rates of substrate-mediated nicotinamide nucleotide transhydrogenation*

Results from the literature were converted into absolute rates at comparable pH and temperatures, arbitrarily assuming a purity of about 0.5% and molecular weights of 10^5 for the steroid dehydrogenases to give consistent units for the constants. The rates of transhydrogenation were then calculated from the equations given in the text in the form where $V = k(E_t)$.

Enzyme	Observed	Calculated	References
Glutamate dehydrogenase	1.5×10^{-6}	8.5×10^{-6}	Holzer & Schneider (1958); Frieden (1959); Olsen & Anfinsen (1953)
3- β -Hydroxy steroid dehydrogenase	1.6×10^{-8}	1.7×10^{-8}	Hurlock & Talalay (1958); Tomkins (1956)
Mixed NAD- and NADP-linked testosterone dehydrogenases:			
Expt. 1	Nil	1.6×10^{-10}	Villee & Spencer (1960)
Expt. 2	1.7×10^{-8}	3.5×10^{-8}	

and of the variables that can be attained under ordinary experimental conditions. The co-substrate of the dehydrogenase reactions is assumed to be present in saturating concentrations.

Table 1 summarizes a comparison of calculated rates of substrate-mediated transhydrogenation with the experimental results for the three applicable cases available in the literature. The transhydrogenation mediated by lactate dehydrogenase (Holzer & Schneider, 1958) is not suitable for theoretical treatment because of the marked inhibition of this enzyme by its substrate. The agreement between the values observed experimentally and those predicted on this theoretical basis suggests that the theory is of general applicability. The kinetics of direct activation of enzymes follows a Michaelis-Menten function of the activator concentration and has been discussed by several authors (cf. Ogston, 1955).

MATERIALS AND METHODS

Enzymes. Oestradiol-17 β dehydrogenase was prepared from human placenta by the procedure of Jarabak *et al.* (1962). The preparation was carried through to the end of stage 5 of the published procedure and the resulting stable enzyme was used for the kinetic experiments.

For comparison with the stage 5 enzyme, simple ammonium sulphate precipitates of the soluble enzymes from human term placenta were prepared as follows (all operations were performed at 0–2°). Placentas were placed on ice within 5 min. of delivery. The placental parenchyma was dissected away from gross connective tissue and was homogenized in 2 vol. of a solution of 0.25 M-sucrose in 5 mM-sodium phosphate buffer, pH 7.0, containing EDTA (1 mM). Homogenization was performed on 100 g. lots of placenta in the 1-quart-size Waring Blender for 60 sec. at 60 v. The resulting homogenate was centrifuged for 20 min. at 5000g. The supernatant fluid was decanted and recentrifuged for 1 hr. at 57000g. The resulting clear supernatant solution was dialysed overnight against 10 vol. of the homogenizing medium. The dialysed solution was then fractionated with solid ammonium sulphate. Quantities of ammonium sulphate were calculated from the Table provided by Umbreit, Burris & Stauffer (1957). Sufficient 3.75 M-ammonium sulphate solution, pH 6.8, was added to bring the concentration of the salt in the enzyme solution to 1.2 M, the addi-

tion being made over a 20 min. period with continuous stirring. Stirring was continued for a further 20 min., and the solution was then centrifuged at 8000g for 20 min. The supernatant fluid was decanted and brought to 1.6 M with respect to ammonium sulphate by addition of the solution as before. After centrifugation the precipitated protein was suspended in 20% (v/v) glycerol in 25 mM-sodium phosphate buffer, pH 7.0, containing EDTA (1 mM), to give a final volume about 2% of that of the original homogenate. Transhydrogenation assays of this material were completed within 6 hr. after suspension of the precipitate, to avoid possible loss of labile enzymic activity.

Enzyme assays. Enzyme activities were determined by measuring the formation of reduced nicotinamide nucleotide in a Beckman model DK-2 recording spectrophotometer at a wavelength of 340 m μ and a scanning speed of 1 in./min. The temperature of the cell compartment was maintained at $25 \pm 0.5^\circ$. All solutions except the enzyme were brought to this temperature before addition to the incubation mixture.

(a) Dehydrogenase activities. The reaction was carried out in a total volume of 15.0 ml. (light-path 50.0 mm.). Each cuvette contained 600 μ moles of tris-HCl buffer, pH 7.4, 50 μ moles of MgCl₂, various quantities of oestradiol-17 β or oestrone dissolved in 0.5 ml. of propylene glycol-ethanol (1:1, v/v), enzyme and either 1.5 μ moles of NAD⁺ or 0.5 μ mole of NADPH. The nicotinamide nucleotide was omitted from the blank; the reaction was started by the addition of enzyme. Preliminary experiments showed that these concentrations of nicotinamide nucleotide were sufficient to saturate the system; more nicotinamide nucleotide did not cause the reaction to go faster, nor was the amount large enough to cause substrate inhibition of the reaction. All the reaction velocities were calculated from the initial linear portion of the extinction recording, and the amount of enzyme was made sufficiently small to ensure absolute proportionality between reaction rate and amount of added enzyme at a given steroid concentration. Separate control experiments demonstrated that the observed rate of reaction was unaffected by the organic solvents used to maintain the steroids in true solution. No oxidation or reduction of nicotinamide nucleotide occurred in the absence of added steroid.

(b) Transhydrogenation activity. The reaction was carried out in a total volume of 3.0 ml. (light-path 10.0 mm.). Each cuvette contained 300 μ moles of tris-HCl buffer,

pH 7.4, 10 μ moles of $MgCl_2$, 10 μ moles of glucose 6-phosphate, 5 Kornberg units of glucose 6-phosphate dehydrogenase, 0.01 μ mole of NADP, 1 μ mole of NAD^+ , various amounts of oestradiol-17 β , oestrone or oestradiol-oestrone mixtures in propylene glycol-ethanol (1:1, v/v) and enzyme. NAD^+ was omitted from the blank cuvette and the reaction was initiated by the addition of NAD^+ to the experimental vessel. Control experiments showed that these concentrations of nicotinamide nucleotides gave maximum values for the velocity of NADH production; other experiments demonstrated that the presence of the constant amounts of organic solvents used did not influence the rate of the transhydrogenation. Appropriate controls for the various reagents and ancillary enzymes were included in each set of determinations. The rate of NADH production was calculated from the initial linear portion of the extinction recording; sufficiently small samples of enzyme were taken to ensure that the rate of reaction was absolutely proportional to the amount of enzyme added at a given steroid concentration.

Reagents. Glucose 6-phosphate dehydrogenase was type V from Sigma Chemical Co., St Louis, Mo., U.S.A.; occasional lots of this enzyme preparation catalysed the slow reduction of NAD^+ in the presence of glucose 6-phosphate, and where necessary this blank was subtracted from the observed reaction velocity. Nicotinamide nucleotides were purchased from Sigma Chemical Co. Steroids were obtained from commercial sources and were verified as to identity and purity by paper or thin-layer chromatography in at least two different solvent systems. All solutions were prepared in glass-redistilled water; the ammonium sulphate used was the 'enzyme grade' sold by Mann Research Laboratories, New York, N.Y., U.S.A. Other chemicals were American Chemical Society specification Analytical Reagent quality or better. Organic solvents were purified as necessary by conventional procedures and redistilled before use.

RESULTS

Kinetic constants for the stage 5 enzyme acting as a dehydrogenase. Dehydrogenase activity was determined in the described assay system at a variety of steroid concentrations (oestradiol, 0.1–33 μ M; oestrone 0.1–10 μ M) in the direction of oestradiol oxidation with NAD^+ as hydrogen acceptor and in the direction of oestrone reduction with NADPH as the hydrogen donor. The results were analysed by the statistical procedure described by Wilkinson (1961), and the results are collected in lines 1, 2 and 3 of

Table 2. An analysis of variance of s/v as a function of s for both reactions showed no statistically significant deviations from linearity, demonstrating that the system follows strict Michaelis-Menten kinetics over the range of substrate concentrations used. An estimate of the Michaelis constant for NADPH in the direction of oestrone reduction was also obtained. In the determination of this constant 125 mg. of purified human serum albumin was added to the standard reaction mixture to give complete solution of the certainly saturating concentration of 0.1 mM-oestrone used and the concentration of NADPH was varied. Since the calculated K_m for NADPH was near the lowest practicable limit of concentration of reduced nucleotide when the spectrophotometric method was used, the value was verified in the following way. Reaction mixtures were prepared that contained, in a total volume of 3.0 ml., 300 μ moles of tris-hydrochloric acid buffer, pH 7.4, 10 μ moles of magnesium chloride, 25 mg. of human serum albumin, 0.3 μ mole of oestrone, 1 μ mole of glucose 6-phosphate, 5.0 units of glucose 6-phosphate dehydrogenase and the placental enzyme preparation. The reaction was initiated by the addition of NADPH in amounts sufficient to give final concentrations of 0.33 μ M-, 0.1 μ M- and 0.033 μ M-nicotinamide nucleotide and the vessels were incubated at 25° for exactly 20 min. At the end of the incubation the reaction was stopped by placing the vessels in a boiling-water bath for 5 min. After cooling, samples were taken for determination of the amount of glucose 6-phosphate remaining by spectrophotometric measurement of its reducing power in the presence of excess of NADP and glucose 6-phosphate dehydrogenase (Kornberg & Horecker, 1955). Under the experimental conditions described, the rate of disappearance of glucose 6-phosphate in this regenerative assay is identical with the rate of oestrone hydrogenation. At the concentrations of NADPH used, the experimentally observed reaction rates were always within one standard error of the value calculated from the spectrophotometrically determined constants for the same enzyme preparation.

Table 2. *Kinetic constants for purified human-placenta oestradiol dehydrogenase*

Values for K_m and V are given as means $\pm$ S.E.M.

Reaction	Variable	$10^7 \times K_m$ (M)	V (μ moles of reduced nicotinamide nucleotide/min./mg. of protein)
1. NADPH + oestrone $\rightarrow$ NADP + oestradiol	NADPH	7.9 ± 1.1	—
2. NADPH + oestrone $\rightarrow$ NADP + oestradiol	Oestrone	1.6 ± 2.5	41.5 ± 5.8
3. NAD^+ + oestradiol $\rightarrow$ NADH + oestrone	Oestradiol	28 ± 3	127.8 ± 4.1
NADPH + NAD^+ $\rightarrow$ NADP + NADH			
Oestradiol or oestrone:			
4.	Observed	1.5 ± 0.2	29.6 ± 1.2
5.	Calculated (see the text)	7.1 ± 9.0	26.6 ± 0.8

From a comparison of the value for this constant given in Table 2 (line 1) with the concentration of NADPH used in the standard transhydrogenation assay, it is evident that the latter amount is almost sufficient to saturate the dehydrogenation reaction in the direction of oestrone reduction. The necessary 21% correction in the calculated maximal velocity of substrate-mediated transhydrogenation has been made wherever required in the subsequent calculations.

Kinetic constants for substrate-mediated transhydrogenation by stage 5 enzyme. Measurements of the rate of transhydrogenation catalysed by the stage 5 enzyme were made with various amounts of added oestrone, oestradiol or oestrone-oestradiol mixtures calculated to give final concentrations from 25 to 750 μM . The enzyme preparation contains a small amount of oestrone or oestradiol (or both), so that appreciable rates of transhydrogenation are observed even when no oestrogen is added. An estimate of the concentration of this endogenous oestrogen was made by extracting a portion of the enzyme successively with ethanol, acetone and methylene chloride, treating the solvent-soluble residue with ^{14}C -labelled acetic anhydride of high and known specific activity, adding carrier oestrone acetate and oestradiol acetate, purifying the mixture by solvent partition and thin-layer chromatography and determining the amount and the specific activity of the isolated pure derivative. The analysis was controlled by carrying known similar amounts of steroid through the entire procedure. From these results the total oestrone plus oestradiol concentration in the enzyme solution used was calculated to be 50 μM . The dehydrogenase assays were performed with very small amounts of enzyme solution, so that the endogenous steroid could be neglected in those assays. The transhydrogenation assays required larger amounts of enzyme and it was desirable to evaluate the kinetics at low steroid concentrations; the concentration of steroid present during the assay was therefore corrected for the amount contributed by the enzyme preparation. The correction was always less than 10%, and the transhydrogenation assays were thus done at effective steroid concentrations of 27 to 752 μM . Analysis of the results in the form of s/v as a function of s gave results statistically indistinguishable from a straight line, the least-squares quadratic equation for the results being:

$$s/v = 11.7 + 0.0029s + 0.0000024s^2$$

where the units of s are μM and of v are units of enzyme activity/mg. of protein. Analysis of the errors associated with this equation gave a variance of 37.716 (27 degrees of freedom) for the deviations from linear regression and 37.460 for the deviations from curvilinear regression, which is a statistically

non-significant decrease. Further calculation (Wilkinson, 1961) gave the observed values for K and V shown in Table 2 (line 4). Line 5 of Table 2 gives the calculated values of the same constants, obtained by substituting the dehydrogenase constants for this enzyme preparation into the formulae already given. The observed and calculated values are statistically indistinguishable. The results of the kinetic measurements for this enzyme preparation are therefore consistent with the hypothesis that the whole of the experimentally observed transhydrogenation catalysed by the purified dehydrogenase is the result of substrate-mediated dehydrogenase-catalysed transfer of hydrogen from one nicotinamide nucleotide to another (Jarabak *et al.* 1962).

Dehydrogenation and transhydrogenation reactions catalysed by crude ammonium sulphate precipitates of placenta. Experimental measurements of transhydrogenation activity were made on enzyme preparations from five placentas, exactly as described for the measurements with the purified enzyme. The endogenous steroid concentration of a pooled sample of these preparations, measured by isotope dilution, gave a value of 10 μM , so that the transhydrogenation measurements were made at effective steroid concentrations from 30 to 700 μM . The results obtained were plotted in the form $1/v$ as a function of $1/s$ and gave a smooth non-linear curve, grossly concave downwards. This result made it clear that transhydrogenation by these preparations did not conform to simple Michaelis-Menten kinetics, and the results were further analysed as described below.

These same placental preparations were assayed for dehydrogenase activity at a variety of steroid concentrations in both the direction of oestradiol oxidation and oestrone reduction, by using experimental procedures identical with those employed with the purified dehydrogenase. The Michaelis constants calculated by Wilkinson's (1961) procedure from these results for the dehydrogenase activity of the crude placental preparation are given in Table 3 (lines 2 and 3). The value for NADPH-linked dehydrogenase activity in this preparation is not statistically different from that found for the same constant in the purified preparation (line 2 in Table 2), whereas that for NAD-linked activity is only one-third as great. The more reliable values obtained with the purified preparation were used in subsequent calculations. The maximal velocities, expressed per mg. of protein, were lower as anticipated for these impure preparations.

The rate of substrate-mediated transhydrogenation calculated from the kinetic constants for dehydrogenase activity in the impure preparations was compared with the experimentally observed rate of transhydrogenation in the same preparations. A consistent excess of the observed over the calcu-

Table 3. *Kinetic constants for crude ammonium sulphate precipitates of human term placenta*Values for K_m and V are given as means $\pm$ S.E.M.

Reaction	Variable	$10^8 \times K_m$ (M)	V (μ moles of reduced nicotinamide nucleotide/min./mg. of protein)
2. NADPH + oestrone $\rightarrow$ NAD ⁺ + oestradiol	Oestrone	32 ± 17	4.6 ± 0.5
3. NAD ⁺ + oestradiol $\rightarrow$ NADH + oestrone	Oestradiol	100 ± 21	32 ± 2
4. NADPH + NAD ⁺ $\rightarrow$ NADP ⁺ + NADPH	Oestradiol or oestrone (substrate-mediated transhydrogenation: calculated)	$15 \pm 2^*$	$3.3^\dagger$
5.	(Residue after correction for substrate-mediated transhydrogenation)	1.8 ± 0.8	2.6 ± 0.2

* From line 4 in Table 2.

† Calculated from dehydrogenase V values above.

lated rate at all concentrations of steroid suggested that the observed non-linearity of the transhydrogenation reciprocal plot might be due to the presence of more than one transhydrogenating system in the crude enzyme preparation (Reiner, 1959). The rates of substrate-mediated transhydrogenation for the crude enzyme were calculated at various steroid concentrations by using the experimentally determined K for substrate-mediated transhydrogenation given in Table 2 (line 4) and the maximal velocities determined experimentally in the dehydrogenase assays of the crude enzyme preparations given in Table 3 (lines 2 and 3). (Use of the calculated values of K for substrate-mediated transhydrogenation derived from the kinetic constants of either the crude or purified enzyme preparation in this further analysis of the results did not appreciably alter the final results.) The resulting values were subtracted from the observed velocities of transhydrogenation. The velocity of the transhydrogenation reaction unaccounted for by dehydrogenase-catalysed substrate-mediated transhydrogenation gave a statistically linear plot of s/v as a function of s . These residuals were fitted to the Michaelis-Menten function by the method of least squares. The results of the calculations are set out in Table 3 (line 5). The error associated with the K for transhydrogenation that is not substrate-mediated is large because of the large number of calculations involved, but the value of the K for the non-substrate-mediated process is clearly statistically distinct from the apparent K for the substrate-mediated process. The value is also statistically different from zero.

DISCUSSION

Various possible difficulties in these experiments must be examined. Substrate concentrations were

of necessity low, which led to greater than desirable variability in the analyses, principally because the observed changes in extinction were small. Thus evaluation of the results gave rather large estimates of error for some of the constants, and these errors are further magnified by the arithmetical manipulations used. The calculated K for non-substrate-mediated transhydrogenation is slightly below the lowest concentration of steroid employed experimentally. Measurements made at lower concentrations were too inaccurate to be of any value. Statistical treatment of the results assists in overcoming this defect, but precise measurement of the constant will not be possible until the protein responsible for the non-substrate-mediated transhydrogenation has been obtained in a purer state. Nevertheless, the final results for the calculated constants and their associated errors do not permit the interpretation that the dehydrogenase activity of the crude enzyme preparation is sufficient to account for the observed transhydrogenation activity.

The results would be vitiated if there were any significant degree of non-specific (i.e. non-catalytically active) binding of the steroid to the proteins in the enzyme preparation. The amount of bound steroid under the experimental conditions used in the enzyme assays was determined directly by equilibrium dialysis with radioactive steroids, and it was shown that this factor could be neglected.

At higher concentrations of steroid than those used in the experiments described inhibition of transhydrogenation was observed, but this effect could not be examined quantitatively because the limited solubility of the steroid precluded accurate enzyme assay at the necessary concentrations.

The observed non-linearity in the inverse plot of the transhydrogenation assays with steroid concentration might be due to binding of an appreciable fraction of the added steroid to the enzyme at the

lower concentrations of steroid rather than to the presence of more than one transhydrogenating system in the crude preparations. (That is, the kinetic assumption that the absolute concentration of substrate is considerably greater than that of the enzyme might not have been valid in these experiments.) This possibility was examined by plotting the results in the form $v/(V-v)$ as a function of s , which gives a linear plot under these conditions (Reiner, 1959). This function of the velocities did not give a linear relationship for the results and the possibility was discarded.

Multiple interacting active sites on the proteins responsible for steroid dehydrogenation could give non-linearity of the inverse plots of the transhydrogenation reaction; but this is impossible because the results obtained with the stage 5 enzyme preparation are uniformly linear. Similarly, non-linearity of the inverse plot of transhydrogenation activity due to endogenous steroid cannot be a factor because the amount of endogenous steroid was determined directly and corrected for. [In previous experiments (Hagerman & Vilee, 1957) the correction for endogenous steroid was made indirectly from the results themselves.]

The various kinetic constants obtained in the present investigations may be compared with previous values in the literature. The Michaelis constant for NADPH in the reaction of oestrone reduction was reported as less than $10^{-6}M$ (Talalay, Hurlock & Williams-Ashman, 1958), in agreement with the present values. The Michaelis constant for oestradiol in the reaction of NAD^+ reduction was reported to be about $3.3 \times 10^{-6}M$ (Langer, Alexander & Engel, 1959), compared with our value of $2.8 \times 10^{-6}M$. The value for the apparent Michaelis constant of the transhydrogenation reaction given in the present paper is not significantly different from that which we previously published, even though the previous experiments were interpreted before the discovery of the complexities of the reactions involved (Hagerman & Vilee, 1957). The value obtained indirectly for the placental enzyme is identical with that reported for a similar enzyme found in human myometrium (Abe *et al.* 1964).

Our experiments make it clear that nearly half of the oestrogen-dependent transhydrogenation activity present in crude extracts of human term placenta cannot be accounted for by substrate-mediated dehydrogenase-catalysed transhydrogenation, in agreement with our previous conclusion that placenta contains a specific oestrogen-dependent transhydrogenase. Jarabak *et al.* (1962) concluded on the basis of studies on the purification of

the dehydrogenases, that all of the transhydrogenation could be accounted for by the measured dehydrogenase activities. Our results show that accurate assays for the oestrogen-dependent transhydrogenase in the presence of the dehydrogenase activities require a correction of the observed transhydrogenation activity for the substrate-mediated contribution to the total. The correction can be calculated from independent measurements of the (maximal) dehydrogenase activities, or from measurements of the total transhydrogenation activity at several steroid concentrations, and calculation with the aid of the Michaelis constants given.

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