THE THERMODYNAMICS OF THE REACTION OF PYRUVIC ACID WITH REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE*

by

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INTRODUCTION

In recent years, there has been a substantial increase in the amount of free energy data pertinent to reactions of biological interest[1,2]. However, for a complete thermodynamic analysis, this information should be correlated with the necessary thermochemical values. It was this consideration which prompted the calorimetric study of the reaction of pyruvate with reduced diphosphopyridine nucleotide, DPN (red), as catalyzed by lactic acid dehydrogenase, LAD, to give lactate and oxidized diphosphopyridine nucleotide, DPN (ox), represented by the following relationship:

$$(1) \qquad H^+ + \text{pyruvate} + \text{DPN (red)} \xrightarrow{\text{(LAD)}} \text{lactate} + \text{DPN (ox)}.$$

This specific system was selected since the standard free energy change, ΔG°, is known[3,4,5]. One could then calculate the standard enthalpy change, ΔH°, and the standard entropy change, ΔS°, for the over-all reaction and for the half-reactions involved.

EXPERIMENTAL

1. *Calorimeter.* The calorimeter was a small silvered Dewar flask fitted with a ground glass evacuated stopper containing three inlet tubes through which passed a thermistor, the enzyme delivery tube, and a non-inductively wound manganin wire resistance coil.

This assembly was mounted on a frame submerged in a thermostat whose temperature was maintained at 25.00° C with temperature fluctuations not exceeding 0.001° C. This could be rotated through an arc of thirty degrees at a rate of 30 ± 4 cycles per minute, mixing was further facilitated by the presence of a small Pyrex bead.

The calorimeter or heater resistance, R_h, was used to determine the energy produced by the reaction. This resistor was made from manganin wire (BS 28) to form a hollow bifilarly wound helix 15 mm in diameter and 35 mm in length exposing a surface of 33 sq.cm hence permitting extremely rapid heat transfer. Copper current and potential leads (AWG #30 and #36) were silver soldered to the coil, and then coated with G.E. adhesive 7031, Formvar, and Dow Corning Silicone Resin #935. It was annealed for six weeks at 125° C prior to incorporating in the calorimeter. The resistance was 25.335 ± 0.002 ohms***. A similar resistor sealed in a Pyrex test tube suspended in the thermostat served as the standard reference, R_s, with a resistance of 31.541 ± 0.002 ohms.

The temperature change in the calorimeter was determined with a thermistor (W.E. #9A) with a resistance of 32,000 ohms at 25° C.

* This work was supported in part by a contract with the Office of Naval Research.

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*** All electrical units are absolute. One calory (thermochemical) = 4.1840 joule. 0° C = 273.160° K[6].

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The leads from these were thermally grounded to the calorimeter cover and the usual precautions followed to reduce any additional heat losses.

The enzyme delivery tube 8 mm o.d., 6 mm i.d., and 23 mm in length sealed with paraffin wax. The enzyme was expelled by manipulating a rod. Heat loss was minimized by passing the rod through three snugly fitted rubber gaskets.

The heat capacity of the calorimeter was equivalent to 12 g of water when charged with 25.00 g air-free distilled water.

2. *Heat loss.* An index of heat loss, valid when the temperature differential between the calorimeter and its environment is small, given by the following equation:

$$\frac{dT}{dt} = k(T_j - T_c) + d \tag{2}$$

where dT/dt is the rate of heat change, T_j and T_c are the temperatures of calorimeter jacket and calorimeter respectively, k is the transfer coefficient, and d is a non-temperature dependent term. This relationship has been adequately discussed by both WHITE[9] and STURTEVANT[10] therefore, only a few of the germane features will be mentioned. The heat loss can be minimized if the factors k, d, and $(T_j - T_c)$ are small. This was accomplished for the initial two terms by construction while the latter term prior to the start of the experiment was equal to 0.10° C and during the progress of the reaction would decrease since the process was exothermic.

3. *Electrical circuits.* Two independent circuit networks were employed, one for calibration and the other for thermometry. The first circuit was similar in principle to the one described by STURTEVANT[10]. A Weston D-C milliammeter, Model 622, was incorporated to obtain "rough" voltage checks and to indicate any malfunction in this section. The voltages were measured with a Leeds and Northrup Type K-1 potentiometer with recalibrated standard cells and resistances.

The circuit for thermometry was a Wheatstone bridge specifically designed to measure small resistance changes in a thermistor. A critically damped d'Arsonval galvanometer, sensitivity of 0.0003 micro-amperes, was used. The customary precautions were followed to obtain maximum accuracy[11,12]. The temperature coefficient of the thermistor was established for the range of 24.7 to 25.3° C using a Beckman thermometer[13]. The resistance of the thermistor in this range obeys the relationship

$$R = R_\infty \exp B/T \tag{3}$$

where

R = thermistor resistance at temperature T
R_∞ = thermistor resistance at infiniter temperature
B = a constant characteristic of a given thermistor
T = temperature, in degrees Kelvin
exp = Naperian logarithm base.

The rate of change of resistance with temperature was found to be 1574 ohms per degree[7]. Therefore by measuring resistance changes to within ± 0.1 ohms the temperature change could be determined with an uncertainty of 0.00006_4° C.

4. *Calibration and reproducibility.* The values for the resistances R_s and R_h were obtained potentiometrically using a recalibrated National Bureau of Standards type resistor as reference. It was observed over a 10 weeks period that the ratio of the voltages across R_s to R_h when R_h was immersed in the reaction solution was 1.24498 with a standard deviation of 0.000049 thus demonstrating the overall stability of the system.

5. *Reagents.* The buffer consisted of analytical grade disodium phosphate and potassium dihydrogen phosphate which had been dried for 24 hours at 115° C. The sodium pyruvate used was doubly recrystallized from aqueous solution by the addition of *iso*propyl alcohol.

The lactic acid dehydrogenase, LAD, (lot no. 23.211) and DPN(red) were obtained from Sigma Chemical Co. The latter material was 0.72 ± 0.025 pure as determined by spectrophotometric assay at 340 millimicrons using the value of $6.22 \cdot 10^6$ cm²/mole for the extinction coefficient of DPN(red)[14]. Possible contaminants were water, barium carbonate, adenine, and DPN(ox), none of which could interfere or produce spurious effects in the calorimetric measurements.

6. *Procedure.* A weighed amount of sodium pyruvate was dissolved in phosphate buffer. The temperature of this solution was adjusted to 25.0° C while the temperature of the calorimeter was brought to 24.5° C. A measured quantity of DPN (red) was introduced into the calorimeter, followed immediately by the addition of 24.97 ml of the pyruvate solution. Previously 31 ± 1 microliters of LAD solution (9.2 micrograms/microliter buffer) were added with a micropipet to the enzyme holder. The calorimetric experiment usually could be initiated about a hour after charging. The addition of

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0.031 ml of the enzyme to 24.97 ml reaction solution introduced a neglibly small heat effect. The reaction was completed within 25 to 30 minutes after the introduction of the enzyme. It was demonstrated by the calorimetric data which were corroborated by spectrophotometric studies that there was no demonstrable reaction prior to the addition of the enzyme. The heat of reaction was determined by simulating the experiment with the sole exception that DPN (ox) was substituted for DPN (red)*. The reaction curve could be matched by three or four voltage settings during the calibration with each voltage being checked by triplicate readings.

7. *Error.* The systematic errors in this study were reduced by the comparative technique of calibration, whereby errors attributed to thermistor heating, temperature lag, timing, heat loss, *etc.* were duplicated and thereby minimized. The most significant source of error is attributed to the time required in switching from one voltage to another which might take up to two or three seconds. By graphical integration it can be shown that the net maximum error due to this is less than 0.4%. The error introduced by the heat of solution of the enzyme and of complex formation was considered to be inconsequential[15,16].

The fact that DPN (red) was quantitatively oxidized was established from the equilibrium constant, K, for reaction (1) where

$$K = \frac{(\text{lactate})\,(\text{DPN (ox)})}{(\text{pyruvate})\,(\text{DPN (red)})\,(\text{H}^+)} \tag{4}$$

had been found[3,4] to be about $2.5 \cdot 10^{11}$. Under the experimental conditions over 99.99% of the DPN (red) would be oxidized upon attainment of equilibrium.

RESULTS AND DISCUSSION

Due to the limited amount of DPN (red) available, only two thermochemical measurements were made. The data assembled in Table I are self-explanatory except for the Q terms which will be clarified shortly. The total volume of the system was 25.00 ml and it was buffered at pH 7.3 with 0.15 molar phosphate buffer. The pH remained constant as determined with a Beckman model H pH meter. The DPN (red) concentration was calculated on the basis that 72% of the material added as such was in the reduced state. The reaction temperature was between 24.9 and 25.0° C. The system which contained 0.206 millimoles DPN (red) produced a heat equivalent, Q_c, of -2.02 ± 0.025 calories, while Q_c for the other experiment with 0.180 millimoles DPN (red) was -1.76 ± 0.015 calories.

Q_c may be considered as being produced primarily by two effects, namely,

$$Q_c = Q_r + Q_h \tag{5}$$

where Q_r is the heat due to reaction (1), and Q_h is the energy attributable to the ionization of the buffer. A minor energy contribution owing to the transformation of the reactants with a given ionization contant to products with different constants, *e.g.*, lactic to pyruvic acid, may be neglected at this pH. To evaluate Q_h, for every Δn moles of DPN (red) oxidized the corresponding number of moles of hydrogen ions must be removed. From Table II it is seen that their source will be the dihydrogen phosphate ions with a heat of ionization of 822 calories per mole.

* In subsequent measurements, the enzyme was not added since its contribution was neglible.

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TABLE I

SUMMARY OF CALORIMETRIC EXPERIMENTS

Experiment	*38*	*42*
pyruvate (initial concentration)	0.981 millimoles	0.993 millimoles
DPN(red) (initial concentration)	0.206 millimoles	0.180 millimoles
LAD	285 + 9 micrograms	285 + 9 micrograms
Q_s	−2.02 ± 0.025 cal	−1.76 ± 0.015 cal
Q_d	0.169 cal	0.148 cal
Q_r	−2.19 ± 0.025 cal	−1.91 ± 0.015 cal
ΔH_r	-10.6_3 kilocal	-10.6_1 kilocal

TABLE II

IONIZATION CONSTANTS AND MOLAL HEATS OF IONIZATIONS

	pK 25° C	ΔH_i° *cal./mole*
1. Pyruvic acid[17]*	2.490	2460**
2. Lactic acid[18]	3.862	−99
3. Phosphoric acid[18]		
1st dissociation	2.124	−1,773
2nd dissociation	7.206	822
4. Ammonium ion #6 position of the adenine group		
a. DPN(ox)[19]	3.9	
b. adenosine triphosphate[20]	4.00	
c. adenosine diphosphate[20]	3.95	
d. adenosine monophosphate[20]	3.74	

* This indicates the literature source.

** The molal heat of ionization of weak electrolytes is a function of the temperature; this value is calculated for the temperature range between 25° C and 37° C[17,18].

The enthalpy change for reaction (1), ΔH_r, was found to be -10.6_3 and -10.6_1 kilocalories/mole for the respective experiments. This excellent agreement is obviously fortuitous, in subsequent calculations even though the value of -10.6_2 kilocalories/mole is used it must be recognized there exists an uncertainty of 2½% due to the difficulty in determining the DPN (red) concentration.

THERMODYNAMICS

The standard enthalpy change, ΔH°, for this system is given by equation (6)

$$\Delta H^\circ = \Delta H_r + \sum \bar{L}\text{reactants} - \sum \bar{L}\text{products} \tag{6}$$

which states that ΔH° is equal to ΔH_r and the difference between the summations of the relative partial molal enthalpies of the reactants and the products. It may be assumed that the contribution of any of the relative partial molal enthalpy terms will be small[21,22]* and furthermore that their cumulative effect will be relatively insig-

* The magnitude to be expected for these partial molal enthalpies may be inferred from data cited in these references.

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nificant since they tend to cancel. Thus the value for ΔH° is equal to —10.6 kilocalories/mole for this system.

The standard free energy change, ΔG°, calculated by BURTON AND WILSON[5] of —15.55 kilocalories/mole will be used in the subsequent calculations. This value compares favorably with those of —15.65 and —15.50 kilocalories/mole computed from NEILAND's[4] and RACKER's[3] data in systems of 0.3 and 0.04 molar ionic strength.

The standard entropy change, ΔS°, was obtained by substituting the appropriate values in the familiar relationship

$$\Delta G = \Delta H - T\Delta S \tag{7}$$

giving a value for ΔS° equal to 16.54 calories/degree mole.

From fundamental principles, it is evident that reaction (1) is the sum of the following two half reactions

$$2H^+ + 2e + \text{pyruvate} = \text{lactate} \tag{1a}$$

$$\text{DPN(red)} + H^+ = \text{DPN(ox)} + 2H^+ + 2e, \tag{1b}$$

and that the thermodynamic functions ΔG°, ΔH°, and ΔS° are likewise the sum of the corresponding terms for the half reactions. Recently the standard free energies for these respective half reactions which will be designated as ΔG_1° and ΔG_2° have been evaluated by BURTON and associates[5].

The value for ΔS_1° may be established from the temperature dependence of the standard reduction-oxidation potential, $\delta \bar{E}/\delta T$. Using the value of —0.715 millivolts/degree obtained by BARRON AND HASTINGS[23], ΔS_1° was calculated utilizing equation (8)

$$\Delta S^\circ = nF \frac{\delta E}{\delta T} \tag{8}$$

where n is the number of electrons and F is the Faraday constant. A value for ΔS_1° of —32.9 calories/degree mole was obtained. This result may appear to be open to criticism on the grounds that it was based on values for ΔG_1° which were approximately 10% higher than those obtained more recently by BURTON AND WILSON[5]; however, since the value for $\delta \bar{E}/\delta T$ is a relative measurement, it is probably more reliable than the individual free energy terms involved.

With this information the remainder of the thermodynamic functions were calculated and assembled in Table III. The values for the standard free energy change and enthalpy change for reaction (1) are estimated to be reliable to within 0.3 units, while no attempt was made to estimate the accuracy of the remainder of the values.

TABLE III

SUMMARY OF THERMODYNAMIC FUNCTIONS*

(at 25° C)

Reaction	ΔG° *Kilocal/mole*	ΔS° *cal/degree mole*	ΔH° *Kilocal/mole*
1a	10.3_2[5]**	-32.9[23]	-20.1_2
1b	5.2_2[5]	49.4	9.5$_0$
1	15.5_5[5]	16.5$_4$	-10.6_2

* No correction was made for the differences in ionic strength.

** This indicates the literature source.

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An indication of their validity may be obtained from inspection of the following derived values. BARRON AND HASTINGS[23] found ΔH_1° to be —21.64 kilocalories/mole, this when corrected for the difference in the free energy term used is —20.2 kilocalories/mole which is in excellent accord with that reported here. The entropy change for the reduction of liquid acetaldehyde is of the same magnitude as that given by reaction (1a).

It is of interest to note that in reaction (1a) that the large negative enthalpy change favors this process while the negative entropy change opposes it; however, in reaction (1b) the converse is the case.

ACKNOWLEDGMENTS

I wish to acknowledge my indebtedness to Dr. CHALMERS L. GEMMILL for having suggested this problem and his generosity in providing the equipment and laboratory facilities necessary for this project.

The author wishes to thank the General Electric Co. (courtesy of Dr. WARREN DE SORBO) for the gift of adhesive 7031 and the Formvar; the Dow Corning Co. for contributing the Silicone Resin #935 and Rohm and Haas Co. for presenting the Acryloid B-4.

In addition, I wish to express my thanks to Mrs. ULLA KATZ for assistance in typing and proofreading.

SUMMARY

An isothermally jacketed calorimeter was constructed in which temperature changes could be measured to within 0.0001° C. The heat of reaction of pyruvate with reduced diphosphopyridine nucleotide catalyzed by lactic acid dehydrogenase to give lactate and oxidized diphosphopyridine nucleotide was measured with this apparatus. The enthalpy change for this reaction was found to be $-10.6 \cdot 10^3$ calories per mole of DPN oxidized. This datum, correlated with results of equilibrium measurements, permitted the calculation of the standard enthalpy change, standard free energy change, and standard entropy change for the over-all reaction, and also for the half reactions involved.

RÉSUMÉS

Un calorimètre isothermiquement isolé, dans lequel des changements de température peuvent être déterminés à 0.0001° C près, a été construit. La chaleur de la réaction du pyruvate avec le diphosphopyridine nucléotide réduit, catalysée par la lactique déshydrogénase pour donner du lactate et du diphosphopyridine nucléotide oxydé, a été déterminée à l'aide de cet appareil. La variation d'enthalpie de la réaction est de $-10.6 \cdot 10^3$ calories par mole de DPN oxydé. Ce résultat, rapproché des résultats des mesures à l'équilibre, permet de calculer la variation standard d'enthalpie, la variation standard d'énergie libre, la variation standard d'entropie pour la réaction globale, et également pour les deux demi-réactions mises en jeu.

ZUSAMMENFASSUNG

Es wurde ein isothermisch isolierter Kalorimeter hergestellt, in welchem es möglich ist, Temperaturschwankungen in einer Grössenordnung von weniger als 0.0001° C zu messen. In diesem Apparat wurde die Reaktionswärme der von Milchsäuredehydrogenase katalysierten Reaktion zwischen reduziertem Diphosphopyridinnucleotid und Brenztraubensäure zu oxydiertem Diphosphopyridinnucleotid und Milchsäure gemessen. Es wurde eine Enthalpievariation von $-10.6 \cdot 10^3$ Kalorien pro Molekül oxydierten DPN's festgestellt. Dieses Ergebnis, im Zusammenhang mit Gleichgewichtsbestimmungen, ermöglichte es, die Standardwerte für die Variationen von Enthalpie, freier Energie und Entropie, sowohl für die Gesamtreaktion, als auch für beide Teilreaktionen zu errechnen.

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Received August 25th, 1954