THERMOCHEMICAL INVESTIGATION OF THE ENZYMIC OXIDATION OF THE FORMATE ION *

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In connection with the growing need for the thermodynamic description of chemical processes occurring in biological systems there is at present developing a new line of direct calorimetric investigation of enzymic reactions. The studies that have been carried out in this region are concerned mainly with hydrolytic reactions [2-5]. Examples of other types of enzymic reactions investigated calorimetrically are the reaction of an oxalacetate with reduced nicotinamide adenine dinucleotide (NADH) catalyzed by malic dehydrogenase [6] and the phosphorylation of glucose in presence of adenosine triphosphate (ATP) catalyzed by hexokinase [7]. Data were published recently on the calorimetric investigation of reactions catalyzed by NAD-dependent oxyreductases [8]. Although the need for thermodynamic data on biological systems is exceptionally great, the number of investigations whose results could be applied in thermodynamic calculations for such systems is very small.

In the present work we carried out a microcalorimetric determination of the enthalpy of oxidation of the formate ion with the use of the enzyme formic dehydrogenase in presence of the oxidized form of nicotinamide adenine dinucleotide (NAD) as cofactor in a phosphate buffer solution. An integral part of this work was the independent determination of the value of the enthalpy of reduction of NAD to NADH required for the calculations, for the relevant data in the literature [9-11] are contradictory. The reduction of NAD was conducted with hydrogen dissolved in phosphate buffer in presence of the enzyme hydrogenase.

The enthalpy of reduction of NAD to NADH in solution can be regarded as a key thermodynamic quantity, for NAD is a cofactor of many (~250) enzymes which take part in oxidation–reduction reactions of organic compounds. Our direct determination of this quantity had the object of establishing a reliable value for it.

EXPERIMENTAL

For the calorimetric determinations we used LKB microcalorimeters (Sweden): an LKB-2107-111 rotating batch-type calorimeter for the investigation of enzymic reactions, and an LKB-2107-121 flow-type calorimeter for the auxiliary determination of the enthalpy of ionization of the buffer solution. Both calorimeters were of the heat-conducting type with a differential method of measurement. The signal from the differential detector thermopile of microcalorimetric cells (working and comparison cells) was recorded after amplification on an automatically recording LKB-2066 potentiometer giving integral and differential recordings, which enabled us to obtain data both on the summative heat liberation in the experiment and on the heat output during the experiment. The temperature of the main block (aluminum, 10 kg) of the microcalorimeter was regulated with the aid of an electric heater fed from a special control system, which shortened the time required for the attainment of thermal equilibrium in the instrument. The calorimeter was mounted in an air thermostat (± 0.02°C). The microcalorimetric system was calibrated with an electric current with the aid of a built-in heater (~50 Ω). Characteristics of instruments: minimum detectable pulse of heat 200 μJ, maximum zero drift 0.5 μV in 8 h, time constant 155 sec for the glass cell of the batch-type calorimeter and 75 sec for the flow-type system. The reaction cell of the batch-type microcalorimeter was a glass vessel in the form of a parallelepiped (10 × 40 × 40 mm) containing a partition reaching to $^2/_3$ of the height of the vessel. The solutions of the reactants were introduced into the two compartments of the cell. The reaction was initiated by mixing the solutions, effected by the rotation of the microcalorimeter. The comparison cell was constructed analogously. The flow-type microcalorimeter was provided with heat exchangers for the equalizing of the temperature of the solutions. The reaction cell of this instrument was a metal tube, diameter 1 mm and total internal volume 1 cm^3, formed into a spiral. Before entering the reaction cell, the two solutions which were to be mixed were pumped through heat exchangers (LKB-12000 pumps) in which they acquired the same strictly definite temperature.

* The results of this work were reported briefly in [1].

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TABLE 1. Results of Experimental Determination of the Enthalpy of Reduction of NAD ($\Delta H_{(1)}$) Dissolved in a Buffer Solution with Hydrogen[a]

Expt.	Heat liberation q_c in calibration in arbitrary units (0.03125 J introduced)	Heat liberation in expt.		NADH concn. in final soln.		$\Delta H_{(1)}$ (25°C), kJ/mole
		arbitrary units (q_{expt})	J (Q)	optical density	c, mM	
1	169.0	183.0	0.03385	1.28	0.206	—27.41
2	171.5	145.0	0.02644	1.05	0.169	—26.07
3	171.0	163.5	0.02987	1.11	0.178	—27.99
4	171.5	185.5	0.03381	1.31	0.211	—26.69
5	169.5	98.0	0.01807	0.67	0.108	—27.91
6	170.0	169.0	0.03109	1.125	0.181	—28.62
Mean value						—27.4±1.1

Note. a) $\Delta H_{(1)} = -10^3 \cdot Q/c \cdot V_{total}$, kJ/mole; Q = heat liberated in experiment, J; c = concentration of NADH in final solution, mM; V_{total} = number of milliliters of solution in which reaction occurs (2.00 + 4.00 = 6.00 ml). In expts. 2 and 5 the solution was saturated with hydrogen outside the calorimeter; in the other experiments the saturation of the solution was conducted in the calorimeter (bubbling of hydrogen for 2 min); $Q = 0.03125 q_{expt}/q_c$.

Determination of the Enthalpy of Reduction of NAD with Hydrogen. We used samples of NAD-dependent hydrogenase enzyme (Enzyme Catalog 1.12.1.2) isolated from a strain of hydrogen bacteria by a method including fractionation with ammonium sulfate and chromatography on diethylaminoethylcellulose [12]. In their characteristics the samples of hydrogenase were close to those described in the literature [13]. They contained other proteins as impurity, but the samples did not have NADH-dehydrogenase and -oxidase activity. The hydrogenase was stored in the frozen state in 0.050 M potassium phosphate buffer and was thawed in portions immediately before the experiment.

Preparation of Solutions. a. 0.050 M potassium phosphate buffer solution: A 6.8045-g (0.050-mole) portion of cp KH_2PO_4 was dissolved in distilled water in a 1-liter measuring flask and the pH of the solution was brought to 7.20 ±0.05 by the addition of cp KOH.

b. A concentrated solution of the enzyme in 0.050 M potassium phosphate buffer (pH 7.2) was diluted before the experiments with buffer solution to the required concentration of the enzyme, and in this form the solution of the enzyme was used in the experiments.

c. The NAD solution for the experiments was prepared by dissolving the required weight of NAD in 0.050 M potassium phosphate buffer solution (pH 7.2).

TABLE 2. Results of the Determination of the Enthalpy of the Enzymic Oxidation of the Formate Ion ($\Delta H'_{(3)}$) at pH 6.44[a]

Expt.	Heat liberation in calibration		Heat liberation in expt.		NADH concn. in final soln.		$\Delta H'_{(3)}$ (25°C), kJ/mole
	arbitrary units (q_{arb})	J (q_c)	arbitrary units (q_{expt})	J (Q)	optical density	mM	
1	116.5	0.06012	133.9	0.06912	4.67	0.751	—15.36
2	117.4	0.06025	130.4	0.06690	4.66	0.749	—14.90
3	117.7	0.06025	135.2	0.06920	4.66	0.749	—15.40
4	116.9	0.06012	133.3	0.06858	4.68	0.753	—15.19
5	118.4	0.06012	134.4	0.06824	4.68	0.753	—15.10
6	118.8	0.06012	137.7	0.07000	4.66	0.749	—15.56

Note. a) $\Delta H'_{(3)} = -10^3 \cdot Q/cV$ kJ/mole; Q = heat liberated in experiment, J; V = volume of final solution, ml; c = NADH concentration in final solution, mM.

TABLE 3. Results of the Determination of the Heat of Mixing of the Original Solutions ($\Delta\Delta H'_{(3)}$) at pH 6.44[a]

Expt.	Heat liberation (q) in calibration		Heat liberation in expt.		Correction to $\Delta H'_{(3)}$ ($\Delta\Delta H'_{(3)}$), kJ/mole
	arbitrary units	J	arbitrary units	J	
1	117.4	0.06012	−2.5	−0.00130	−0.29
2	118.1	0.06012	7.1	0.00360	0.79
3	118.0	0.06025	6.7	0.00343	0.75
4	118.0	0.06025	1.6	0.00084	0.17
5	117.9	0.06012	10.3	0.00527	1.17
6	118.0	0.06025	−0.3	−0.00017	−0.04

Note. a) Enthalpy of enzymic oxidation of formate ion $\Delta H_{(3)} = \Delta H'_{(3)} - \Delta\Delta H'_{(3)} = -14.85 \pm 0.60$ kJ/ mole.

The sequence of operations in the determination of the enthalpy of the reaction under investigation was as follows: On one side of the partition in the reaction vessel of the LKB-2107-111 microcalorimeter we introduced 2.00 ml of a 0.050 M potassium phosphate buffer containing 3 mg/ml of protein with hydrogenase activity, while on the other side we introduced 4.00 ml of 0.050 M potassium phosphate buffer containing NAD at a concentration of 0.001 M; before the experiment the solution was saturated with hydrogen. The solutions were mixed on the attainment of temperature equilibrium. The progress of the reaction was followed from the change in heat output. The end of the reaction was determined by the establishment of a stable base line on the recording band of the LKB-2066 automatically recording potentiometer, and also more accurately with the aid of an integrator.

To the thermal effect found we made a correction for the mixing of the original solutions. The correction was determined by mixing the above-described solutions, but without NAD in the second solution, in the comparison vessel. The mixing was conducted simultaneously with the occurrence of the main reaction in the reaction vessel. The measurements were conducted at 25°C.

The determination of the concentration of NADH in the final solution was conducted on a flow-type LKB Uvicord 3 spectrophotometer at 340 nm. The extinction coefficient of NADH was taken to be 6.22 mmole·liter^{-1}. The thermal effect of the reaction under investigation was about 0.02-0.03 J with a calorimetric sensitivity of the instrument of about 0.0004 J. The results of the experiments are given in Table 1.

The amounts of heat liberated are stated in Table 1 in arbitrary units (obtained with the aid of the integral record on the LKB-2066 automatically recording potentiometer) and also in joules.

The calibration of the microcalorimeter was effected by the passage of an electric current before and after each experiment. In the calibration 0.03125 J of heat was introduced through the heater into reaction vessel (dosage from the control unit of the calorimeter). The results of the determination of optical density are given (Table 1) in optical-density units (Table 1) and in weight units (mmole·liter^{-1}).

The equation for the reaction being studied of the reduction of NAD with dissolved hydrogen in the buffer solution characterized above has the form:

$$NAD^+ + H_2 \text{ (aq. soln.)} = NADH + H^+. \quad (1)$$

Measurements were made at pH 7.20 ±0.05 and 25°C. For reaction (1) of the hydrogenation of NAD we obtained the following value for the enthalpy: $\Delta H_{(1)} = -27.4 \pm 1.1$ kJ/mole; the confidence interval is given with a probability of 95%.

To the experimentally determined value of the enthalpy of reaction we made corrections for the enthalpy of solution of hydrogen in water, equal to -3.8 ± 0.8 kJ/mole [14] and for the enthalpy of the ionization of the buffer, equal to -3.97 ± 0.25 kJ/mole. The latter was determined experimentally in an LKB-2107-121 flow-type microcalorimeter with equal rates of flow of the two solutions (5.5 ml/h). In this we mixed two solutions: The first was 0.1 M potassium phosphate buffer; the second was 0.00400 M HCl. From the thermal effect found for the mixing of these solutions the heat of dilution of 0.1 M phosphate buffer was automatically subtracted. The enthalpy of ionization (-3.97 ± 0.25 kJ/mole) was calculated from the results of 6 experiments.

After the two above-indicated corrections had been made in ΔH_1, Eq. (1) was transformed into the standard form of the oxidation–reduction reaction of NAD:

$$NAD^+ + H_2(gas) = NADH + H^+. \quad (2)$$

Here, hydrogen is in the gaseous form, but the remaining substances are in a buffer solution with an enthalpy of ionization of zero.

For the oxidation of the formate ion we used the enzyme NAD-dependent formic dehydrogenase (Enzyme Catalog 1.2.1.2.) isolated from a strain of methylotrophic bacteria [15]. The preparations of the enzyme used in the experiments were homogeneous according to the results of analytical disk electrophoresis and high-velocity sedimentation. The enzyme was stored at +4°C in 0.050 M sodium phosphate buffer containing 0.010 M EDTA. The added EDTA has a stabilizing action on the enzyme in the water-soluble form.

In the determination of the enthalpy of the reaction investigated in the LKB-2107-111 microcalorimeter on one side of the partition in the reaction vessel we introduced 2.00 ml of a solution containing 0.050 mole of sodium phosphate buffer, 0.30 mole of sodium formate, and 0.010 mole of EDTA per liter and 0.5 mg/ml of the enzyme, and on the other side of the partition 4.00 ml of a solution containing, per liter, 0.050 mole of sodium phosphate buffer, 0.30 mole of sodium formate, 0.010 mole of EDTA, and 0.001 mole of NAD (the solutions were prepared as in the case of the potassium phosphate buffer). The comparison vessel contained sodium phosphate buffer (0.050 M). After the attainment of thermal equilibrium the solutions were mixed. The progress of the reaction was followed as described above. To the thermal effect found a correction was made for the mixing of the original solutions, determined in special experiments in which the same solutions were mixed as in the performance of the reaction, but the second solution did not contain NAD (the comparison vessel contained 0.050 M sodium phosphate buffer). The measurements were conducted at 25°C.

The determination of the enthalpy of the enzymic oxidation of the formate ion was conducted at four different pH values: 6.02 ±0.05, 6.44 ±0.05, 7.48 ± 0.05, 8.01 ±0.05. Typical results of the determinations at pH 6.44 are given in Table 2.

The thermal effect at pH 6.44, as can be seen from Table 2, and also at other pH values was of the order of 0.04-0.08 J. Six experiments were carried out at each pH value. In Table 2 the heats of reaction are given in arbitrary units (obtained with the aid of the integral record on the LKB-2066 automatically recording potentiometer), and also in joules.

The microcalorimeter was calibrated by an electric current before or after each experiment. In the calibration 0.04-0.08 J of heat was introduced through the heater into the reaction vessel. The results of the calibration are presented in Table 2 in arbitrary units and in joules. The results of the calibration were reproducible within 1-2%.

The NADH concentration in the final solution was determined spectrophotometrically at a wavelength of 340 nm. The results of the measurements of the NADH concentration are given in Table 2 in optical-density and gravimetric (mM) units.

The results of the experiment on the determination of the heat of mixing of the original solutions at pH 6.44 are also given in Table 3. Table 3 also gives values of the heat liberated in experiments on the calibration of the microcalorimeter with an electric current in arbitrary units and in joules. The heat of mixing the original solutions was +0.005 to −0.001 J. Six experiments were carried out at each pH value.

In special experiments we determined the enthalpy of ionization of the buffer solution used in an LKB-2107-111 microcalorimeter. In these determinations on one side of the partition in the glass reaction vessel we introduced 2.00 ml of 0.0020 -0.0040 N H_2SO_4, and on the other we introduced 4.00 ml of a solution containing, per liter, 0.0750 mole of sodium phosphate buffer, 0.450 mole of sodium formate, and 0.0150 mole of EDTA. In the comparison vessel on one side of the partition we introduced 2.00 ml of doubly distilled water, and on the other side 4.00 ml of a solution containing, per liter, 0.0750 mole of sodium phosphate buffer, 0.450 mole of sodium formate, and 0.0150 mole of EDTA. The values found for the enthalpy of ionization of the buffer solution at pH values of 6.43 and 7.46 were −7.66 ±0.60 kJ/mole and −7.53 ±0.55 kJ/mole respectively, which indicates the practical absence of dependence of the enthalpy of ionization of the buffer on pH.

DISCUSSION OF RESULTS

The enthalpies of the enzymic oxidation of the formate ion in the solution characterized above with allowance for the heat of mixing of the original solutions at pH values of 6.02, 6.44, 7.48, and 8.01 are equal respectively to −15.19 ±0.75, −14.85 ± 0.60, −14.02 ± 0.70, −14.18 ±0.80 kJ/mole; the confidence interval is given with a probability of 95%.

It was important to determine in what form carbon dioxide separates in the reaction: as the gas, or as a combined form in aqueous solution. We showed that the thermal effect of the reaction studied depends weakly on the pH of the medium (in the change in the pH of the medium from 6 to 8 the enthalpy of the reaction changes only by 0.8 kJ/mole), and the enthalpy of ionization of the buffer solution is practically equal to the enthalpy of the first-stage ionization of carbonic acid [14]. If in the course of the reaction carbon dioxide were obtained in various forms (partially in the bound form in aqueous solution), then values of the enthalpy of reaction at various pH values should differ by several kJ/mole. The results of the calculation of the state of carbon dioxide in the solutions investigated on the basis of its known thermodynamic functions, and also the weak dependence of the enthalpy of the oxidation of the formate ion on the pH of the medium, enable us to maintain that in the final state of the solutions investigated carbon dioxide was completely present in the combined form.

In view of the above considerations, at pH 8 the equation of the reaction under investigation can be written as follows:

$$HCOO^- + NAD^+ + H_2O = NADH + HCO_3^- + H^+. \quad (3)$$

At this pH carbonic acid is completely dissociated. At other pH values, apart from the bicarbonate ion, considerable amounts of undissociated carbonic acid molecules will also be present.

On the basis of the values obtained of the enthalpy of the enzymic oxidation of the formate ion with the use also of data in the literature on the enthalpies of formation of aqueous solutions of the formate ion and of carbonic acid and the products of its dissociation [14] we calculated the enthalpy of the oxidation–reduction reaction of nicotinamide adenine dinucleotide in accordance with Eq. (2). As a result of calculations for reaction (2) at pH values of 6.02, 6.44, 7.46, and 8.01 we obtained values of $\Delta H_{(2)}$ of -28.20 ± 1.5, -27.87 ± 1.5, -27.24 ± 1.5 and -27.40 ± 1.5 kJ/mole respectively.

The values of the enthalpy of reaction (2) of the oxidation–reduction of NAD found by two independent methods [direct experimental determination (-27.2 ± 1.5 kJ/mole) and calculation from ΔH of the enzymic oxidation of the formate ion (-27.6 ± 1.5 kJ/mole)] at pH 7 agree closely with one another. The average value of $\Delta H_{(2)}$ is -27.4 ± 1.5 kJ/mole. The quantity $\Delta H_{(2)}$ depends only weakly on the pH of the medium (diminishes in absolute value by 0.8 kJ mole when the pH changes from 6 to 8).

The enthalpy of reaction (2) of the oxidation–reduction of NAD is a key thermochemical quantity. By the use of the value of $\Delta H_{(2)}$ at 298.15 K and pH 7 = -27.4 ± 1.5 kJ/mole we may calculate enthalpies of a wide range of reactions catalyzed by NAD-dependent enzymes.

CONCLUSIONS

1. Enthalpies were determined experimentally of reactions of the two-stage process of the enzymic oxidation of the formate ion to hydrogen and carbon dioxide directly under conditions under which this process can be effected in practice in a fuel element.

2. The values obtained of the enthalpies of reduction of NAD dissolved in a buffer solution with hydrogen ($\Delta H_{(1)}$) and of the reduction of NAD with the formate ion in a buffer solution ($\Delta H_{(3)}$) may be applied in thermochemical calculations on biological systems.

3. The enthalpy of the reduction of NAD with gaseous hydrogen ($\Delta H_{(2)}$) was reliably established: This is a key thermochemical quantity for reactions catalyzed by NAD-dependent enzymes.

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SYNTHESES FROM DIMETHYLPYRAZOLES

III. DERIVATIVES OF VINYL- AND p-DIVINYL-BENZENES

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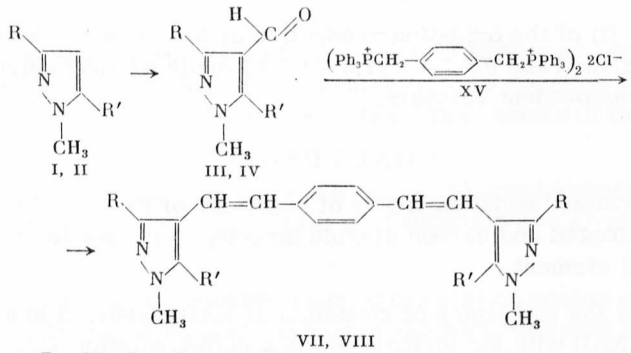
Stilbene and p-divinylbenzene derivatives have luminescence properties and find application as optical brightening agents, luminescent additives to pigments [1], etc. Continuing our search for ways of utilizing 1,3- and 1,5-dimethylpyrazoles (I) and (II), we have synthesized derivatives of styrene and p-divinylbenzene from them. For this purpose we formulated the compounds (I) and (II) by the Vilsmeier–Haack reaction, which resulted in the synthesis of 1,3-dimethylpyrazole-4-carboxaldehyde (III) and 1,5-dimethylpyrazole-4-carboxaldehyde (IV). The possibility in principle of synthesizing analogs of stilbene from pyrazole was shown by the reaction of the compound (IV) with benzyltriphenylphosphonium chloride (V), as a result of which we obtained 1,5-dimethyl-4-styrylpyrazole (VI). From the pyrazole carboxaldehydes (III) and (IV) by the Wittig reaction [3] we synthesized chromatographically pure 4,4'-(p-phenylenedivinylene)bis[1,3-dimethylpyrazole] (VII) and 4,4'-(p-phenylenedivinylene)bis[1,5-dimethylpyrazole] (VIII) [Eq. (1)].



$R = CH_3$, $R' = H$ (I, III, VII); $R = H$, $R' = CH_3$ (II, IV, VIII). (1)

The quantitative compositions of the compounds (VI)-(VIII) were confirmed by their mass spectra: The M^+ ions were at 198, 318, and 318 respectively. The question of the structures and configurations of these compounds was resolved with the aid of their IR and UV spectra.

In the IR spectra of the compounds (VI)-(VIII) (Fig. 1) we can distinguish several regions of absorption. In the region 690-1000 cm^{-1} bands are present which characterize the type of substitution in the benzene ring, the vibrations of the pyrazole rings, their CH bonds, and the configuration of CH = CH groups. In the spectrum of the compound (VI) intense bands at 697 and 755 cm^{-1} correspond to out-of-plane deformation vibrations of the benzene ring and are typical for a monosubstituted benzene (690-710 and 730-770 cm^{-1} [4-6]). The substitution of the benzene ring in the 1,4 positions in the compounds (VII) and (VIII) is confirmed by bands at 815-820 cm^{-1} (800-860 cm^{-1} [4-6]). To the in-plane and out-of-plane deformation vibrations of the pyrazole ring there correspond bands in the regions 780-790 cm^{-1} (790 cm^{-1} [7]) and 860-865 cm^{-1} (865 cm^{-1} [8]) respectively.

According to [9, 10], absorption associated with presence of cis-CH = CH groups in the cis,trans-p-di-

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