4. The results also indicate the formation of a ternary complex between magnesium, adenosine triphosphate and 8-hydroxyquinoline.

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Enthalpies of Hydrolysis of Glutamine and Asparagine and of Ionization of Glutamic and Aspartic Acids

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Thermodynamic data for glutamine are of special biochemical interest because in the glutamine synthetase reaction (Elliott, 1953) the synthesis of glutamine is coupled with the hydrolysis of adenosine triphosphate. Levintow & Meister (1954) showed that this reaction is readily reversible, and found that the equilibrium constant is 1.2×10^3 . If the free energy of hydrolysis of glutamine were known, it would be possible to evaluate, in a simple manner, the free energy of hydrolysis of adenosine triphosphate.

There are no data in the literature for the free energy of hydrolysis of glutamine. The glutaminase- or acid-catalysed hydrolysis of glutamine is considered practically irreversible, and its equilibrium constant and free energy appear therefore not to be readily measurable by chemical analysis of reactants and products. For an approach from thermal data, heat of combustion and low-temperature heat-capacity data are available for glutamic acid, but not for glutamine. Levintow & Meister (1954) and M. F. Morales (appendix to Levintow & Meister, 1954) have therefore assumed that the hydrolysis of glutamine is thermodynamically analogous to that of certain dipeptides or of asparagine. The free energy of hydrolysis of these compounds can be calculated from available

heat of combustion and heat-capacity data (Borsook & Huffman, 1938).

In order to examine the assumed analogy between glutamine and asparagine, we have directly measured, in two steps, the enthalpy changes associated with the transformation of glutamine and asparagine into the undissociated glutamic and aspartic acids.

EXPERIMENTAL

Method of heat measurements

Heats of reaction were measured by dissipation microcalorimetry (Benzinger & Kitzinger, 1954, 1956; Kitzinger & Benzing, 1955), with quantities of reactants ranging from 1 to 16 μ moles. The solutions are placed in glass or Teflon vessels of approx. 16 ml. capacity. In 'bicompartimented' vessels, similar quantities of the two solutions (8 ml. each) can be kept separated by a circular partitioning wall during the initial equilibration period. In 'drop' vessels, droplets of one solution are measured with an Agla microsyringe into small dimple-shaped recesses and thus kept apart from the main bulk (16 ml.) of the other solution. The larger volumes are measured by suitable hypodermic syringes or pipettes. Solutions are placed in the vessels with polyethylene tubing attached to the syringes or pipettes.

After thermal equilibrium has been attained, the solutions are mixed by rotation of the entire calorimeter, thus

initiating the reaction. The resulting heat change induces a rapid flow of heat through a thermopile of 10 000 junctions between the reaction vessel and a metal block, which serves as a heat sink. The potential derived from this pile is amplified and continuously recorded over time by a recording potentiometer. The thermal e.m.f. shows the rate of heat flow at any time during the experiment. Total heat (flow rate $\times$ time) is represented by the area under the heat-flow curve and is evaluated with a planimeter.

Calibrations may be performed in two ways: (a) electrically, by sending known constant currents or short pulses through precisely determined resistance wires which are immersed in the water-filled calorimetric vessel; (b) chemically, by mixing known quantities of HCl with an excess of NaOH. Procedures, results and standard deviations of such tests were described by Kitzinger & Benzinger (1955). They were repeated after the transfer of an instrument from Washington to Oxford. The value of $94\,000\,\mu\text{V cal.}^{-1}\text{ sec.}^{-1}$ was obtained in the electrical tests. In the chemical calibrations, 0.1 ml. portions of 0.117 N-HCl were added to 15 ml. of 0.01 N-NaOH in the drop vessels and 160.5, 160.2 and 160.0 mcal. were evolved in three separate tests. Corrections were applied for the addition of 0.10 ml. of HCl to 15 ml. of water (2.56 and 2.62 mcal. evolved in two tests) and for the addition of 0.10 ml. of water to 15 ml. of NaOH (1.01 mcal. absorbed). Thus the heat of neutralization is 158.7 mcal. corresponding to $\Delta H = -13\,560\text{ cal./mole}$ (295° K). The accepted value (Harned & Owen, 1950) is $-13\,720$ at 293° K, and this corresponds to $-13\,620$ at 295° K because of a temperature coefficient of $-50\text{ cal./}^\circ\text{K}$. This agreement of 0.44% is of the order to be expected from the instrument.

The small corrections applied in these chemical calibrations and also in the measurements of the heats of ionization (see Results section) compensate for any dilution heats and for any heat changes due to interactions between the reagents and the glass surfaces of the reaction vessels.

Materials

Glutaminase. This was prepared from *Clostridium welchii* (SR 12) by the method of Hughes & Williamson (1952). In each experiment 0.15 ml. of the final solution was used. This amount of enzyme had sufficient activity to catalyse the hydrolysis of $16\,\mu\text{moles}$ of glutamine in 16 ml. of solution (0.01 N-KCl, 0.01 M-sodium acetate buffer, pH 4.5) within 1 hr.

Asparaginase. This was prepared from guinea-pig serum according to Meister (1955). In each experiment 0.60 ml. of the final preparation was used and this hydrolysed $16\,\mu\text{moles}$ of substrate in 16 ml. of solution (0.01 M-sodium borate buffer, pH 8.5) within 1 hr.

L-Glutamine. This was a laboratory preparation from mangel-wurzels according to the methods of Vickery, Pucher & Clark (1936). The chemical analysis indicated 98.5% of the theoretical N and 100.4% of the theoretical NH_3 , after acid hydrolysis.

L-Glutamic acid and L-asparagine. These were obtained from British Drug Houses Ltd.

L-Aspartic acid. This was obtained from L. Light and Co., Ltd.

Ammonia. This was determined by steam-distillation *in vacuo* according to Parnas & Heller as described by Krebs (1935) and measured in the Beckman spectrophotometer at 475 m μ after nesslerization.

Total nitrogen. This was determined by digesting with H_2SO_4 containing sodium selenate for 1 hr., followed by distillation of NH_3 *in vacuo*.

pH. This was measured with a glass electrode with the model G Beckman pH meter calibrated according to B.S. 1647:1950.

RESULTS

Hydrolysis of glutamine

The heat changes associated with the hydrolysis of glutamine are shown in Table 1. The experiments were carried out with 15 ml. of 0.01 M-sodium acetate buffer (pH 4.5), 0.01 N-KCl and 0.30 ml. of glutaminase in the main space of the calorimetric vessel. Appropriate quantities of 0.1 M-glutamine solution were placed in the drop recesses. The total heats evolved after the mixing of the substrate drops with the buffered-enzyme solution are shown in the second column of Table 2. The heat production ceased after 30–60 min., depending upon the quantities of substrate.

Completeness of the enzymic reaction was checked by calorimetric or by chemical analyses. Ammonia was estimated in the solution remaining in the calorimeter at the end of the first and last experiments shown in Table 1. The amount found ($16.2\,\mu\text{moles}$) agrees with the amount of glutamine introduced ($16.0\,\mu\text{moles}$) within the limits of error. In the one experiment with a larger amount of glutamine ($32.0\,\mu\text{moles}$) the hydrolysis was not

Table 1. Heat of hydrolysis of the amide group of glutamine: $\text{glutamine} + \text{water} \rightarrow \text{glutamate}^{+--} + \text{NH}_4^+$

0.1 M-Glutamine was added to 15 ml. of 0.01 M-acetate buffer, pH 4.5, containing 0.2 ml. of the glutaminase preparation. The ΔH_1 values of column 3 apply to the transformation of glutamine into ammonium glutamate dissolved in buffer at pH 4.5.

Glutamine hydrolysed (μmoles)	Heat evolved (mcal.)	ΔH_1 observed (cal./mole)
27.5*	143.5	-5220
8.0	41.5	-5190
2.0	10.5	-5250
8.0	42.6	-5325
12.0	63.7	-5310
16.2*	86.0	-5310

Mean $\pm$ s.e. -5270 ± 55

* NH_3 was determined chemically (see text).

$160\,\mu\text{l.}$ of 0.1 M-ammonium glutamate was added to 15 ml. of acetate buffer plus glutaminase as above.

Ammonium glutamate added (μmoles)	Heat evolved (mcal.)	ΔH_2 observed (cal./mole)
160	1.78	-110

$\Delta H_1 - \Delta H_2 = -5160\text{ cal./mole}$. This value is for the reaction in dilute aqueous solution at pH 7.0.

complete, as only 27.5 μ moles of ammonia and the corresponding quantity of heat, 143.5 mcal., were found. In four other experiments with less than 16 μ moles of glutamine, it was confirmed that the hydrolysis had been complete by transferring the reaction mixture to another calorimetric vessel and adding fresh enzyme. No heat change was then found except for the small initial heat of mixing. The heat change observed ($\Delta H_1 = -5270$ cal./mole) applies to the transformation of aqueous 0.1M-glutamine into ammonium glutamate dissolved at 0.001–0.06M in a solution containing 0.01M-sodium acetate buffer (pH 4.5), 0.01M-KCl and enzyme.

The enthalpy change on adding 0.16 ml. of 0.1M-ammonium glutamate (previously adjusted to pH 7.0) to the buffer-enzyme solution was also measured and found to be 1.78 mcal. evolved or –110 cal./mole. The difference

$$\Delta H_1 - \Delta H_2 = -5160 \text{ cal./mole}$$

is the enthalpy change for the conversion of 0.1M-glutamine into dilute ammonium glutamate at pH 4.5 followed by the conversion of this ammonium glutamate into 0.1M-solution at pH 7; in other words ($\Delta H_1 - \Delta H_2$) is the heat of hydrolysis of 0.1M-glutamine to form 0.1M-ammonium glutamate at pH 7.

Table 2. *Heat of hydrolysis of the amide group of asparagine:*

40–160 μ l. of 0.1M-asparagine was added to 15 ml. of 0.01M-borate buffer, pH 8.5, containing 0.6 ml. of asparaginase. The ΔH_1 values of the third column apply to the transformation of aqueous asparagine into ammonium aspartate dissolved in buffer at pH 8.5.

Asparagine (μ moles)	Heat evolved (mcal.)	ΔH_1 observed (cal./mole)
4.0	20.4	–5100
2.0	10.5	–5250
1.0	5.2	–5200
8.0	40.4	–5050
16.0	81.8	–5120
Mean $\pm$ S.E.		–5140 $\pm$ 85

160 μ l. of aqueous 0.1M-ammonium aspartate solution was added to 15 ml. of borate buffer plus asparaginase as above.

Ammonium aspartate (μ moles)	Heat absorbed (mcal.)	ΔH_2 observed (cal./mole)
8.0	4.36	550
8.0	4.70	590
Mean		570

$\Delta H_1 - \Delta H_2 = -5710$ cal./mole. This value is for the reaction in aqueous 0.1M-solution at pH 7.0.

Hydrolysis of asparagine

Upon addition of asparagine to a solution of asparaginase in borate buffer at pH 8.5, a substantial absorption of heat was first observed, followed by the heat production of the enzymic hydrolysis. This was presumably due to ionic interactions between the asparagine and buffer since a similar heat absorption (830 cal./mole of asparagine) occurred in the absence of enzyme without, of course, the subsequent heat production due to hydrolysis. The initial heat absorption is reversed in the course of hydrolysis and therefore the final net production of heat obtained from the calorimeter records does not require correction for the interaction of asparagine and buffer. It is, however, necessary to measure the heat change on mixing ammonium aspartate with the enzyme-buffer solution as was done with ammonium glutamate in the experiments with glutamine. The enthalpy changes observed (Table 2) were

$$\Delta H_1 = 5140 \pm 85 \text{ cal./mole}$$

for the conversion of aqueous 0.1M-asparagine into ammonium aspartate dissolved in 0.01M-borate buffer (pH 8.5) containing asparaginase, and $\Delta H_2 = 570$ cal./mole for the addition of aqueous 0.1M-ammonium aspartate to the mixture of buffer and enzyme. Thus the heat of asparagine hydrolysis is

$$(\Delta H_1 - \Delta H_2) = -5710 \text{ cal./mole (pH 7.0).}$$

The completeness of hydrolysis in these experiments was established by the consistency of the heat changes over a 16-fold change of substrate concentrations (Table 2).

Association of glutamate⁺⁻⁻ and H⁺ ions

The transformation of glutamate⁺⁻⁻ ions into the zwitterionic glutamic acid was carried out in the microcalorimeter by adding HCl to excess of sodium glutamate, 0.01M, pH 6.0 (Table 3). After the heat change had been measured, the final pH was determined so that the amount of HCl reacting could be calculated. As shown in Table 3, the amounts of HCl not reacting with glutamate⁺⁻⁻ were negligibly small. The corrections from Table 5 were applied as shown in Table 3. These corrections were not necessary for the bicompartmented reaction vessels. The values obtained for the enthalpy changes due to the association of glutamate⁺⁻⁻ and H⁺ ions are similar in the different experiments of Table 3 despite the different types of reaction vessel or concentrations of HCl. The mean ($\pm$ S.E.M.) of these values is -950 ± 30 cal./mole.

Association of aspartate⁺⁻ and H⁺ ions

In Table 4 the corresponding experiments with aspartic acid are summarized, as carried out with drops of 70–140 μ l. of 0.117N-HCl added to 15 ml. of 0.01M-sodium aspartate. The quantity of H⁺ not reacting with the aspartate was again calculated from the pH. The observed heat of association of aspartate⁺⁻ and H⁺ ions to form aqueous aspartic acid is $\Delta H = -1530 \pm 35$ cal./mole.

DISCUSSION

The enthalpy changes/mole that have been observed in this investigation are as follows:

Table 3. *Heat of association of glutamate⁺⁻ and H⁺ ions to form glutamic acid*

In experiments with 'bicompartimented' vessels, 5.5–8.0 ml. of 4.1 mN-HCl was placed in one compartment and the other compartment was filled with a similar volume of 0.01M-sodium glutamate. In experiments with 'drop' vessels, 0.117N-HCl was used in small amounts, as shown in column 1, while the larger space of the vessel was filled with 15 ml. of 0.01M-sodium glutamate. The amounts of H⁺ ion which had reacted were calculated from the amount of HCl added and the pH changes. For the corrections applied in column 5 see Table 5.

HCl added		H ⁺ ion reacting (μ moles)	Heat evolved (mcal.)	Heat corrected for dilution of HCl (mcal.)	- ΔH (cal./mole)	Type of vessel employed
(ml.)	(μ moles)					
8.0	32.8	32.5	30.1	No corr.	930	Teflon 'bicompartimented'
7.1	29.1	27.3	26.2		930	
7.0	28.7	27.0	24.6	No corr.	910	Glass 'bicompartimented'
5.5	22.5	21.2	22.9		1080*	
0.198	23.2	22.8	24.4	21.8	950	Glass 'drop'
0.100	11.7	11.6	13.3	11.9	1020	
0.050	5.85	5.8	6.1	5.4	930	
				Mean $\pm$ S.E.	950 $\pm$ 30	

* Omitted from average but included in S.E.

Table 4. *Heat of association of aspartate⁺⁻ and H⁺ ions to form aspartic⁺⁻ acid*

Experiments were carried out in calorimetric vessels with drops of 0.117N-HCl and 15 ml. of 0.01M-sodium aspartate, as described for the analogous experiments with glutamate in Table 3. For the corrections applied in column 4 see Table 5.

HCl added (μ moles)	H ⁺ ion reacting (μ moles)	Heat evolved (mcal.)	Heat corrected for dilution of HCl (mcal.)	- ΔH (cal./mole)
11.70	11.60	19.0	17.5	1500
7.03	7.00	11.9	11.0	1570
14.05	13.70	22.6	20.8	1520
Mean $\pm$ S.E.				1530 $\pm$ 35

Table 5. *Corrections applied in 'drop' experiments of Tables 3 and 4*

Procedure	Heat (mcal.)	Heat/mole of HCl (cal.)
Addition of 100 μ l. of 0.117N-HCl to 15 ml. of water	2.6	220 evolved
Addition of 100 μ l. of water to 15 ml. of 0.01M-sodium glutamate	1.4	100 absorbed
Total correction, to be subtracted:		120 evolved
Addition of 100 μ l. of 0.117N-HCl in 15 ml. of water	2.6	220 evolved
Addition in 100 μ l. of water of 15 ml. of 0.01M-sodium aspartate	1.05	90 absorbed
Total correction, to be subtracted		130 evolved

[The errors quoted are standard errors; those for reactions 1 and 3 were obtained by adding an estimated 14 cal. to the s.e.m. of ΔH_1 (Tables 2 and 3) in order to take account of the expected possible errors in the measurement of the smaller heat change, ΔH_2 .]

It was expected that if there was a close thermodynamic analogy between asparagine and glutamine, the heats of their hydrolysis to form the acids would be closer together than would the heats of hydrolysis to form the aspartate^{+--} and glutamate^{+--} ions. However, when the heats of hydrolysis to form the acids are calculated from the above data, it is seen that there is a considerable difference between the values for the two amides:

The corresponding value for asparagine is -7240 cal. In view of these unexpected differences it does not seem that a satisfactory value for the free energy of hydrolysis of glutamine can be obtained by analogy with the data for asparagine. More data are therefore needed to obtain an accurate value for the free energy of hydrolysis of adenosine triphosphate by a calculation based on the equilibrium of the glutamine synthetase reaction.

Previous values for the free energy of hydrolysis of asparagine have been based on the heats of combustion and the entropy data for the reactants. Thus the values given by Borsook & Huffman (1938) give $\Delta G = -3260$ cal. for reaction (3) and slightly modified data given by Burton (1957) give -3440 cal. M. F. Morales (appendix to Levintov & Meister, 1954) obtained the value of -3560 cal., also based on the data of Borsook & Huffman (1938). Instead of the heats of combustion, a value (-3610 cal.) which is probably more accurate can now be obtained from the directly determined heats of reactions (3) and (4). The ancillary data used to obtain this new value are the entropies of crystalline asparagine and aspartic acid, their heats of solution, the free energy of solution of asparagine (Borsook & Huffman, 1938) and the free energies of solution and ionization of aspartic acid (see Burton & Krebs, 1953).

The value found for the heats of reactions (3) and (4) (total $\Delta H = -7240$ cal.) is consistent with the available data for the heats of combustion and solution of anhydrous asparagine and of aspartic acid (Borsook & Huffman, 1938). These data give the value of $\Delta H = -7300$ cal./mole for the hydro-

lysis of aqueous asparagine and the subsequent association of an H^+ ion with the aspartate^{+--} ion. Another value (-6870 cal./mole) may be calculated with the combustion and solution data for asparagine monohydrate (Borsook & Huffman, 1938) instead of the corresponding data for the anhydrous compound.

The values found for the heats of ionization of the secondary carboxyls of glutamic and aspartic acids are similar in magnitude to those derived from titration curves at different temperatures (see Borsook & Huffman, 1938; Smith & Smith, 1942). There are, however, considerable differences and the values obtained in this work by direct heat measurement are considered to be more reliable.

SUMMARY

1. Direct measurements are described of the heats evolved upon the enzymic hydrolysis of the amide groups of glutamine and asparagine in aqueous solution. At pH 7, the ΔH values are -5160 ± 70 and -5710 ± 100 cal./mole respectively.

2. The heats of ionization of the secondary carboxyl groups of glutamic and aspartic acids have also been measured; the respective ΔH values are 950 ± 30 and 1530 ± 35 cal./mole.

3. The values found for asparagine are consistent with the independent values for the heats of combustion and solutions of asparagine and aspartic acid.

4. In view of the unexpected difference between the heats of hydrolysis of the two amides and also between the two heats of ionization, a satisfactory value for the free energy of hydrolysis of glutamine cannot be obtained by analogy with the data for asparagine. More data are therefore needed to obtain an accurate value for the free energy change of hydrolysis of adenosine triphosphate by a calculation based on the equilibrium of the glutamine synthetase reaction.

We wish to thank Professor Sir Hans Krebs, F.R.S., in whose Laboratory this work was done and for his interest throughout. We are greatly indebted to Dr K. Burton for suggesting this problem, for his advice during the course of the work and especially for the preparation of the Discussion. We are also grateful to Dr D. E. Hughes for his advice on the preparation of glutaminase.

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Free-Energy Changes of the Glutaminase Reaction and the Hydrolysis of the Terminal Pyrophosphate Bond of Adenosine Triphosphate

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In the preceding paper, Kitzinger & Hems (1959) found differences between the heats of hydrolysis of the amide groups of asparagine and glutamine and concluded that it was not reasonable to assume that the free energies of hydrolysis were closely similar. ΔG^0 for the hydrolysis of glutamine could not therefore be estimated from the available value for asparagine. As previously discussed, the interest of free-energy data for the hydrolysis of glutamine is that they allow of a simple calculation of the free energy of hydrolysis of the terminal pyrophosphate group of adenosine triphosphate.

For the combined determination of the free energy, heat and the entropy changes of an equilibrium reaction with one analytical method of general applicability, Benzinger (1956) suggested making two calorimetric measurements with reactants and products. In the present study, this procedure was extended to study a reaction that goes practically to completion. By measuring the heat produced when glutaminase was added to ammonium glutamate solutions, the reversibility of the glutaminase reaction could be demonstrated. An important advantage of such a calorimetric procedure is that small chemical changes can be followed whatever is the original composition of the reaction mixtures. It was therefore possible to study the formation or disappearance of small amounts of glutamine in mixtures which contained 1000–3000 times as much ammonium glutamate as glutamine.

The free-energy change for the hydrolysis of glutamine has been combined with data for the glutamine synthetase reaction (Levintow & Meister, 1954; Varner & Webster, 1955) to obtain the free energy of hydrolysis of adenosine triphosphate.

The measurements with glutaminase have been briefly reported in an earlier paper (Benzinger & Hems, 1956). These experimental results can now be considered in conjunction with experimental values for the activities of ammonium glutamate in aqueous solution [R. A. Robinson personal communication (1958)] and also with recent values for the formation constants of the magnesium complexes of adenine nucleotides (Burton, 1959).

EXPERIMENTAL

Heat measurements

The microcalorimeter was the same as described in the preceding paper (Kitzinger & Hems, 1959). In each experiment, 16 ml. of the substrate solution (a known mixture of ammonium glutamate, KCl and glutamine) was measured into the main compartment of a microcalorimetric drop vessel (Kitzinger & Benzinger, 1955) and two 0.08 ml. drops of the glutaminase solution (20 mg./ml.) were measured into each of two dimpled recesses of the vessel. The calorimeter was assembled and, when thermal equilibrium was attained, the enzyme and substrate were mixed by rotating the entire calorimeter. The rate of heat flow from the calorimeter vessel and across the thermopile of 10 000 junctions was continuously recorded (Figs. 1, 3 and 4).

In the later experiments of this work, the enzyme was dissolved in ammonium glutamate plus KCl of the same composition as the principal substrate solution. This procedure eliminated most of the mixing heat which occurred with enzyme dissolved in water. Chemical equilibrium was attained in the small volume of enzyme and ammonium glutamate before it was mixed with the bulk of the ammonium glutamate.

The chemical and electrical calibrations carried out between the experiments are described in the preceding paper (Kitzinger & Hems, 1959).