TABLE III

BENZOYL DERIVATIVES OF AMINO ACIDS

Substituted N-benzoyl-2-amino-4-pentenoic acid	M.p., °C.	Formula	Calculated, % C	Calculated, % H	Calculated, % X	Found, % C	Found, % H	Found, % X
4-Chloro	126.5–127.5	$C_{12}H_{12}NO_3Cl$	56.81	4.77	13.98	57.15	4.56	14.00
4-Bromo	141–142	$C_{12}H_{12}NO_3Br$	48.34	4.06	26.81	48.64	4.21	27.16
4-Iodo	144–146	$C_{12}H_{12}NO_3I$	41.76	3.51	36.77	42.03	3.92	37.34
cis-5-Chloro	169–170	$C_{12}H_{12}NO_3Cl$	56.81	4.77	13.98	56.92	4.85	...
trans-5-Chloro	173.5–175	$C_{12}H_{12}NO_3Cl$	56.81	4.77	13.98	56.96	4.82	...
5-Bromo	166–169	$C_{12}H_{12}NO_3Br$	48.33	4.06	26.81	48.73	4.32	...
4,5-Dibromo	163–164	$C_{12}H_{11}NO_3Br_2$	38.22	2.94	42.39	38.46	2.80	42.22
4,5-Dichloro	159.5–160.5	$C_{12}H_{11}NO_3Cl_2$	50.02	3.85	24.61	49.82	3.72	...
5,5-Dichloro	172.5–173.5	$C_{12}H_{11}NO_3Cl_2$	50.02	3.85	24.61	50.17	3.81	24.79

hydroxide, and treated with an equal volume of ethanol. After standing at −15° overnight, the product was filtered to give 8.8 g. (48% yield) of light colorless flakes. A small sample was recrystallized three times from dilute ethanol to give the analytical sample.

2-Amino-4,5-dibromo-4-pentenoic Acid.—A solution of 20.0 g. of 2-amino-4-pentynoic acid in ethanol was prepared by warming a suspension of the amino acid in absolute ethanol and adding the minimum amount of concentrated hydrobromic acid. To this solution was added 16.0 g. of bromine, and then the mixture was warmed, with stirring, for two hours. After this period, the solution was almost colorless. It was then neutralized with concentrated ammonium hydroxide, at which time a considerable amount of darkening occurred. An equal volume of acetone was added and the solution was held at −15°. The precipitate which formed was filtered to give 12.2 g. of nearly white product. The mother liquors yielded 6.0 g. of slightly less pure product. These crops represent a 67% yield. An analytical sample was obtained after three recrystallizations from dilute ethanol.

Preparation of Benzoyl Derivatives of the Amino Acids.—A modification of the Schotten–Baumann reaction was used. One equivalent of the amino acid was dissolved in a small amount of water containing three equivalents of sodium hydroxide. This was cooled to 0°, and 1.5 equivalents of benzoyl chloride was added dropwise, with vigorous stirring. After stirring for an additional 10 minutes, the mixture was treated dropwise while still cold with concentrated hydrochloric acid until it was acidic to congo red test paper. The mixture was filtered and the solid dried. This solid was suspended in a small amount of hot benzene and filtered. Ether could not be used since the products were too soluble in this solvent. The residue was washed with small amounts of hot benzene, dried, and crystallized twice from ethanol–water to give the analytical samples. The melting points and analyses of these derivatives are shown in Table III.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DARTMOUTH MEDICAL SCHOOL, AND THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY, NO. 1582]

The Enthalpy Change in the Hydrolysis of Creatine Phosphate

BY MARTIN GELLERT AND JULIAN M. STURTEVANT

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The enthalpy change on hydrolysis of creatine phosphate has been measured calorimetrically. The hydrolysis was carried out at *p*H 8.0 and 25° by the combined action of the enzymes ATP[1]-creatine transphosphorylase (in the presence of ADP) and myosin and led to the result $\Delta H = -9.0 \pm 0.5$ kcal. per mole. This quantity was found to be essentially independent of ionic strength.

In view of the importance of creatine phosphate in the chemical events associated with muscle action, considerable interest attaches to the thermodynamics of its hydrolysis under physiological conditions. Previous determinations of the heat of hydrolysis of CrP involve considerable uncertainty, either because the reaction was carried out in complex muscle extracts of unknown composition[2] or because they depend on measurements at *p*H values far removed from neutrality.[3]

In the present work, the heat of hydrolysis of CrP is measured directly at *p*H 8.0 and 25°. The hydrolysis is carried out, in the presence of ADP, by combined use of the enzymes ATP-Cr transphosphorylase and myosin ATPase. The transphosphorylase catalyzes the reaction

$$CrP + ADP + H^+ \rightleftarrows Cr + ATP \qquad (1)$$

the equilibrium constant of which is close to unity at *p*H 8.0. The basis for writing reaction 1 with a hydrogen ion on the left side will be presented later. Myosin catalyzes the reaction

$$ATP + H_2O \rightleftarrows ADP + HPO_4^= + H^+ \qquad (2)$$

which proceeds essentially to completion at *p*H 8.0. Thus the over-all reaction

$$CrP + H_2O \rightleftarrows Cr + HPO_4^= \qquad (3)$$

is also carried essentially to completion. This fact, coupled with the fact that the reactions are sufficiently rapid so that practically complete hydrolysis could be achieved within a period suitable for direct calorimetry, simplified the analytical determination of the extent of reaction at the end of the calorimetric experiment.

Experimental

Five-hour extracted myosin B was prepared by the method of Botts and Morales.[4] ATP-Cr transphosphorylase[5] and

(1) The following abbreviations are used: AMP, ADP, ATP, adenosine mono-, di- and triphosphate, respectively; Cr, creatine; CrP, creatine phosphate; tris, tris(hydroxymethyl)-aminomethane.

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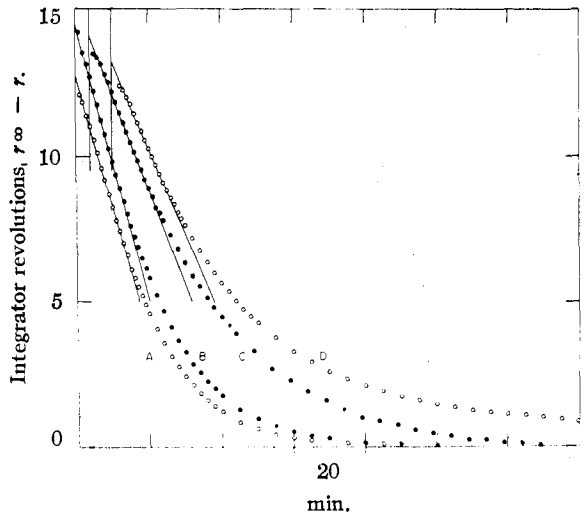

Fig. 1.—The heat evolution in the hydrolysis of CrP as a function of time. The heat evolution is expressed in the form of integrator revolutions.[6] Curves A, B, C and D refer to the runs of the same designations in Table I.

a chromatographically purified sample of CrP were kindly supplied by Professor L. Noda. ADP was obtained from the Sigma Chemical Co.; on incubation with myosin it showed no detectable release of inorganic phosphate, thus demonstrating the absence both of ATP from the ADP, and of ATP-AMP transphosphorylase from the myosin. Other chemicals were of reagent grade and were used without further purification.

The calorimetric apparatus and method have been described previously.[6] The twin calorimeters were each loaded with an ADP + CrP solution in one compartment and myosin + ATP-Cr transphosphorylase in the other. After 12 hr. for temperature equilibration, the solutions in one calorimeter were mixed, and the evolution of heat was recorded. When the reaction was judged complete (3–4 hr.), the solutions in the second calorimeter were mixed and the heat evolution again recorded. The contents of both calorimeters were then assayed for creatine, by the method of Ennor and Stocken,[7] as a check on the completeness of the CrP hydrolysis. The solutions in each calorimetric experiment immediately after mixing had the following composition: 5.0×10^{-4} *M* CrP, 5.0×10^{-4} *M* ADP, 0.001 *M* $CaCl_2$, 1 mg. per ml. of myosin, and 0.002 mg. per ml. of ATP-Cr transphosphorylase, in 0.045 *M* tris buffer, *p*H 8.00. In addition, the solutions in Runs A and B contained 0.15 *M* KCl and those in Runs C and D 0.60 *M* KCl. The volume of each solution was 28.0 ml. The experiments were performed at 25.00 ± 0.05°.

Results and Discussion

As was expected, the instantaneous heat effect accompanying mixing the reactants was very small, since the solutions to be mixed were at the same *p*H and all solute species which underwent dilution on mixing were present in very low concentrations. Figure 1 illustrates the heat evolution as a function of time in each of the runs, starting at the instant of mixing; integrator revolutions[6] (proportional to heat evolution) are plotted against time, r being the reading of the integrator output shaft at time t and r_∞ being the value at the completion of the reaction. The readings have been corrected for calorimetric drift. Since two enzymic reactions are involved, it is not unexpected that the processes follow obviously complex kinetics. The first half, with the possible exception of the first few per cent., follows apparent zero order kinetics; since calorimetric limitations preclude getting reliable readings within less than two minutes after initiation of the reaction, it is possible that there is a small undetected lag phase at the start of the reaction. Evaluation of the total heat effect involves extrapolation to $t = 0$, and doubt as to how this extrapolation should be performed contributes some uncertainty to the results.

Table I summarizes the results of the calorimetric experiments. The analytical results in the third column indicate that complete hydrolysis was obtained, the theoretical yield of Cr being 14.0 micromoles. The last column gives ΔH_{obsd}, the observed heat effect in cal. per mole of Cr formed. Inclusion of uncertainty in the calorimeter calibrations (cal. per integrator revolution) and in the buffer correction (see below) leads to an estimated uncertainty interval of ±500 cal. per mole.

TABLE I

THE HEAT OF HYDROLYSIS OF CREATINE PHOSPHATE AT *p*H 8.00, 25°

Run	Conc. KCl, mole/l.	Creatine prod., μmole	Obsd. heat evol., cal.	$-\Delta H_{obsd}$, cal./mole
A	0.15	14.0	0.116	8290
B	.15	13.7	.134	9780
C	.60	14.2	.131	9220
D	.60	14.1	.120	8510
			Mean	8950
			Std. error of mean	340

If protons are liberated or absorbed during the reaction, a corresponding absorption or liberation of protons by the buffer will also take place. Since the heat of ionization of the buffer is 11600 cal. per mole,[8] it is essential that a careful determination of ϕ_H, the number of moles of protons produced per mole of CrP hydrolyzed, be made. The corrected heat of hydrolysis will then be given by the expression[9]

$$\Delta H_{hyd} = \Delta H_{obsd.} - \phi_H \Delta H_b \qquad (4)$$

where ΔH_b is the heat of hydrolysis of the buffer acid. A value of ϕ_H can, in principle, be calculated from the ionization constants of CrP, Cr and H_3PO_4. However, since the conditions of our experiments differ (particularly in the presence of Ca^{++} and of rather high protein concentrations) from those under which the ionization constants have been determined, it was decided to measure ϕ_H directly.

Two types of experiments were performed. In the first type, the same reaction mixture, except for omission of the buffer, as used in the calorimetric experiments was incubated at 25° until the reaction was sensibly complete (approximately 4 hr.). During this time the system was kept CO_2-free by a flow of nitrogen. At the end of the reaction the very small amount of acid or base needed to return the *p*H to 8.00 was determined. The average of three experiments gave $\phi_H = 0.00 \pm 0.05$.

In the second type of experiment, a solution containing ADP and CrP at the concentrations used in

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the calorimetric experiments was incubated at 25° with ATP-Cr transphosphorylase, in the absence of buffer but in the presence of Ca^{++} and KCl as in the calorimetric runs. The *p*H was maintained at 8.00 by adding KOH from a microburet. After some time the enzyme was inactivated by adding *p*-chloromercuribenzoate, and the solution was analyzed for Cr. Two experiments of this type gave *for reaction 1*

$$\phi_H = -0.98 \pm 0.02$$

The published value[8] of ϕ_H for reaction 2 under our experimental conditions is 1.00. These two figures give $+ 0.02 \pm 0.02$ for ϕ_H for reaction 3.

Since ϕ_H differs from zero by less than the experimental error, the correction term $\phi_H \Delta H_b$ in equation 4 has been taken to be zero. Thus the final value for the heat of hydrolysis of CrP is $\Delta H_{hyd} = -9000 \pm 500$ cal. per mole.

The heat of hydrolysis of CrP is thus notably higher than the values found, under similar conditions, for ATP[8,9] (− 5000 cal. per mole) and inorganic pyrophosphate[10] (−5800 cal. per mole). In terms of the heat available on hydrolysis, CrP may thus be considerd a "high-energy phosphate" in a rather more real sense than the polyphosphates. On the other hand, this need not be true of the more significant standard free energy of hydrolysis. In fact, equilibrium studies[11] on reaction 1 indicate that the standard free energy of hydrolysis of CrP is quite close to that of ATP.

Since the standard free energies of hydrolysis of ATP and CrP are nearly equal and the enthalpy of hydrolysis[12] of CrP is much more negative than that of ATP, the standard entropy of hydrolysis of CrP must be more negative than that of ATP by approximately 13 entropy units per mole. In view of the multiply charged character and the unknown extent of binding of Ca^{++}, of the various solute species, and the fact that three molecules are formed in the hydrolysis of ATP whereas only two are formed in the hydrolysis of CrP, it is not possible to give an interpretation of this difference in entropies.

Utilization of the data of Bernhard[13] on the ionization constants and heats of ionization of the intrafibrial buffers of mammalian muscle permits an estimate of the "physiological" heat of hydrolysis of CrP. On the assumption that ΔC_p for the hydrolysis of CrP is zero, the value obtained for 37° and *p*H 6.9 is $\Delta H_{hyd} = -6900$ cal. per mole.

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Synthetic Work Related to Arginine-Vasopressin[1]

BY ROLF O. STUDER[2] AND VINCENT DU VIGNEAUD

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A new synthetic route to arginine-vasopressin was investigated. The approach involved the coupling of either S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine or S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine by the azide method with L-prolyl-L-arginylglycinamide hydrobromide, which was synthesized by using nitroarginine as an intermediate. The resulting crude protected nonapeptide hydrobromide was treated with sodium in liquid ammonia to remove the protecting groups and then oxidized by aeration. The biologically active material thus obtained was purified by countercurrent distribution followed by electrophoresis and ion-exchange chromatography. The active product proved to be identical with natural arginine-vasopressin in potency, electrophoretic mobility, paper chromatography and ion-exchange chromatography on IRC-50 resin.

In earlier synthetic studies[3,4] on arginine-vasopressin, the protected pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[5] was coupled with the monohydrobromide of the tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide[6] according to the tetraethyl pyrophosphite method to give the protected nonapeptide amide. Also, the more highly protected pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-*o*-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine was used. In both cases, treatment of the protected nonapeptide amides with sodium in liquid ammonia and oxidation of the resulting sulfhydryl compounds gave active products, which after purification by countercurrent distribution and electrophoresis possessed a lower specific activity than the natural arginine-vasopressin. The lower activity of the products was probably due to the formation of an anhydro compound as a contaminating product in the coupling reaction of the carboxyl group of asparagine.[7,8]

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(2) Appreciation is expressed to the Stiftung für Stipendien auf dem Gebiete der organischen Chemie, Zürich, Switzerland, for a Stipendium.

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