# MOLAR ENTHALPY CHANGE FOR HYDROLYSIS OF PHOSPHORYLCREATINE UNDER CONDITIONS IN MUSCLE CELLS

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ABSTRACT The enthalpy change for the hydrolysis of phosphorylcreatine (PCr) by hydrochloric acid or by alkaline phosphatase was observed at 0, 25, and 37°C. The value for ΔH is $-44$ kJ $mol^{-1}$ under alkaline, $Mg^{2+}$-free conditions and is almost independent of temperature, ionic strength, and concentration of reactants. In muscle the reaction is accompanied by a transfer of protons from the buffers (largely histidine) to orthophosphate, release of $Mg^{2+}$ from PCr, and binding of $Mg^{2+}$ to orthophosphate. Measurements are reported of the heats of these processes. The calculated value of the overall heat of hydrolysis of PCr (including these processes) at pH 7, pMg 3 is $-35$ kJ $mol^{-1}$.

## INTRODUCTION

In skeletal muscle cells the major chemical reactions that occur during brief contractions are ATP cleavage and the immediate rephosphorylation of ATP by the creatine kinase reaction (5, 39). The net result of these processes is hydrolysis of phosphorylcreatine (PCr). However "energy balance" experiments have been used to show that other energy yielding processes also occur both during isometric contraction (40) and during shortening (41). The study of these processes is of continuing physiological interest (17). The idea of energy balance experiments is to subtract from the observed energy output of contracting muscle the amount due to the measured PCr hydrolysis. For this purpose it is essential to know accurately the amount of energy produced by this reaction under the conditions inside muscle cells (i.e., pH, pMg, temperature, ionic strength, reactant and product concentrations). It is the object of this paper to report measurements of this quantity. A short account of a part of this work has been published previously (7).

PCr hydrolysis, as it occurs in muscle cells, can be represented by the following equations, in which the heavy arrows show the direction in which the process proceeds in muscle during contraction.

$$PCr^{2-} \longrightarrow Cr + HPO_4^{2-} \quad (1)$$

$$HPO_4^{2-} + H^+ \rightleftharpoons H_2PO_4^- \quad (2)$$

$$\text{histidine} + H^+ \rightleftharpoons \text{histidine } H^+ \quad (3)$$

$$HPO_4^{2-} + Mg^{2+} \rightleftharpoons MgHPO_4 \quad (4)$$

$$PCr^{2-} + Mg^{2+} \rightleftharpoons MgPCr \quad (5)$$

Part of the orthophosphate formed in reaction 1 is protonated in reaction 2. The protons used are provided by the buffer, reaction 3, which in muscle near pH 7 is generally assumed (6, 8) to be principally histidine groups in carnosine and proteins. Both PCr and orthophosphate combine with $Mg^{2+}$, so reaction 1 is accompanied by the shifts shown in the equilibrium positions of reactions 4 and 5. The enthalpy change observed per mole of PCr hydrolyzed in this system ($\Delta H_{obs}$) can be calculated (1) from the molar enthalpy change for all these reactions ($\Delta H_1$, $\Delta H_2$, etc.) and the equilibrium constants for reactions 2, 4, and 5 ($K_2$, $K_4$, and $K_5$). In previous measurements of the enthalpy change for hydrolysis of PCr (3, 4) reactions 2 and 3 were largely avoided by using alkaline conditions, but the $Mg^{2+}$ binding reactions were not considered separately. Previous attempts to calculate $\Delta H_{obs}$ for the conditions in muscle (5, 6) have also neglected this reaction, and in addition, used an incorrect value for the enthalpy of protonation of orthophosphate ($\Delta H_2$), as will be described.

## METHODS

Two calorimeters were used: a batch microcalorimeter (LKB Instruments, Inc., Gaithersburg, MD; for experiments at 25 and 0°C) and a Calvet MS70 (SETARAM, Lyon, France) microcalorimeter for experiments at 37°C. Both instruments were calibrated by Joule heating.

Experiments for acid hydrolysis of PCr were made by adding 0.11 ml of 80 mM $Na_2PCr$ (pH 8) to the calorimeter cell containing 5 ml of 0.1 N HCl (and in some experiments also 0.56 mM ammonium molybdate). The contents of the calorimeter cell were stirred every 100 s by vertical movement of a perforated Teflon disk. Six or eight additions were made at 2-h intervals. The results for the first addition were more variable than the others and were not used. In control experiments the PCr solution was

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replaced by a solution of the products of hydrolysis: 80 mM Cr + 80 mM $Na_2HPO_4$.

For experiments on enzymatic hydrolysis of PCr, at 37°C, alkaline phosphatase from *Escherichia coli* was used (Sigma Chemical Co., St. Louis, MO; P4252, type III). The enzyme is supplied in ammonium sulfate, and just before the experiment 21 U of enzyme was mixed with an equal volume of buffer (25 mM Tris, pH 8) and passed through a short column of Sephadex G-25 to remove ammonium sulfate. The enzyme was then added to 4.0 ml of 25 mM Tris, pH 8, in the calorimeter cell. The experiments were then carried out as described for acid hydrolysis except that the first addition of PCr solution was only 0.03 ml. The results used are based on the second addition (0.11 ml). Although further additions were made, the PCr hydrolysis following them was too slow to provide useful results.

For enzymatic hydrolysis experiments at 25 and 0°C, the buffer solution contained 200 mM KCl and 10 mM carnosine (pH 8) instead of Tris. The PCr and 'products' solutions were 10 mM and also contained 200 mM KCl. A single addition of PCr (0.5 ml) was made, in the side compartment of the cell, and the reaction was initiated by rotation of the calorimeter block.

To obtain the total heat production, we integrated calorimeter records above a line joining the initial and final baselines. For observation of the time course of the initial phase of enzymatic hydrolysis, however, an extrapolation of the initial base line was used, and the records were then corrected for the delay in heat flow out of the cell by the method of Hill (11), using a time course of heat flow determined from observations of the neutralization of Tris by HCl.

In all these experiments the exact amount of PCr or products added was determined by analysis of the calorimeter cell contents for PCr, Cr, and creatinine at the end of the experiment. Parallel experiments, outside the calorimeters, but at the same temperature and using the same solutions, were carried out to establish the time course of PCr hydrolysis and the amount of creatinine formed. Free creatine and total creatine were measured by the method of Ennor (10) and creatinine by the alkaline picrate method (9).

Experiments on ion binding reactions (i.e., reactions 2–5) were made by a titration technique using a modification to the LKB calorimeter. A motor driven micrometer syringe mounted inside the calorimeter airbath was used to make successive 10 $\mu$l additions of titrant to a titrand solution (normally 2 ml) in the calorimeter cell. Additions were made at 15-min intervals and followed immediately by rotation of the calorimeter block to mix the reactants. The addition process itself caused some heat production, largely due to the compression of air in the calorimeter cell. The size of this artefact was determined by making additions of 0.1 M HCl to 2 ml of the same 0.1 M HCl solution. The resultant heat was 1.2 ± 0.06 mJ (mean ± SEM). This quantity was therefore subtracted from all of the observations.

## Sign Convention

The thermodynamic sign convention is used throughout, that is negative values of $\Delta H$ represent heat produced during reaction. The forward direction of the reaction is taken as that from left to right as the reactions are written in this paper.

# RESULTS

## Hydrolysis of PCr (Reaction 1)

The enthalpy change for this reaction can be found from measurements of the heat produced ($h_e$) during the hydrolysis of PCr catalyzed by acid or by alkaline phosphatase. In either case an appropriate control observation is the heat ($h_c$) produced when a solution of Cr + $HPO_4^{2-}$ is added to the catalyst. If the composition of the final solution is the same when reactants are added as when products are added, then the difference, $h_e - h_c$, represents the enthalpy change for reaction 1, under the conditions in the reactant solution before mixing with the catalyst. Under the conditions we have used, pH 8, in the absence of $Mg^{2+}$, the process occurs mostly as in reaction 1.

## Acid Hydrolysis

These experiments were carried out only at 37°C, because the process is too slow at lower temperatures for satisfactory calorimetric observations, that is repeatable observations could not be made over the many hours required for the reaction to complete. Measurements of the appearance of free creatine showed that acid hydrolysis of PCr in 0.1 N HCl at 37° occurred exponentially with a time constant of 30 min. In the calorimetric experiments heat production was therefore measured over a period of 120 min, by which time the calorimeter baseline had returned to its original level. The results are given in Table I.

It is well known that acid hydrolysis of PCr produces

TABLE I
OBSERVATIONS USED TO DETERMINE $\Delta H$ FOR HYDROLYSIS OF PCr AT pH 8*

| | T | $h_e$ | $h_c$ | $h_e - h_c$ | % Creatinine formation |
|---|---|---|---|---|---|
| | °C | | | | |
| Acid hydrolysis | | | | | |
| For 100% hydrolysis at 120 min | | | | | |
| Without molybdate | 37 | 36.1 ± 0.3 (45) | −6.0 ± 0.2 (38) | 42.0 ± 0.4 | 6.4 |
| With molybdate | 37 | 43.7 ± 1.6 (19) | −5.8 ± 0.3 (26) | 49.5 ± 1.7 | 53.0 |
| Corrected for creatinine formation | 37 | | | 41.0 ± 0.5 | |
| Enzymatic hydrolysis | | | | | |
| For 100% hydrolysis at 120 min | 37 | 44.5 ± 0.5 (7) | 1.3 ± 0.3 (6) | 43.2 ± 0.6 | |
| For 98% hydrolysis at 120 min | 25 | 42 ± 2 (11) | 3.0 ± 0.5 (8) | 38 ± 2 | |
| For 70% hydrolysis at 180 min | 0 | 34 ± 5 (10) | 0 ± 1 (10) | 34 ± 5 | |
| From regression of data in Fig. 1 and using controls in lines above | 37 | 43.6 | 1.3 | 42.3 | |
| | 0 | 43 | 0 | 43 | |

$h_e$ is the heat produced on adding PCr to catalyst; $h_c$ is the heat produced on adding Cr + $HPO_4^{2-}$ to catalyst. The units are kJ per mol of product, and the results are expressed as mean ± SEM, with the number of observations in parentheses. *Approximately reaction 1, but see text.

creatinine as well as creatine (28). In our experiments at the time when PCr hydrolysis was almost complete (120 min at 37°C), 6.4 ± 0.5% of the hydrolysis products was creatinine (mean and SEM from five observations). To find the contribution of this creatinine formation to the observed heat production we made another series of experiments in which ammonium molybdate was added to the acid solution used to hydrolyze PCr. In agreement with the results of Barker et al. (28) we found that, in the presence of molybdate, 53% of the hydrolysis product was creatinine. The observed heat production was greater than in the experiments without molybdate by 7.5 ± 1.8 kJ (mol PCr hydrolyzed)$^{-1}$. Thus formation of creatinine from creatine produces 16.1 ± 3.4 kJ mol$^{-1}$, and the heat observed in the experiment without molybdate should be corrected by 1.0 ± 0.2 kJ mol$^{-1}$, for the effect of creatinine formation.

## Enzymatic Hydrolysis

Analyses showed that the enzymatic hydrolysis of PCr did not proceed exponentially under the conditions used in our calorimetric experiments. At 37° 95% of the hydrolysis occurred in 45 min and had reached 97.5% by 90 min. Heat production was measured over a period of 120 min and compared, on the assumption that the reaction was by then complete, with the amount of PCr hydrolyzed. The result (43.2 ± 0.6 kJ mol$^{-1}$, Table I) is rather greater than that obtained in the acid hydrolysis experiments (41.0 ± 0.5 kJ mol$^{-1}$) as is discussed below.

In similar calorimetric experiments at 25°C PCr hydrolysis was found to be 98 ± 3% complete after 120 min and at 0.2°C the reaction was 70 ± 5% complete after 180 min. The observed heats were compared with the amount of PCr found to have been hydrolyzed in the samples used for calorimetry, with the results given in Table I. The precision obtained by these measurements of heat produced over a 120- or 180-min period was not good enough to give useful information about the temperature dependence of the heat of reaction 1. We have therefore used the observations at 0.2°C and at 37°C to compare the time course of heat production, after correction for instrumental lag, with the time course of PCr hydrolysis, measured in parallel experiments outside the calorimeter. The results are shown in Fig. 1. At both temperatures the heat produced is proportional to the amount of PCr hydrolyzed. The slope of the two lines in Fig. 1 is very similar, suggesting that ΔH is almost independent of temperature, as would be expected (12) for a reaction in which there is no change in the number of charged particles.

In the experiment at 37°C there is an excess amount of heat produced at the times, after 45 min, when PCr hydrolysis is >95% complete (* in Fig. 1). This probably represents the heat from some process other than PCr hydrolysis. If so, the inclusion of this heat production would explain why the result based on total heat for enzymatic hydrolysis is somewhat greater than that for acid hydrolysis. However, the slope of the line in Fig. 1

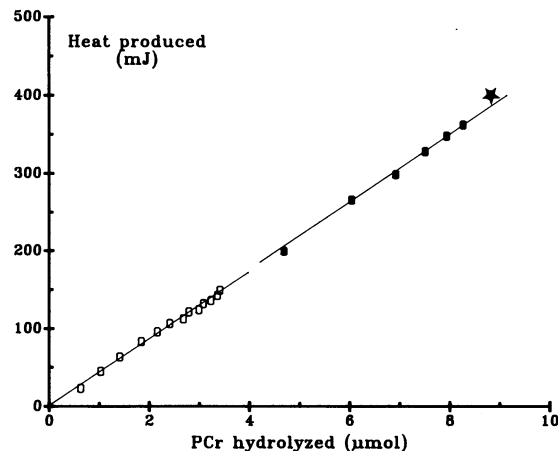



FIGURE 1 Heat produced and PCr split measured in parallel experiments. (○) From an experiment at 0°C. The first two points refer to measurements at 5 and 10 mins after the reaction was initiated, subsequent points are for successive 10-min intervals. The amount of PCr initially present was 5.2 μmol. (●) From the mean of seven experiments at 37°C. The points refer to measurements made at 5-min intervals, except for *, which is for 30 min after the previous point. The amount of PCr present initially was 9.2 μmol.

provides a value of $\Delta H_1$, uncontaminated by this process. After subtraction of the heat of the appropriate control observations, this gives a value which agrees reasonably with that obtained by acid hydrolysis, although it is less precise. It seems best therefore to use the value obtained by acid hydrolysis and to take $\Delta H_1$ as independent of temperature.

A correction to the value of $\Delta H_1$ is needed to allow for the fact that at pH 8.0, 5.8% of the orthophosphate formed in the reaction will be in the form $H_2PO_4^-$, and thus the process occurring does not correspond exactly to reaction 1. The correction, calculated as described by Alberty (1), is −3.1 kJ mol$^{-1}$. The corrected $\Delta H_1$ value for 80 mM reactant concentration is −44.1 ± 0.5 kJ mol$^{-1}$.

The dependence of $\Delta H_1$ on the concentration of reactants and products was investigated by comparing the heat of dilution of PCr and of Cr + $HPO_4^{2-}$ solutions. This was done by flow calorimetry (at 37°C). Solutions (80, 40, and 20 mM) were diluted with equal volumes of water. The dilution heats observed were <0.6 kJ mol$^{-1}$ for each step and differed between reactant and product solutions by <0.3 kJ mol$^{-1}$. The $\Delta H_1$ values in Table I refer to 80-mM solutions. The corrections required to obtain values for 40, 20, and 10 mM solutions are +0.26, +0.46, and +0.32 kJ mol$^{-1}$. Similar experiments were carried out to find the influence of ionic strength on $\Delta H_1$. Reactant or product solutions (50 mM) were mixed with KCl solutions (0.2, 0.4, and 0.6 M), using the mixing of the same KCl solution with water as the baseline. The heats observed were <0.5 kJ mol$^{-1}$. The $\Delta H_1$ value for 50 mM refers to an ionic strength of 0.15 M. The correction to obtain a value for ionic strength of 0.3, 0.5, and 0.7 M are −0.42, −0.13, and +0.28 kJ mol$^{-1}$. Thus the corrections to $\Delta H_1$ both for ionic

strength and for reactant and product concentrations within these ranges are <1%, and no more than the standard error of the best estimate of $\Delta H_1$. Therefore they can be considered negligible.

## Heat of $Mg^{2+}$ Binding (Reactions 4 and 5)

Most of these experiments were done using titration calorimetry at 25°C and at 0.2°C. Successive additions of 1 M $MgCl_2$ were made to solutions containing 30 mM $KH_2PO_4$, $Na_2HPO_4$, or $Na_2PCr$. The heat produced comes from the dilution of the $MgCl_2$ and the binding of $Mg^{2+}$ to ligand. As described, for example by Tyrell and Beezer (13), these experiments can provide values of both the heat of reaction, ΔH, and the equilibrium constant, *K*. Accordingly curves were fitted to the results to find best values of $\Delta H$ and *K*. These curves fitted the observations closely ($r > 0.999$) when the total amount of added $MgCl_2$ was less than about three times the amount of ligand present. At higher concentrations of $MgCl_2$ a systematic deviation appeared, particularly in experiments at low ionic strength. This is probably due to the formation of complexes other than the 1:1 association of $Mg^{2+}$ with the phosphate, but fortunately the association constant for these complexes is low enough to be negligible under physiological conditions. Accordingly only results for $MgCl_2$ concentrations less than three times ligand concentration were used.

When the ligand in these experiments was $Na_2HPO_4$ a slow endothermic process occurred on addition of $MgCl_2$ besides the normal endothermic binding process. This process, presumably a precipitation, was avoided by adding a small quantity (0.003 M) of $KH_2PO_4$ to the ligand solution. A correction had then to be made to the calorimetric results to allow for the heat from binding of $Mg^{2+}$ to $H_2PO_4^-$. As this binding is weaker and less endothermic than that to $HPO_4^{2-}$ the correction is small, <2%, and its exact value has little effect on the final results.

The results of these $Mg^{2+}$ binding experiments are given in Table II *A*, the values of *K* are compared with published results using other techniques. In view of the difficulty of obtaining accurate values of *K* the agreement seems reasonable. Results were obtained for ligand solutions with and without added KCl. As expected the value of *K* was less in solutions of higher ionic strength but there were only slight changes in the value of $\Delta H$.

A few observations of $Mg^{2+}$ binding heats were made at 37°C using flow calorimetry. $Mg^{2+}$ and $Na_2PCr$ (or $Na_2HPO_4$) were mixed in five different ratios from 0.25:1 to 4:1 and the results were analyzed in the same way as the titration experiments, giving the result for $HPO_4^{2-}$ included in Table II *A*. With PCr however, anomalous results were obtained. Considerably less heat absorption per mole of PCr is observed when PCr is mixed with an equimolar amount of $Mg^{2+}$ than when there is a fourfold excess of PCr. This suggests that the binding of a second $Mg^{2+}$ to MgPCr is an exothermic process. Further work would be needed to clarify this point. Thus it is not possible to obtain from these observations data for the 1:1 interaction of $Mg^{2+}$ and PCr which is presumably the only one of importance in muscle, where PCr is in at least 10-fold excess over $Mg^{2+}$.

From the observations in Table II *A* the change in heat capacity ($\Delta Cp = d\Delta H/dT$) for these reactions can be calculated. The values of $\Delta Cp$ are positive, as would be expected for reactions in which there is a decrease in the number of charged species (12).

## Heats of Protonation (Reactions 2 and 3)

These experiments were also made by the titration techniques. As all these binding reactions were strong (pK 6 to pK 8) equilibrium constants could not be obtained. Heats were observed for five successive additions of acid (0.1 M HCl) to base. Each addition was equivalent to one-tenth of the total base present, 25% of which had been neturalized before the start of the observations. A small correction was

TABLE IIA
HEATS OF MAGNESIUM-PHOSPHATE BINDING

| | This work | | | | Other reports | |
|---|---|---|---|---|---|---|
| Orthophosphate | | | | | | |
| Temperature (°*C*) | 0 | 25 | 25 | 37 | 25 | 25 |
| Ionic strength (*M*) | 0.25 | 0.075 | 0.25 | 0.15 | 0.16 | 0.20 |
| Heat of binding | +4.8 | +7.3 | +8.0 | +11.0 | — | — |
| Log K | 1.4 | 2.2 | 1.7 | 2.1 | 1.6 | 1.9 |
| Reference | | | | | 31 | 32 |
| Phosphorylcreatine | | | | | | |
| Temperature (°*C*) | 0 | 25 | 25 | 37 | 25 | |
| Ionic strength (*M*) | 0.25 | 0.075 | 0.25 | — | 0.10 | |
| Heat of binding | +2.3 | +4.7 | +4.9 | See text | — | |
| Log K | 1.6 | 2.1 | 1.6 | — | 1.6 | |
| Reference | | | | | 33 | |

The calorimetric determinations were made by titration calorimetry (see Methods) and are means of results for at least four experiments. Values of $\Delta H$ are given in kJ $mol^{-1}$.

TABLE IIB
HEATS OF PROTONATION

| | This work | | | | Other reports | | | |
|---|---|---|---|---|---|---|---|---|
| Tris at 25°C | | | | | | | | |
| Ionic strength (*M*) | | 0.005 | 0.05 | | 0.01 | 0.015 | 0.10 | |
| Heat of protonation | | −45.7 | −47.1 | | −45.7 | −47.4 | −47.5 | |
| Reference | | | | | 34 | 35 | 37 | |
| $Na_2HPO_4$ at 25°C | | | | | | | | |
| Ionic strength (*M*) | 0.015 | 0.09 | 0.14 | 0.24 | 0.1 | 0.025 | 0.01 | 0 |
| Heat of protonation | −4.4 | −4.4 | −4.8 | −4.2 | −5.1 | −4.6 | −3.3 | −4.1 |
| Reference | | | | | 38 | 38 | 38 | 39 |
| $Na_2HPO_4$ at 0°C | | | | | | | | |
| Ionic strength (*M*) | | 0.25 | 0.15 | | 0 | | | |
| Heat of protonation | | −10.1 | −11.2 | | −9.5 | | | |
| Reference | | | | | 39 | | | |
| Carnosine and histidine at 25°C | | | | | | | | |
| Ionic strength | | 0.15 | 0.25 | | 0.16 | | | |
| Heat of protonation | | −30.3* | −29.5‡ | | −31.6 | | | |
| Reference | | | | | 40 | | | |
| Carnosine at 0°C | | | | | | | | |
| Ionic strength (*M*) | | 0.005 | 0.25 | | | | | |
| Heat of protonation | | −34.5 | −31.6 | | | | | |

The calorimetric determinations in this work were made by titration calorimetry (see Methods). Values are given in kJ $mol^{-1}$. Each is the mean of eight or more observations with SEM < 0.3 kJ $mol^{-1}$. *Carnosine. ‡Histidine.

TABLE IIC
DATA FOR THE CALCULATIONS OF THE VALUE OF $\Delta H_{obs}$ AS A FUNCTION OF pH AND pMg

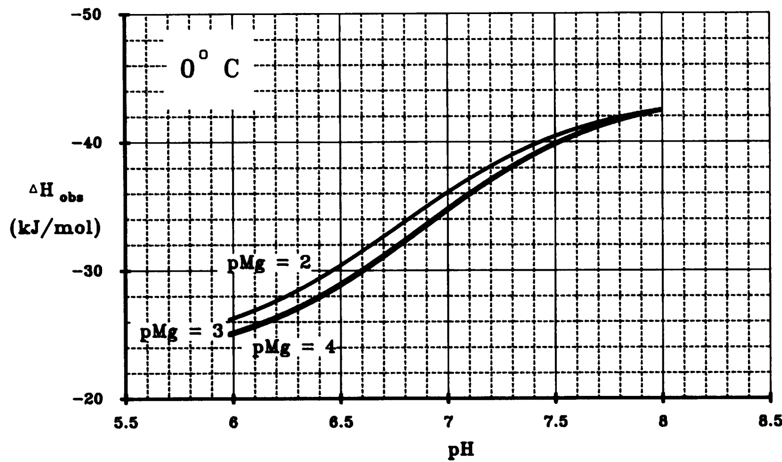
| Temperature (°C) | 0° | 25° | 37° |
|---|---|---|---|
| $\Delta H_1$ (Splitting of phosphorylcreatine) | −44.1* | −44.1* | −44.1* |
| $\Delta H_2$ (Protonation of orthophosphate) | −10.6‡ | −4.5‡ | −1.5§ |
| $pK_2$ | 6.89ǁ | 6.77ǁ | 6.75ǁ |
| $\Delta H_3$ (Protonation of histidine) | −32.0‡ | −29.9‡ | −28.9§ |
| $\Delta H_4$ (Magnesium binding to orthophosphate) | 4.6‡ | 7.8‡ | 11.2‡ |
| $pK_4$ | 1.5‡ | 1.8‡ | 1.9§ |
| $\Delta H_5$ (Magnesium binding to phosphorylcreatine) | 2.3‡ | 4.9‡ | 6.2§ |
| $pK_5$ | 1.7‡ | 1.7‡ | 1.8§ |

*From the acid hydrolysis experiments in Table I, corrected for the formation of creatinine, and for the incomplete ionization of orthophosphate in these experiments.
‡Interpolated values from Table II, *A* and *B*, for an ionic strength of 0.2 M.
§Extrapolated from the values in Table II, *A* and *B*, assuming the heat capacity to be independent of temperature.
ǁFrom reference 37.

made to all the observations for the heat of dilution; the value of this correction was found by making additions of HCl to KCl solution of the same ionic strength as the base solutions used. Results are given in Table II *B* and compared with those of other workers. The agreement is satisfactory, particularly in the case of Tris, for which good calorimetric data are available. For carnosine and phosphate, observations were made at 0.2° as well as 25°C. As expected the heat capacity changes, calculated from the $\Delta H$ values at the two temperatures, are positive. There is little evidence that the heat of protonation of carnosine or phosphate is dependent on ionic strength.

## Calculation of $\Delta H_{obs}$

Fig. 2 shows the calculated values of $\Delta H_{obs}$, the enthalpy produced by PCr hydrolysis and the accompanying $Mg^{+2}$ and $H^+$ reactions (reactions 1, 2, 3, 4, and 5) under various conditions. The calculations are for an ionic strength of 0.2 M, which is probably close to the in vivo value (19). The pH inside frog muscle cells is probably about 7 (14–16) and the pMg value about 3 (18, 21). The values of $\Delta H_{obs}$ for these values of ionic strength, pH and pMg are −34.6 at 37°C, −35.0 at 25°C, and −34.8 kJ $mol^{-1}$ at 0°C. The fact that $\Delta H_{obs}$ is almost independent of temperature under



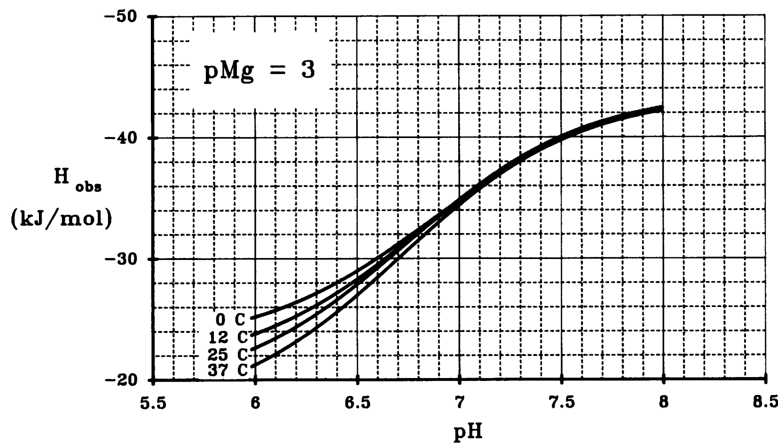




FIGURE 2 $\Delta H_{obs}$ calculation for the data in Table II C. (*A*) The value is shown as a function of pH for three different values of pMg. (*B*) value is shown as a function of pH for four different temperatures. Standard error of the experimental determinations on which $\Delta H_{obs}$ is based is 0.5 kJ/mol at pH 8 and increased to 0.7 kJ/mol at pH 6.

these conditions is coincidental; for instance at pH 6 $\Delta H_{obs}$ changes by 10% over this temperature range (Fig. 2).

## DISCUSSION

The value of $\Delta H_1$ for PCr splitting found by us ($-44 \pm 0.6$ kJ $mol^{-1}$) is greater than those previously reported. Gellert and Sturtevant (3) give a value of $-38 \pm 2$ for pH 8, pMg 3 in Tris buffer. The value of $\Delta H_{obs}$ calculated from our results for these conditions is $-41$ kJ $mol^{-1}$, which is not significantly different from their result. However our result is significantly different from that of Pin (4): $-36 \pm 1$ kJ $mol^{-1}$ at pH 9; as his result, like ours, is apparently obtained by comparison with control experiments using the products of hydrolysis, it refers to Mg-free conditions, under which we would expect to obtain the full value of $\Delta H_1$. This suggests that the process actually occurring at pH 9 is not the same as our reaction 1, presumably because of an extra ionization of either creatine or phosphocreatine. For physiological purposes however it seems justifiable to neglect this ionization under strongly alkaline conditions. The pioneering work of Meyerhof and Schultz (20) should also be mentioned. They measured the heat produced when PCr was hydrolyzed by a "second water extract of muscle." The pH and pMg values in this extract are not known, but probably the conditions were mildly alkaline and the concentration of $Mg^{2+}$ low. Under these conditions the expected value of $\Delta H_{obs}$ is $\sim -40$ to $-43$ kJ $mol^{-1}$ (Fig. 2). The value obtained by Meyerhof and Schultz was $-46$ kJ $mol^{-1}$. In view of the correction that had to be made in their experiments for the heat from glycolysis concurrent with PCr splitting, the agreement is close. Meyerhof states elsewhere (2) that errors of 4–6 kJ $mol^{-1}$ cannot be excluded.

Carlson and Siger (5) and Woledge (6) have previously attempted to establish the value of $\Delta H_{obs}$ for in vivo conditions. Their results, $-40$ and $-38$ kJ $mol^{-1}$, respectively, are more negative than the value of $-35$ kJ $mol^{-1}$ suggested in this paper. The reason for the discrepancy is that both papers used a value for heat of protonation of phosphate ($\Delta H_2$) at 0°C of $-33$ kJ $mol^{-1}$ from Bernhard (22). As has been discussed elsewhere (17, 23) this value was obtained by differentiation of inadequately precise pK values; it disagrees with calorimetric determinations of the quantity and should be disregarded. With this single exception, all experimental determinations of the relevant $\Delta H$ values thus seem in reasonable agreement, and the value of $\Delta H_{obs}$ can therefore be regarded as well established. The value of $\Delta H_{obs}$ recommended by Curtin and

Woledge (8), $-30$ kJ $mol^{-1}$, is smaller than that given here largely because they used a smaller value for $\Delta H_1$.

In addition to $Mg^{2+}$ muscle contains appreciable amounts of $Ca^{2+}$ and $Zn^{2+}$ (19). The total concentrations are 10 and 1 mM, respectively. Although both can form complexes with phosphates (24) it seems unlikely that the presence of either can influence significantly the enthalpy change for PCr hydrolysis. In the case of $Ca^{2+}$ this is because the ion is largely sequestered in the sarcoplasmic reticulum. Although it is released upon stimulation of the muscle, the free $Ca^{2+}$ concentration does not rise above $10^{-4}$ M (25). Since the binding constant for the formation of $CaHPO_4$ is $\sim 10^{-2}$ M, no significant amount of this complex can form. $Zn^{2+}$ binds to $HPO_4^{2-}$ somewhat more strongly than $Ca^{2+}$ does, but binds much more strongly to histidine (24). As there is a much higher concentration of histidine groups than of $Zn^{2+}$ in muscle, it seems unlikely that the free $Zn^{2+}$ level can be high enough for Zn-phosphates to be formed.

The idea that the imidazole group is the effective buffer in muscle is based on the fact of its large concentration and near neutral pK. About 10 mM histidine is present in the dipeptide carnosine, and another 25 mM in the contractile proteins. It has therefore been assumed in this work that the intracellular buffers can be regarded as predominantly imidazole groups in histidine, and that the heat of dissociation of the group is constant, regardless of whether the histidine is part of a large molecule. Mihalyi (26) has shown, by comparison of titration curves of myosin at different temperatures that nearly all the imidazole groups are free to act as buffers and that the heat of protonation of the groups has approximately its usual value. Although small concentrations of other buffer substances such as phosphate and $MgATP^{2-}$ are known to be present they are thought to contribute <5% of the buffer capacity. In some experiments with living muscle, a bathing solution buffered with $HCO_3^-$ and gassed with 5% $CO_2$ is used (8). In this solution $HCO_3^-$ will provide ~17% of the internal buffer capacity. This will have the effect of increasing the value of $-\Delta H_{obs}$ by ~1 kJ $mol^{-1}$.

Recently Curtin (16) has measured the buffer power of live frog muscle fibers over a range of intracellular pH ($pH_i$) values. For $pH_i$ below 7 the measured buffer power was similar to that predicted on the above assumptions about the nature of the intracellular buffers. However for $pH_i$ values between 7 and 7.5 the measured buffer power considerably exceeded that predicted. There must therefore be additional buffers acting within this pH range. The discovery of these buffers introduces extra uncertainty into the calculation of $\Delta H_{obs}$ for in vivo conditions in this pH range. For example at pH 7.3 Curtin's results show that 60% of the total buffering is due to these unknown buffers; if, to take extreme values, the heat of protonation was $-70$ (or 0) kJ $mol^{-1}$, then $\Delta H_{obs}$ would be $-36$ (or $-39$), rather than the $-37.5$ kJ/mol suggested here. The uncertainty in $\Delta H_{obs}$ is less both at higher $pH_i$ values, because the extent of the buffer reaction is less, and at lower $pH_i$ values where the unknown buffers contribute less of the total buffer power.

## Recovery Ratios

A measurement that is often made on muscle (for examples see references 27 and 34) is of the ratio of heat produced in the initial processes, due largely to PCr splitting, to that produced during the recovery period, due to the oxidative resynthesis of PCr. The value of this ratio is very sensitive to the value of $\Delta H_{obs}$ (17) because an increase, for example, in $\Delta H_{obs}$ not only increases the heat produced in the initial period but also decreases that produced in the recovery period when PCr splitting is reversed. Godfraind-De Becker (27, 34) has shown that when muscles are acidified by $CO_2$ the recovery ratio is increased. This would be the result if $\Delta H_{obs}$ fell which, as the measurements reported in this paper show, is what would be expected.

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