Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 5958–5962, October 1981
Biochemistry

Application of differential scanning microcalorimetry to the study of cellular processes: Heat production and glucose oxidation of murine macrophages

(scanning calorimetry/glucose metabolism/drug effects)

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Contributed by Julian M. Sturtevant, March 13, 1981

ABSTRACT **Differential scanning microcalorimetry provides a noninvasive method for studying heat evolution in living cells. We used this technique to measure the heat evolved by thioglycollate broth-elicited mouse macrophages, and the effects of NaF, KCN, cycloheximide, and cytochalasins B and D on this parameter. The total heat evolved in the interval 10–37°C scanned at 1°C min^{-1} ranged from 300 to 2500 × 10^{-12} cal (1 Cal = 4.184 J) per cell, depending on cell density, glucose concentration, and the presence or absence of various drugs.**

Calorimetry provides a noninvasive method for studying the overall metabolic activity of living organisms. Calorimetric measurements of cellular metabolism are based upon the principle that all cellular metabolic activities are accompanied by characteristic heat effects.

Microcalorimetry for studying cellular metabolism has been limited to the use of batch and flow microcalorimeters and has required large numbers of cells (1–10 × 10^9 cells) for reproducible measurements (see ref. 1 for review). Differential scanning microcalorimetry offers a more sensitive method for studying cellular metabolism in living cells. In a differential scanning calorimeter, a sample and a suitable reference material are heated in separate chambers at a constant rate. A feedback system maintains an essentially zero temperature difference between the chambers and provides an output that measures the excess power requirement of one chamber relative to the other one. Integration of this excess power over a time interval gives the heat change in this interval. The heat effects arising from movement or settling of the cell suspension in the chamber of the calorimeter are reduced or eliminated by allowing the settling to occur at approximately 5°C before starting the scan. The rate of heat evolution is then measured during a scan from this low temperature, at which there is very little metabolic activity, to 37°C or to any other desired temperature limit.

Our motive in measuring the heat produced by macrophages stems from our interest in the energetics of phagocytosis. The experiments reported here describe the use of differential scanning microcalorimetry to measure the heat produced by nonphagocytosing macrophages. They show that the rate of heat evolution by these cells is correlated with the concentration of glucose in the medium and with the rate at which these cells oxidize glucose to lactic acid. Studies to be reported elsewhere describe the heat evolution and glucose oxidation by phagocytosing macrophages.

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MATERIALS AND METHODS

Fetal bovine serum was purchased from Flow Laboratories (McLean, VA), [U-^{14}C]glucose from Amersham, Nelson Collins strain mice from Rockefeller University, Brewer's thioglycollate broth from Difco, trypan blue from GIBCO, and cytochalasins B and D from Aldrich.

Preparation of Thioglycollate-Elicited Peritoneal (TEP) Cells. Peritoneal exudate cells from mice injected intraperitoneally with Brewer's thioglycollate broth 4 days in advance were harvested in Dulbecco's phosphate-buffered saline, pH 7.2, as described (2). The cells were suspended in this saline containing 5% dialyzed fetal bovine serum, which had been dialyzed against the saline as described (2) to remove glucose.

Calorimetric Measurements. A differential scanning microcalorimeter designed by Privalov (3) was used. The calorimeter was precooled to 5°C before the macrophages were loaded into the sample chamber. The reference chamber contained the corresponding solution without the cells. The chambers in the calorimeter have ≈1-ml volume and provisions are made for the application of 1-atmosphere (1.013 × 10^5 Pa) of excess pressure of N_2 on the chambers to prevent the formation of gas bubbles. The cells were allowed to settle in the sample chamber for 10 min before the scan was initiated. Recording was started at 10°C and continued to 37°C at a rate of temperature increase of 1°C min^{-1}. After the temperature scan, the cells were removed from the sample chamber, and cell viability was determined (4) and found to be 90% or greater. Furthermore, 98% of the cells that were initially loaded into the calorimeter were recovered after the scan, indicating that cell lysis did not occur.

ATP and Creatine Phosphate. ATP and creatine phosphate were determined (2) before and after a simulated calorimetric scan in medium containing 5.5 mM glucose.

Glucose Metabolism. Many of the biochemical determinations reported were carried out under simulated calorimetric conditions; that is, TEP cells were loaded into 12 × 75 mm test tubes at 5°C in a Lauda temperature bath. The temperature of this bath was then increased at the same rate as that of the calorimeter by means of an electronic temperature scanner. For comparison, some of the biochemical determinations were carried out under isothermal incubation at 37°C. The lactic acid produced was measured in 5.3% perchloric acid extracts of the TEP cells (5). All of the reagents used in this determination were purchased from Sigma. For each lactic acid determination, duplicate standards of lactic acid in 5.3% perchloric acid were

Abbreviation: TEP cells, thioglycollate-elicited peritoneal cells.

measured also. Glucose oxidation to $^{14}CO_2$ from [U-^{14}C]glucose was determined as described (6) by incubating the labeled glucose with TEP cells in specially adapted test tubes (17 × 100 mm); the $^{14}CO_2$ evolved during the scan was trapped on filter paper impregnated with 1 M NaOH (6). The filter paper was then removed and assayed in a liquid scintillation counter (Nuclear-Chicago) using Aquasol (New England Nuclear) as the scintillation fluid.

All biochemical experiments were done at least three times with duplicate samples for each set. The data given are for representative experiments in which the values from the duplicate samples agreed within 10%.

RESULTS

Heat Evolution from Thioglycollate-Elicited Peritoneal Cells. Approximately 85% of the TEP cells were identified as macrophages as judged by morphological criteria (7) and by their capacity to phagocytose antibody-coated erythrocytes (8). These cells were suspended in phosphate-buffered saline/dialyzed fetal bovine serum containing 5.5 mM glucose at cell concentrations of 5, 15, 30, and 60 × 10^6 cells ml^{-1} and scanned in the Privalov calorimeter from 10–37°C at a rate of 1°C min^{-1}. Fig. 1 shows that there is a sharp decrease in heat evolved per TEP cell as the cell density is increased, but that the general shape of the thermogram, including the temperature of maximal heat evolution, 32–34°C, is independent of cell density up to 30 × 10^6 cells ml^{-1}. In all subsequent experiments, unless otherwise noted, this cell density was employed.

It should be pointed out that there is an unavoidable ambiguity in the interpretation of the data from a differential scanning calorimetry experiment. The output has the dimensions either of specific power (e.g., watts or cal min^{-1} per unit of material) or of specific heat [e.g., cal $degree^{-1}$ per unit of material (1 Cal = 4.184 J)], and there is no general basis for deciding which dimension is appropriate in any given case. Thus, the curves in Fig. 1 depict either the variation with temperature of the exothermic power produced by the cells or the variation with temperature of the apparent specific heat of the cells. In either case, an integral over time or over temperature, respectively, has the dimension of energy per unit of material. Repeated baseline scans with phosphate-buffered saline/dialyzed fetal bovine serum in both chambers showed that, within experimental uncertainty, the baseline can be taken to be horizontal.

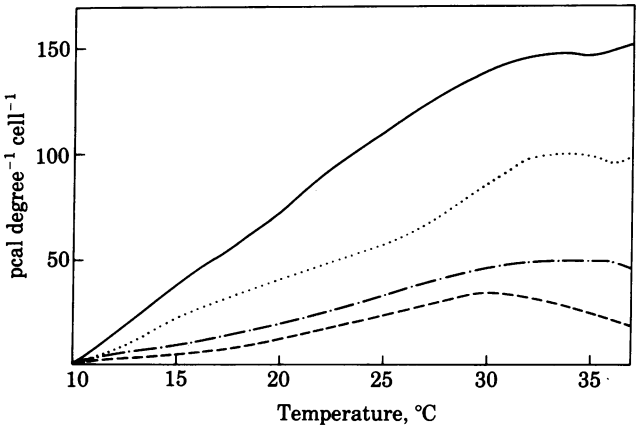

FIG. 1. Heat evolution by TEP cells. The TEP cells, suspended in phosphate-buffered saline containing 5.5 mM glucose and 5% dialyzed fetal bovine serum, were placed into the sample chamber of the calorimeter at 5°C. The reference chamber contained the corresponding solution without the peritoneal cells. Heat effects were measured in the calorimeter during a temperature scan at 1°C min^{-1} from 10–37°C. Curves: —, 5 × 10^6 cells ml^{-1} (2500); ·····, 15 × 10^6 cells ml^{-1} (1300); ·–·–·, 30 × 10^6 cells ml^{-1} (870); ----, 60 × 10^6 cells ml^{-1} (550). The numbers in parentheses were obtained by integrating the area under the corresponding curve; they give the total amount of heat evolved [expressed in picocalories (1 picocalorie = 10^{-12} calories) per cell] during the scan.

The heat evolved by TEP cells during a calorimetric scan increased as the glucose concentration was raised from 0 to 10 mM. The total heat and the temperature at which heat evolution was maximal increased from 335 pcal $cell^{-1}$ and 29°C in the absence of glucose to 890 pcal $cell^{-1}$ and 34°C in medium containing 10 mM glucose (Fig. 2). These results suggest that glucose metabolism is responsible for a major portion of the heat generated by these cells.

Glucose Metabolism in TEP Cells. Evans and Karnovsky (9) have shown that the chief pathway of glucose metabolism in resident mouse peritoneal macrophages is to lactic acid through pyruvic acid. TEP cells incubated in 5.5 mM glucose and scanned from 10–37°C produced 24 nmol of lactic acid per 10^6 cells; this represents the metabolism of 12 nmol of glucose per 10^6 cells. When these cells were incubated in medium containing [U-^{14}C]glucose (final glucose concentration equal to 5.5 mM) during a scan from 10–37°C, 345 pmol of CO_2 were evolved per 10^6 cells. Thus, the amount of glucose oxidized to CO_2 is only 0.5% of the amount metabolized to lactic acid. TEP cells incubated under isothermal conditions (37°C for 30 min) in 5.5 mM glucose produced 76 nmol of lactic acid and 0.8 nmol of CO_2 per 10^6 cells. These results confirm that under both scanning and isothermal (37°C) conditions, lactic acid is the major product of glucose metabolism in TEP cells.

Total lactic acid production, measured after either a simulated or an actual calorimetric scan, and cellular heat production were similarly dependent on the glucose concentration in the medium (Fig. 3). Furthermore, when TEP cells were incubated under isothermal conditions (37°C for 30 min), lactic acid production varied with glucose concentration in the medium (data not shown) in the same way as observed in the scanning experiment shown in Fig. 3.

To determine whether the rates of lactic acid formation and heat evolution change in parallel with one another during a calorimetric scan, we measured the amount of lactic acid produced per each 3°C increase in temperature during a scan of TEP cells from 10–37°C. As shown in Fig. 4, the *rates* of lactic acid formation and of heat evolution were roughly parallel to one another, with maximal rates occurring at about 33°C in both cases. This observation indicates that the shape of the thermograms reflects the rate of glucose metabolism; it reinforces our conclusion that the heat generated by TEP cells is primarily metabolic in origin.

Effects of Cytochalasin B, Cytochalasin D, and NaF on Cellular Metabolism. Cytochalasins B and D inhibit phagocy-

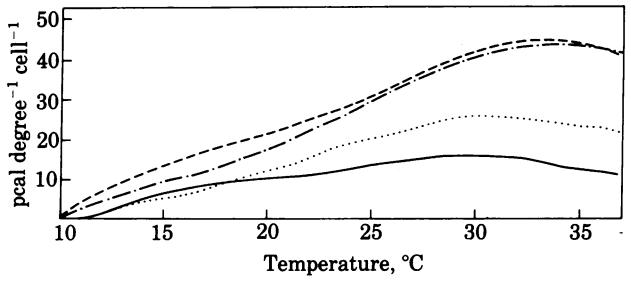

FIG. 2. The concentration of glucose in the medium affects heat evolution by TEP cells. Cell density, 30 × 10^6 cells ml^{-1}. Glucose concentrations: ——, 0.0; ·····, 1.0; ·–·–·, 5.5; ----, 10 mM. Heat effects were measured in differential scanning calorimetry during a temperature scan from 10–37°C at 1°C min^{-1}.

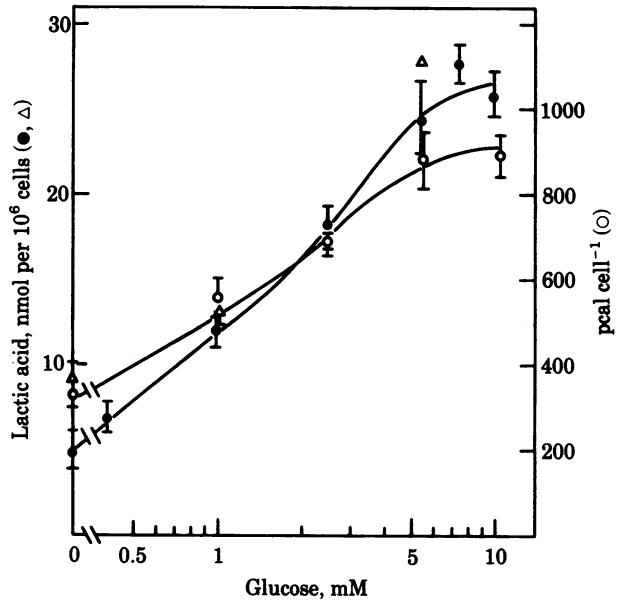

FIG. 3. Heat evolution and lactic acid production by TEP cells suspended in various concentrations of glucose. Heat evolution (○) was measured in differential scanning calorimetry during a temperature scan at 1°C min^{-1} from 10–37°C. After completion of the scan, the cells and medium were collected and analyzed for the lactic acid produced (△) during the scan. Lactic acid production (●) also was determined for TEP cells incubated in test tubes under simulated calorimetric conditions (i.e., under conditions in which the temperature was increased from 10–37°C at the same rate as in differential scanning calorimetry).

tosis through their effects on actin filament polymerization (10, 11). Because these drugs were used in our calorimetric study of phagocytosis, we examined their effects on heat evolution (i.e., shape of the thermogram and total heat evolved) and on lactic acid production in nonphagocytosing cells. Cytochalasin D had no significant effect on the shape of the thermogram (data not shown), on total heat evolution during a temperature scan (Table 1), or on lactic acid production during a 30-min incubation at 37°C (Table 1). However, whereas both cytochalasins B and D disrupt cytoskeletal actin filaments, cytochalasin B has the unique property of inhibiting glucose transport (12). TEP cells treated with cytochalasin B in the presence of 5.5 mM glucose generated 62% less heat and 88% less lactic acid than untreated cells during a temperature scan. These results suggest that the inhibitory effect of cytochalasin B on heat evolution is primarily due to its action on glucose transport.

Studies with NaF further substantiated the observation that

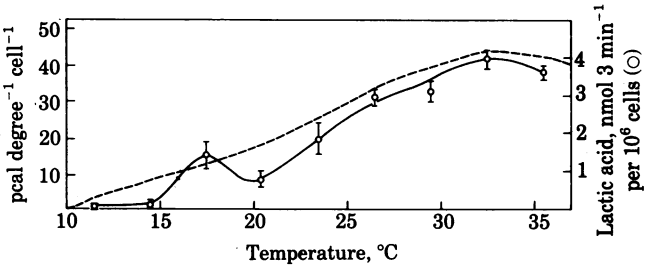

FIG. 4. Comparison of the *rates* of lactic acid production (——) and heat evolution (– – –) by cells during a calorimetric temperature scan. The rate of lactic acid production was calculated for each 3-degree interval during a temperature scan from 10–37°C by measuring the difference in lactic acid production between two samples scanned from 10°C to "X°C", and from 10°C to (X + 3)°C. Each point (○) lies at the midpoint of the 3-degree interval. Glucose concentration, 5.5 mM.

Table 1. The effects of various drugs on heat and lactic acid production by TEP cells

Drug	Conc., mM	Heat production, pcal per cell	Lactic acid production, % of control: Scanning conditions	Lactic acid production, % of control: Isothermal conditions
None		870	100*	100†
NaF	20	282	20	—
Cytochalasin D	0.01	850	93	98
Cytochalasin B	0.01	336	28	11
Cycloheximide	0.07	896	98	—
KCN	1	824	87	—

TEP cells (30×10^6 cell ml^{-1}) suspended in phosphate-buffered saline containing 5.5 mM glucose and 5% dialyzed fetal bovine serum were held at 5°C in the presence or absence of the indicated drug. Heat production was determined in differential scanning calorimetry during a temperature scan at 1°C min^{-1} from 10–37°C; lactic acid was measured under simulated calorimetric scanning conditions and under isothermal (37°C for 30 min) conditions. The data given are the averages of two to five experiments, in which the values agreed within 10%. Conc., concentration.
* 100% = 24 nmol per 10^6 cells.
† 100% = 75 nmol per 10^6 cells.

glycolysis is a major contributor to the heat generated by macrophages. NaF, an inhibitor of glycolysis (13), inhibits heat production by about 68% and lactic acid production by about 80% in TEP cells during a temperature scan (Table 1), whereas cyanide, an inhibitor of oxidative phosphorylation, and cycloheximide, an inhibitor of protein synthesis, had no significant (±6%) effects on either parameter (Table 1).

ATP and Creatine Phosphate Levels. To determine whether the scanning conditions used affect cellular energy metabolism, we measured the ATP and creatine phosphate content of TEP cells before and after a simulated scan from 10°C to 37°C in medium containing 5.5 mM glucose. ATP levels were 0.85 nmol per 10^6 cells before the scan and 0.84 nmol per 10^6 cells afterwards. The corresponding values for creatine phosphate were 1.39 nmol and 1.68 nmol per 10^6 cells. Thus, as measured by ATP and creatine phosphate content, the net energy phosphate stores of TEP cells are virtually unaffected under scanning conditions.

DISCUSSION

It is important that the conditions used in experiments such as those reported here be specified in detail because the amount of heat generated per cell decreases as the cell density increases (Fig. 1). The reason for this is unexplained but may be related to the effect of aggregation of the cells at the bottom of the chamber prior to the scan. If this is correct, then the amount of heat evolved may be affected by the geometry of the calorimeter chamber. In this respect, microcalorimetry of cells in suspension differs markedly from calorimetry of molecules in solution.

Judged by the amount of lactic acid formed, the glucose concentration in the medium decreased less than 1% under conditions in which the glucose concentration in the medium was 0.4 mM or greater. Thus, depletion of glucose from the medium cannot account for the decreased heat production at high cell concentrations. However, it is conceivable that cells in the interior of the cell aggregate experience a significant local decrease in glucose concentration during the scan and that this local decrease in the availability of glucose accounts for the reduction in heat evolution at high cell densities.

Although lactic acid production under scanning conditions was found to parallel reasonably well the heat evolution in the differential scanning calorimetry it must be realized that observations made under scanning conditions cannot be used as

a direct indication of what will be observed under isothermal conditions. For example, under isothermal conditions the rate of lactic acid production was 2-fold greater at 37°C than at 30°C (unpublished data), whereas under scanning conditions the rates at these two temperatures were approximately equal (Fig. 4).

Differential scanning calorimetry offers a noninvasive method for studying the effects of various drugs on the metabolic activity of cells. Drugs which inhibit lactic acid production (e.g., NaF and cytochalasin B) markedly inhibit heat evolution in TEP cells incubated in 5.5 mM glucose. However, drugs that do not inhibit lactic acid production (e.g., cytochalasin D, cycloheximide, and KCN) have no measurable effects on heat evolution in these cells.

We have calculated the fraction of the total heat generated by TEP cells that can be attributed to the formation of lactic acid from glucose, even though the result of this calculation is unavoidably subject to large uncertainty for at least two reasons: (*i*) the intracellular buffering system is unidentified, and we do not know its heat of ionization; and (*ii*) we do not know how ΔH for glucose oxidation to lactic acid changes as a function of temperature. The overall chemical process at pH 8 can be written in the form

$$\frac{1}{2}\,C_6H_{12}O_6(aq) + P_i^{2-} + ADP^{3-} = C_3H_5O_3^-(aq) + ATP^{4-} + H_2O. \quad [1]$$

An estimate of the enthalpy change in this process at 25°C can be made as outlined in Eqs. 2–6. In these equations the enthalpy changes are given in kcal per mol of reaction as written (e.g., for ½ mol of glucose in Eq. 2).

$$\frac{1}{2}\,C_6H_{12}O_6(\alpha,c) + 3O_2 = 3CO_2 + 3H_2O \qquad \Delta H = -334.8 \text{ (ref. 13)} \quad [2]$$

$$\frac{1}{2}\,C_6H_{12}O_6(aq) = \frac{1}{2}\,C_6H_{12}O_6(\alpha,c) \qquad \Delta H = -1.2 \text{ (ref. 14)} \quad [3]$$

$$3CO_2 + 3H_2O = C_3H_6O_3(c) + 3O_2 \qquad \Delta H = +321.2 \text{ (ref. 15)} \quad [4]$$

$$C_3H_6O_3(c) = C_3H_5O_3^- + H^+ \qquad \Delta H = +1.8 \text{ (ref. 16)} \quad [5]$$

$$ADP^{3-} + P_i^{2-} + H^+ = ATP^{4-} + H_2O \qquad \Delta H = +4.9 \text{ (refs. 17 and 18).} \quad [6]$$

The enthalpy in Eq. 3 includes the heat of mutarotation. The enthalpy in Eq. 6 is calculated from the heat of hydrolysis of ATP at pH 8.0 in Tris buffer (17) less the heat of protonation of Tris (18). Summing Eqs. 2–6 gives -8.1 kcal mol^{-1}, with an uncertainty estimated to be ± 1 kcal mol^{-1}.

The cells in our experiments were suspended in phosphate buffer at pH 7.2. We assume the intracellular pH is the same, and ionization corrections must be made for Eq. 6. In making these corrections, we have employed the Henderson–Hasselbach equation with the pK's and enthalpies of ionization given in Table 2. The following equations must be added to Eq. 6:

Table 2. Values of pK′ and $\Delta H_{ionization}$ (19)

	pK′	$\Delta H_{ionization}$, kcal mol^{-1}
P_i	7.2	+0.90
AMP	6.4	−1.8
ADP	7.0	−1.3
ATP	7.0	−1.2

$$0.50\ P_i^{1-} = 0.50\ P_i^{2-} + 0.50\ H^+ \qquad \Delta H = +0.45;\ \Delta H^+ = +0.50 \quad [7]$$

$$0.39\ ADP^{2-} = 0.39\ ADP^{3-} + 0.39\ H^+ \qquad \Delta H = -0.49;\ \Delta H^+ = +0.39 \quad [8]$$

$$0.39\ ATP^{4-} + 0.39\ H^+ = 0.39\ ATP^{3-} \qquad \Delta H = +0.47;\ \Delta H^+ = -0.39 \quad [9]$$

Thus, the total corrections for Eq. 6 are $\Delta H = +0.43$ kcal per mol of ATP formed and $\Delta H^+ = +0.50$. The effect of the 0.50 proton that must be absorbed by the intracellular buffering system is considered below.

Most of the lactic acid produced by the cells is excreted into the extracellular medium through a lactate/proton symport mechanism (20). One proton is transported into the extacellular fluid per mol of lactate released; these protons will be neutralized by the extracellular phosphate buffer with liberation of 0.90 kcal mol^{-1} (Table 2). Thus, the total correction to be added to Eq. 1 is -0.47 kcal mol^{-1} ($-0.90 + 0.43$ kcal mol^{-1}), giving $\Delta H = -8.57$ kcal mol^{-1} for the overall heat of formation of lactate from glucose.

During a scan of 30×10^6 TEP cells in medium containing 5.5 mM glucose, 720 nmol of lactic acid was formed (Table 1, footnote *). This represents the liberation of 6.17 mcal (8.57 kcal mol^{-1} × 720 nmol). The actual heat evolved, as measured by differential scanning calorimetry, was 26.1 mcal per 30×10^6 cells. Thus, the metabolism of glucose to lactate accounts for 24 ± 3% of the heat liberated.

Although this is an accurate assessment of the heat of glycolysis, it is not a complete accounting of the major heat-evolving metabolic processes. Our data show that, under scanning conditions, TEP cells maintain constant levels of ATP and creatine phosphate. Thus, all of the ATP formed according to the reaction in Eq. 1 must eventually undergo hydrolysis to ADP + P_i, AMP + PP_i or AMP + 2 P_i, or some mixture of these products. If the hydrolysis is entirely to ADP + P_i, additional heat evolution as indicated by the reverse of Eq. 6, corrected by the reverse of Eqs. 7–9, will be observed. The effect of the 0.50 H^+ mentioned before (Eqs. 7–9) as having to be absorbed by the intracellular buffering system will be exactly nullified. Thus, the heat evolved by the hydrolysis of ATP to ADP will be -4.9 kcal mol^{-1} [the reverse of Eq. 6; 1 mol of ATP is generated (Eq. 1) and hydrolyzed per mole of lactate formed] plus the heats of ionization of the products, -0.43 kcal mol^{-1} (the reverse of Eqs. 7–9), giving a total of -5.33 kcal mol^{-1}. The overall heat effect due to lactate formation, neutralization of lactic acid by the phosphate buffer in the medium, and ATP hydrolysis to ADP will be -13.9 kcal mol^{-1} ($-8.57 - 5.33$). This is, of course, the same as the enthalpies of reactions 2–5 (-13.0 kcal mol^{-1}) plus the heat of neutralization of lactic acid by the external buffer (-0.9 kcal mol^{-1}). In this case, 30×10^6 cells will be expected to generate 10 mcal (13.9 kcal mol^{-1} × 720 nmol), or about 38% (10/26.1) of the observed heat.

If ATP is hydrolyzed to AMP + 2 P_i, additional heat will be evolved. In the absence of a direct calorimetric determination, we estimate the heat of hydrolysis of ADP to be -4 ± 1 kcal mol^{-1}:

$$ADP^{3-} + H_2O = AMP^{2-} + P_i^{2-} + H^+ \qquad \Delta H = -4 \pm 1\ \Delta H^+ = +1.0 \quad [10]$$

Again we must make corrections for the ionization states existing at pH 7.2 by adding to Eq. 10 the following equations (Table 2):

$$0.50\ P_i^{2-} + 0.50\ H^+ = 0.50\ P_i^{1-}$$

$$\Delta H = -0.45; \Delta H^+ = -0.50 \quad [11]$$

$$0.14\ AMP^{2-} + 0.14\ H^+ = 0.14\ AMP^{1-}$$

$$\Delta H = +0.25; \Delta H^+ = -0.14 \quad [12]$$

$$0.39\ ADP^{2-} = 0.39\ ADP^{3-} + 0.39\ H^+$$

$$\Delta H = -0.49; \Delta H^+ = +0.39 \quad [13]$$

The heat of ionization of the products of these reactions is -0.69 kcal mol^{-1}. The net proton generation (Eqs. **10–13**), + 0.75 H^+, would be absorbed by the intracellular buffering system which we assume to have a heat of ionization similar to that of the imidazole group of proteins (6.5 kcal mol^{-1}), yielding -4.88 kcal mol^{-1} (0.75×6.5). Thus, the additional heat due to ADP hydrolysis would be -9.57 kcal mol^{-1} ($-4.0 - 0.69 - 4.88$), and the total expected heat evolution due to lactate formation and complete hydrolysis of ATP to AMP and 2 P_i would be 16.9 mcal for 30×10^6 cells, approximately 65% (16.9/26.1) of the observed heat.

We recognize that many additional processes take place during the scan which must account for the remainder of the observed heat evolution. Nevertheless, we believe we have considered here the two processes which make the major contributions to the heat evolution.

We are grateful to Virginia F. Kozler for expert technical assistance and to Dr. Ephraim Racker for his thoughtful comments on the manuscript. This research was aided by grants from the National Institutes of Health (GM-04725) and the National Science Foundation (PCM 78–24107) to J.M.S., by grants from the National Institutes of Health (AI-08697) and the Muscular Dystrophy Association to S.C.S., and by a U.S. Public Health Service Fellowship (CA-05724) to J.D.L.

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