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# A thermochemical study of metabolic pathways in activated and triggered 2C11-12 mouse macrophage hybridoma cells ☆

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**Abstract**

Hybridization with lymphosarcoma cells immortalized mouse macrophages for the study of phagocytosis and cytobicidal properties. In the study of macrophage physiology, it became necessary to know more of their cellular metabolism and the changes which occurred when the cells were triggered into the respiratory burst. It was found that, in common with many other types of growing cell, activated 2C11-12 macrophage hybridomas produced considerable lactate under fully aerobic conditions, judging from the highly exothermic CR ratio and subsequent spectrophotometric analysis. Most of both the substrates (glucose and glutamine) was converted to lactate, respectively, by glycolysis and glutaminolysis, in the demand for biosynthetic precursors during growth. Glucose was the more important energy source. Approximately 60% of heat production was explained in terms of enthalpy changes in glucose and glutamine metabolism. It was suspected that fatty acid oxidation from contaminants in the bovine serum albumin needed for cell culture may be important in catabolism.

The respiratory burst was triggered by phorbol-12-myristate-13-acetate and recorded by greatly (5-fold) increased heat production and enhanced chemiluminescence. Oxygen consumption was very rapid and soon led to anoxia in the closed culture system. The calorimetric–respirometric (CR) ratio was less negative and analysis confirmed that there was less lactate production. Radioisotope studies indicated that glycolysis and glutaminolysis

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were less intensive, with respiration of glucose accounting for over 90% of the heat production. The imperative for producing NADPH and cytotoxic oxygen metabolites heavily biased catabolism, reducing the supply of biosynthetic precursors. Known sources of heat production accounted for 87% enthalpy recovery and the remainder may well be caused by fatty acid oxidation.

*Keywords:* Calorimetry; Lactate; Macrophage hybridoma; Metabolic pathway; Respiratory burst

## 1. Introduction

The macrophage, as one of the non-specific effectors of immunity, is characterized by its phagocytic and cytobicidal properties to such as microbes and tumour cells. Engulfment is accompanied by a respiratory burst to produce the potentially lethal oxygen metabolites hydrogen peroxide, the superoxide anion, hydroxyl radical and singlet oxygen [1]. Both phagocytosis and the burst are highly energetic processes, respectively requiring ATP for cytoskeletal movement [2] and NADPH for the reduction of oxygen [3]. Studying the energy requirements of such activities is important to our understanding of them, but fresh macrophages are heterogeneous in type and do not grow in culture without mitogens [4]. Therefore, de Baetselier [5] decided to immortalize them by hybridization with lymphosarcoma cells, selecting clones which retained the immunological properties of phagocytosis and cytotoxicity. Although investigating these aspects of their behaviour is important, it is also vital to relate them to metabolic physiology, especially with regard to the re-acquired ability of growth.

One of the problems in metabolic physiology is making sure that account has been taken of every reaction and pathway. A means of doing so is the enthalpy balance method [3] in which the heat flux, $J_Q$ (W $m^{-3}$) is compared against the overall reaction enthalpy flux $J_{B,r}$, $\Delta_r H_B$ [6] where $J_{B,r}$ is the reaction flux (mol $s^{-1}$ $m^{-3}$) of the substance B in each individual catabolic reaction or series of reactions or pathway (r) and $\Delta_r H_B$ is its molar reaction enthalpy change (J $mol^{-1}$) in a stoichiometric form with the modulus of the reaction r of unity $|\nu_B| = 1$. When the heat flux ($Q$)/enthalpy ($H$) flux ratio (enthalpy recovery), $Y_{(Q/H)} = J_Q/J_H$ [6] equals unity then all the reactions and side reactions in a complex reaction system such as a cell which does not exchange energy in the form of work, have been brought into account. If the enthalpy recovery is greater than unity, then some exothermic reactions remain to be detected; if it is less than unity, then some endothermic ones require to be found in the system.

The most powerful adjunct to calorimetry in constructing an enthalpy balance is measuring oxygen consumption to give the so-called calorimetric–respirometric (CR) ratio [7] which is a heat flux/reaction flux ratio $Y_{(Q/B)} = J_Q/J_B$ [5]. In this case, substance B is oxygen with a flux of $J_{O_2}$. All potential respiratory substrates (amino and fatty acids, carbohydrates) obey Thornton's rule to have similar

theoretical oxycaloric equivalents of −450 kJ per mol $O_2$ (±5%) [7]. The existence and intensity of anaerobic processes can be detected in cells by this ratio.

The general aim of one of the studies in this laboratory is to construct enthalpy balances for a clone of mouse macrophage hybridoma cells 2C11-12 [8], grown under resting conditions and after stimulation to produce a respiratory burst [1,9]. Resting cells [10] and those activated with modulating agents, lipopolysaccharides and interferon-$\gamma$ [11], excreted considerable quantities of lactate even though there were no limitations to the oxygen available in the culture vessels. McKeehan [12] highlighted the possibility that glutamine could be partly oxidized to lactate and termed the pathway glutaminolysis. It is possible, therefore, that the great amount of this metabolite detected in many cell types by calorespirometry [3] and more conventional means [13,14] could have been due to this process. The specific aim of this study has been to construct enthalpy balances for activated mouse macrophage hybridomas and those triggered into the respiratory burst by phorbol-12-myristate-13-acetate. Enthalpy recoveries met with limited success but there was considerable progress with defining the intensity of glutaminolysis and glycolysis participating in the metabolism of cells prior to and during the burst.

## 2. Materials and methods

### 2.1. Cell culture

2C11-12 mouse macrophage hybridoma cells [5] were grown at 37°C in plastic Petri dishes (78 $cm^2$) using RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 2 mmol $dm^{-3}$ glutamine [10] and containing 20 mmol $dm^{-3}$ HEPES (pH 7.2 ± 0.1), 10 kU $dm^{-3}$ polymixin sulphate B [8] and 59 mg $dm^{-3}$ gentamycin. At subculture (72 h), cells were removed from the plastic by mechanical agitation and their number assessed by particle counter (Model D, Coulter, Luton, UK). For serial passage, the cells were diluted to $1.25 \times 10^5$ cells $cm^{-3}$ in fresh growth medium and cultured at 37°C in a humidified atmosphere.

Viability was determined by a dual fluorochromatic method [15,16] using fluorescein diacetate and ethidium bromide [17].

### 2.2. Incubation procedures

All incubations were performed at 37°C in T flasks of several different capacities. Cells in confluent monolayer were washed twice with the incubation medium, which consisted of Dulbecco phosphate buffered saline (PBS) with (per $dm^3$), 5.5 mmol glucose, 2 mmol glutamine, 2 mg bovine serum albumin (BSA), 9.3 mmol $Na_2HPO_4$, 1.5 mmol $KH_2PO_4$ and 20 mmol HEPES; pH 7.2 ± 0.1, 272 mOsm. The cells were then mechanically removed from the plastic, counted and diluted in sufficient medium to give a density of $2 \times 10^6$ $cm^{-3}$. The time of incubation and the exact conditions varied as will be seen later, but all of them were terminated by dilution with 1 part to 5 of 25% (w/v) perchloric acid and cooling the mixture to

0°C. Precipitated protein was removed by centrifugation at 8500*g* for 5 min, followed by storage for the assay of Lowry et al. [18]. When appropriate for analyses, the supernatant was neutralized with KOH and the $KClO_4$ was removed by centrifugation at 8500*g* for 5 min. In some cases, cells were separated from supernatant by centrifugation and the two analysed separately.

### 2.3. Direct calorimetry

Heat flow was registered in an LKB heat conduction flow microcalorimeter (Model 10700-1) with a liquid flow vessel modified from that described earlier [19] by substituting the teflon tubing with thin-walled stainless steel tubing (i.d. 0.9 mm) to give a vessel of 1.36 $cm^3$. Apart from the pump tubing (tygon), all transmission tubing was stainless steel to eliminate oxygen diffusion through the walls.

The cell suspension (100 $cm^3$) was placed in a 250 $cm^3$ Connop flask (straight-sided with three ports) with turbine stirrer. It was overlaid with mineral oil degassed under vacuum [9] and the flask was filled with a constant supply of argon gas [20]. The cells were continuously pumped at a rate of 58 $cm^3\ h^{-1}$ from the vessel to the microcalorimeter by a peristaltic pump (MicroPerpex pump LKB 2132, Pharmacia LKB Biotechnology AB, Bromma, Sweden) located at the outlet connection of the instrument. On most occasions, the suspension was treated with $HClO_4$ rather than being returned to the culture vessel. Exogenous substances were added to the vessel by a Hamilton manual microinjection syringe placed at a port. As a check for the comparability of results, on occasions an oxygen or carbon dioxide electrode was fixed at the central port. To test that, methodologically, the results were correct, some experiments were done with an LKB heat conduction twin ampoule microcalorimeter (Model 2107) [22]. One of the 2 $cm^3$ ampoules was filled with a suspension of cells at a density of $2 \times 10^6$ $cm^{-3}$ in the incubation medium containing 30% (v/v) Percoll (1.050 g $cm^{-3}$). This was the density found to be required in order to make the cells buoyant and thus avoid the anoxic conditions characteristic of the "crowding" effect at the bottom of the ampoule [3]. The second ampoule contained 2 $cm^3$ incubation medium with Percoll and both were equilibrated to 37°C in the calorimeter for 20 min, before the heat plateaux were recorded for 15 min.

The voltage signal of the microcalorimeters were amplified with Keithley 150B microvolt ammeters at a sensitivity of 10 μV. The instruments were calibrated weekly with the in-built 50 Ω electrical resistor and monthly by chemical means ultizing the slow hydrolysis of triacetin, which has a carefully measured enthalpy change [21].

### 2.4. Polarographic procedures

Oxygen consumption and carbon dioxide evolution were determined on parallel samples to those used for flow calorimetry. In both cases, 4 $cm^3$ stirred aliquots were equilibrated in sealed glass vessels for 5 min. When appropriate, reagents were added with a Hamilton syringe and measurements taken over a 30 min period.

Oxygen uptake was measured [22] with a Clark-type electrode in a YSI oxygen monitor modified such that, wherever possible, glass replaced plastic. Electrode limits were set using medium saturated with oxygen (100%) and degassed with 5% (w/v) sodium sulphite. Carbon dioxide was estimated using an Orion $CO_2$ electrode in a Metrohm pH/ion meter (Model 692; V.A. Howe, Banbury, Oxon.). pH was recorded at the beginning and end of the measurements to within $\pm 0.05$, using a pH electrode with the same instrument.

### 2.5. Chemiluminescence

Enhanced chemiluminescence was measured using a Packard 6500 48-channel Picolite luminometer (Packard Instrument Company, Downers Grove, IL, USA). Cells at a density of $2 \times 10^4$ cm$^{-3}$ were suspended in 0.1 mol dm$^{-3}$ veronal-buffered solution, pH 7.6, containing per dm$^3$, 200 μmol $Ca^{2+}$, 500 μmol $Mg^{2+}$, 200 mg BSA, 20 U recombinant interferon (INF-γ-R) and 5.5 mmol glucose [8]. The emissions from 1 cm$^3$ aliquots of the cell suspension were recorded for 30 min after addition of luminol (5′-amino-2,3-dehydro-1,4-phthalazinedione: Boehringer) at a final concentration of 0.1 mmol dm$^{-3}$ and the triggering agent, phorbol-12-myristate-13-acetate at a diluted concentration of 10 ng cm$^{-3}$.

### 2.6. Radioisotopic methods

The evolution of $^{14}CO_2$ by cells was measured in stationary 25 cm$^3$ Erlenmayer flasks containing suspended Eppendorf tubes [23]. At the start of the incubation period, 40 kBq of one of D-[1-$^{14}$C] glucose (specific activity 107 kBq μmol$^{-1}$), D-[6-$^{14}$C] glucose (126 kBq μmol$^{-1}$), D-[U-$^{14}$C] glucose (100 kBq μmol$^{-1}$) or L-(U-$^{14}$C] glutamine (136 kBq μmol$^{-1}$) was introduced into each flask containing 4 cm$^3$ cell suspension at 37°C. The experiment was terminated at 30 min by injecting 2 cm$^3$ 610 mmol dm$^{-3}$ perchloric acid into the flasks. The carbon dioxide so released was absorbed in 1 cm$^3$ hydroxide of Hyamine-10-X (Rohm and Haas, Inc.), contained in the Eppendorf tube. The flask was shaken for 3 h at 37°C and the hyamine was then transferred to a vial containing 15 cm$^3$ toluene-based scintillant and counted to 1% error in an LKB Wallac Rackbeta 1215 liquid scintillation counter.

Condensed $^{14}$C-labelled metabolites were analysed on an isocratic HPLC-system (LKB-Pharmacia Ltd., Milton Keynes, Bucks.). Samples containing $^{14}$C-glucose were separated at 45°C on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) protected by a cation-$H^+$ guard column, using 10 mmol dm$^{-3}$ $H_2SO_4$ as mobile phase (flow rate 0.6 cm$^3$ min$^{-1}$). Preparations with $^{14}$C-glutamine were fractionated at 85°C on an Aminex HPX-87C column protected by a carbo-C guard column, using water as the mobile phase (flow rate 1 cm$^3$ min$^{-1}$).

### 2.7. Spectrophotometric methods

Assays for metabolites and enzymes were performed on neutralized extracts of culture medium and cells using a Gilford 240 spectrophotometer. Apart from

estimations achieved as part of amino acid analysis, glutamine, glutamate, aspartate, serine and alanine were assayed by classic Bergmeyer enzymic methods (see Ref. [24]). Glucose, lactate and pyruvate were determined using Sigma test kits. Enzyme activities were assayed in familiar protocols described by Ardawi and Newsholme [25]. Ammonia was estimated using the indophenol method [26].

### 2.8. Amino acid analysis

The protein (mostly BSA) was removed from cell-free culture medium using sulphosalicylic acid in 0.3 M lithium citrate, pH 1.8, followed by centrifugation at 15000*g* for 10 min. Ammonia was removed by adjusting the pH to 11.5 with 2 M NaOH, followed by evaporation over concentrated $H_2SO_4$. The dried sample was dissolved in lithium buffer made pH 2.2 with 2 M HCl. It was applied to a Bio-Rad A9 column of an LKB 4101 AAA system and the amino acids separated by the usual $Li^+$ buffer system [27].

### 2.9. Chemicals

Except where specifically mentioned, all chemicals were purchased at the highest possible grade from Sigma. Radioisotopes and scintillants were bought from Amersham International plc, Amersham, Bucks. Cell culture media were purchased from Gibco BRL Ltd. (Paisley, Strathchyde) and fetal calf serum from Advanced Protein Products Ltd. (Brierley Hill, West Midlands).

## 3. Results

Measurement of the exchange of amino acids between activated 2C11-12 mouse macrophage hybridoma cells and the complete culture medium (Table 1) showed that only glutamine was utilized to any great extent. There was a net production of

Table 1
Flux of amino acid utilization (negative values) by 2C11-12 mouse macrophage hybridoma cells activated with 1 ng $cm^{-3}$ bacterial lipopolysaccharides and 20 U $cm^{-3}$ recombinant interferon-$\gamma$ (average 5 experiments)

| Metabolite B | $J_B \pm$ SEM/ pmol $s^{-1}$ per $10^6$ cells | Metabolite B | $J_B \pm$ SEM/ pmol $s^{-1}$ per $10^6$ cells |
|---|---|---|---|
| Glutamine | $-23 \pm 2$ | Histidine | $-0.2 \pm 0.03$ |
| Glutamate | $+6 \pm 0.5$ | Isoleucine | $-0.7 \pm 0.06$ |
| Asparagine | $-0.9 \pm 0.1$ | Leucine | $-0.8 \pm 0.08$ |
| Aspartate | $+1 \pm 0.1$ | Lysine | $-0.4 \pm 0.03$ |
| Serine | $+2 \pm 0.1$ | Methionine | $-0.2 \pm 0.02$ |
| Glycine | $-0.3 \pm 0.02$ | Phenylalanine | $-0.2 \pm 0.01$ |
| Alanine | $+3 \pm 0.03$ | Threonine | $-0.4 \pm 0.03$ |
| Arginine | $-0.06 \pm 0.005$ | Valine | $-0.3 \pm 0.02$ |

some amino acids (glutamate by deamination of glutamine and alanine, aspartate and serine by transamination). Perhaps surprisingly, asparagine was utilized to a similar net extent as many of the other amino acids.

The choice of metabolites for analysis in short-term incubations of the cells was based on this information. When glucose and glutamine were present in the medium as the only conventional substrates, assays showed that both were heavily utilized by activated macrophage hybridoma cells (Table 2) with the production of alanine, aspartate, glutamate, lactate, pyruvate, serine and succinate. Ammonia and carbon dioxide were evolved during the incubation and oxygen was consumed under these fully aerobic conditions. The gas exchange ratio was low, $R = 0.92$, but the Lac/$O_2$ ratio reflected the participation of anaerobic processes as demonstrated by the highly negative CR ratio of $-592 \pm 91$ kJ per mol $O_2$ (Table 3). The precise contribution of lactate to the CR ratio was calculated from the equation

$$\Delta_k H_{O_2(ox+Lac)} = \Delta_k H_{O_2} + \text{Lac}/O_2 \times \Delta_k H_{Lac} \quad (1)$$

and shown to be $-553$ kJ per mol $O_2$. Cells suspended in 30% Percoll in an ampoule microcalorimeter had a similar heat flux (32 μW per $10^6$ cells) to a second aliquot of the same cell population (see Table 2) pumped through the flow vessel of a second calorimeter. The oxygen consumption was also similar (51 pmol $s^{-1}$ per $10^6$ cells) to give a CR ratio of $-619 \pm 83$ kJ per mol $O_2$, insignificantly different to that of cells in the flow vessel.

Table 2
Flux of major metabolites in the simple incubation medium containing 2C11-12 mouse macrophage hybridoma cells (average of 5 experiments)

| Metabolite B | $J_B \pm$ SEM/pmol $s^{-1}$ per $10^6$ cells | |
|---|---|---|
| | Activated | Triggered cells |
| Glucose | $-37 \pm 4$ | $-51 \pm 5$ |
| Glutamine | $-23 \pm 2$ | $-19 \pm 2$ |
| Glutamate | $+6 \pm 0.5$ | $+5 \pm 0.05$ |
| Aspartate | $+1 \pm 0.1$ | $+0.5 \pm 0.07$ |
| Serine | $+2 \pm 0.3$ | $+1 \pm 0.01$ |
| Alanine | $+3 \pm 0.2$ | $+3 \pm 0.3$ |
| Pyruvate | $+2 \pm 0.2$ | $+6 \pm 0.4$ |
| Lactate | $+61 \pm 6$ | $+34 \pm 3$ |
| Succinate | $+0.5 \pm 0.3$ | $+0.5 \pm 0.04$ |
| Oxygen | $-57 \pm 4$ | $-109 \pm 7$ |
| Carbon dioxide | $+53 \pm 9$ | $+105 \pm 19$ |
| Ammonia | $+22 \pm 2$ | $+18 \pm 2$ |
| $CO_2O_2$ ratio ($R$) | $0.92 \pm 0.2$ | $0.97 \pm 0.2$ |
| Lac/$O_2$ ratio | $1.07 \pm 0.1$ | $0.31 \pm 0.1$ |
| Ratio 1-$^{14}CO_2$/6-$^{14}CO_2$ | $1.18 \pm 0.2$ | $1.36 \pm 0.2$ |

Table 3
Heat flux $J_Q$ of 2C11-12 mouse macrophage hybridoma cells/μW per $10^6$ cells, experimental heat/$O_2$ flux (CR) ratio $Y_{Q/O_2}$, and the calculated enthalpy/$O_2$ flux ratio or molar enthalpy of catabolic reaction, $\Delta_k H_{O_2(ox+Lac)}$, both in units of kJ per mol $O_2$ (see Table 2)

| | Activated cells | Triggered cells |
|---|---|---|
| Heat | 34 ± 2 | 58 ± 4 |
| Experimental CR ratio | −592 ± 91 | −533 ± 94 |
| Calculated CR ratio | −533 ± 5 | −499 ± 0.2 |

The $CO_2$ ratio of C1/C6 indicated that the pentose phosphate pathway was weak in activated cells.

Stoichiometric calculations on the data in Table 2 revealed that, after allowing for deamination of glutamine, 16.3 pmol $s^{-1}$ per $10^6$ cells of this amino acid was available to the activated cells (Table 4). If all the oxygen were consumed in

Table 4
Stoichiometric calculations for the respiration of glutamine to $CO_2$ and $NH_4^+$

| Theoretical oxygen flux ($J_{O_2}$) | $J_B$/pmol $s^{-1}$ per $10^6$ cells | |
|---|---|---|
| | Activated cells | Triggered cells |
| Gln transamination (tam) flux<br>$J_{tam} = J_{Asp} + J_{Ser} + J_{Ala}$ | 0.83 + 2.67 + 1.5 = 5.0 | 0.47 + 0.76 + 2.6 = 3.83 |
| Gln deamination (dam) flux to Glu<br>$J_{dam} = J_{Glu} = J_{tam}$ | 6.4 − 5.0 = 1.4 | 5.1 − 3.8 = 1.3 |
| Gln oxidation flux<br>$J_{Gln(ox)} = J_{Gln} - J_{Glu}$ | 22.7 − 64 = 16.3 | 18.7 − 5.1 = 13.6 |
| These values are used to calculate the enthalpy change for complete oxidation of glutamine | | |
| Oxygen consumption related to $J_{Gln(ox)}$<br>$O_2$/Gln = 1/0.22 = 4.55 | | |
| $J_{O_2}(Gln) = J_{Gln(ox)} \times O_2/Gln$ | 16.3 × 4.55 = 74 | 13.6 × 4.55 = 62 |
| Actual value | 57 | 109 |
| Complete Gln oxidation based on $O_2$ uptake | 57/4.55 = 12.5 | 109/4.55 = 24 |
| RQ/Gln = 1.11 | | |
| Ammonium balance<br>$NH_4^+/O_2 = 0.44$ | | |
| $J_{NH_4^+}(Gln\text{-}ox) = J_{O_2}(Gln) \times NH_4^+/O_2$ | 74 × 0.44 = 32.6 | 62 × 0.44 = 27.3 |
| $J_{NH_4^+}(theor) = J_{NH_4^+}(dam) + J_{NH_4^+}(Gln\text{-}ox)$ | 1.4 + 32.6 = 34 | 1.3 + 27.3 = 28.6 |
| Actual value | 22 | 18 |

[a] The theoretical oxycaloric equivalent for glutamine $\Delta_k H_{O_2}$ is −452 kJ per mol $O_2$ at pH 7 and an enthalpy of neutralization, $\Delta_b H_{H^+}$ of −8 kJ $mol^{-1}$ [35]. Assuming an enthalpy of neutralization of the intracellular buffer $\Delta_b H_{H^+}$ of −21 kJ $mol^{-1}$ [7] and $H^+/O_2 = 0.46$, the standard oxycaloric equivalent (−452 − (0.46 × −8) = −448 kJ $mol^{-1}$) becomes the actual value −448 + (0.46 × −21) = −456 kJ $mol^{-1}$.

glutamine oxidation, then only 12.6 pmol $s^{-1}$ per $10^6$ cells of it could be fully oxidized and none of the glucose. Furthermore, $CO_2$ production was lower than could be expected from the oxidation of glutamine with a respiratory quotient (RQ) of 1.11. The balance for the ammonium ions also showed that deamination and complete oxidation of glutamine was less than would be suggested by its consumption.

Based on Thornton's rule for the average oxycaloric equivalent of substrates at −450 kJ per mol $O_2$ ± 5% [7], the oxygen flux of 57 pmol $s^{-1}$ per $10^6$ cells (Table 2) would give an enthalpy flux of 25.7 μW per $10^6$ cells and an enthalpy recovery of 1.32. It was necessary to discover both the oxidative pathways responsible for this heat production and the other catabolic reactions indicated by the high value for recovery.

Despite misgivings about results for isotopic trace studies on catabolic pathways, the findings in Table 5 do provide a number of indicators. Most of the glucose was consumed in the glycolytic pathway to form lactate. In addition, the greatest part of the glutamine was converted to lactate, probably by partial oxidation in the glutaminolytic pathway. Some of both the sugar and the amino acid was fully oxidized to carbon dioxide (Table 5) and therefore they could be described as significant energy sources for these cells.

The reaction fluxes shown in Table 5 were used to calculate relevant reaction enthalpy fluxes by multiplication with the appropriate molar reaction enthalpy [28,29] (Table 6). If glucosamine were respired by the cells to the degree indicated in Table 5 by $CO_2$ production, then the reaction enthalpy flux would be 4.6 μW per $10^6$ cells. In the oxidation of the amino acid to lactate and $CO_2$, the values for the two end products did not agree with the stoichiometry. A range is quoted, therefore, which depended on choice of end product; however, the average was used in calculating the total enthalpy flux, including respiration and glycolysis of glucose. The resultant enthalpy recovery of 1.63 underlined the necessity of discovering more of the pathways in these cells.

Table 5
Metabolic flux of [U-$^{14}$C] glucose $J_{Glc}$ in pmol $s^{-1}$ per $10^6$ cells (%) and [U-$^{14}$C] glutamine $J_{Gln}$ in pmol $s^{-1}$ per $10^6$ cells (%) [a] by 2C11-12 mouse macrophage hybridoma cells in standard incubation medium (average of 4 experiments)

| | Activated cells | | | | Triggered cells | | | |
|---|---|---|---|---|---|---|---|---|
| | $J_{Glc}$ | | $J_{Gln}$ | | $J_{Glc}$ | | $J_{Gln}$ | |
| Lactate | 55 ± 5 | (73) | 9 ± 1 | (38) | 30 ± 2 | (30) | 4 ± 0.5 | (21) |
| Pyruvate | 0.5 ± 0.1 | (0.7) | 1 ± 0.1 | (5) | 6 ± 0.5 | (6) | 0.2 ± 0.1 | (0.9) |
| Carbon dioxide | 23 ± 2 | (10) | 10 ± 1 | (9) | 90 ± 7 | (29) | 7 ± 1 | (7) |
| Glutamate | – | | 7 ± 0.5 | (29) | – | | 5 ± 0.5 | (26) |
| Total | | (83.7) | | (81) | | (65) | | (54.9) |

[a] Calculated from specific activity of relevant substrate.

Table 6
Flux of 2C11-12 mouse macrophage hybridoma cells calculated from the measured reaction flux (see Table 5) and theoretical molar reaction enthalpy for each reaction pathway

| | Molar reaction enthalpy $\Delta_r H_B$/ kJ mol$^{-1}$ [a] | Reaction enthalpy flux $J_Q$/ μW per $10^6$ cells [b] | |
|---|---|---|---|
| | | Activated cells | Triggered cells |
| Actual heat production | | 34 | 58 |
| Calculated from data in Table 5 for | | | |
| (a) Oxidation of | | | |
| (i) Gln to $CO_2$ | −2084 | −(4.6) | −(2.5) |
| (ii) Gln to Lac [c] | −695 | −3.5–6.3 | −2.4–2.8 |
| (iii) Gln to Pyr | −890 | −0.2 | −0.05 |
| (iv) Glc to $CO_2$ | −3018 | −11.6 | −45.3 |
| (b) Glycolysis of | | | |
| (i) Glc to Lac | −153 | −4.2 | −2.3 |
| Total | | −20.9 | 50.25 |

[a] Calculated from Wilhoit [28] and Bäckman [29]. [b] The SI unit for enthalpy flux and scalar heat flux is W m$^{-3}$. These measurements are expressed "per $10^6$ cells" and, assuming the cells are of constant volume, are taken to be equivalent to flux. See Ref. [6]. [c] Based on $CO_2$ end product (low figure) and Lac end product (high).

In order to provide evidence for the existence of the glutaminolytic pathway in the activated hybridoma cells, they were assayed for some of the enzymes characteristic of this oxidative route which involves the second half of the tricarboxylic acid cycle. Many of the enzymes were highly active (Table 7) and certainly sufficient to produce the pyruvate. Its conversion to lactate is promoted by the high ratio of LDH to PDH.

When activated cells in a closed system were triggered into the respiratory burst by phorbol-12-myristate-13-acetate (PMA), the effect was manifested as a considerable but temporary increase in enhanced chemiluminescence (Fig. 1). This stimulation was arrested within 12 min and had completely disappeared at 36 min. Its general form was repeated in the continuous trace of heat flux which also had the same ephemeral nature, being at its peak within 10 min of the moment of trigger and absent by 30 min (Fig. 1). The reason for the transitory nature of the burst is seen in Fig. 2 which shows that the trigger so stimulated oxygen consumption that there was none left dissolved in the incubation medium at 20 min. Despite this exhaustion $^{14}C$-labelled $CO_2$ continued to be produced at a similar rate by the cells until the end of the experimental period of 30 min (see Fig. 3). The rate of evolution of $^{14}CO_2$ from carbon-C6 slowed 20 min after adding the PMA, so increasing the C1/C6 ratio at the point of oxygen depletion.

Table 7
Activities of some catabolic enzymes in activated 2C11-12 mouse macrophage hybridoma cells at 25°C (1 cell = $4.9 \times 10^{-10}$ g protein)

| Enzyme | Activity/ pmol $s^{-1}$ per $10^6$ cells |
|---|---|
| Acetoacetyl-CoA thiolase | 2.6 |
| Citrate synthetase | 15.6 |
| Glutaminase | 11.7 |
| Hexokinase | 10.4 |
| Lactate dehydrogenase (LDH) | 94.6 |
| $NAD^+$-linked malate dehydrogenase (MDH) | 56.1 |
| Oxoglutarate dehydrogenase | 1.7 |
| Phosphoenolpyruvate carboxykinase (PEPCK) | 1.3 |
| Pyruvate kinase (PK) | 39.6 |
| Pyruvate dehydrogenase (PDH) | 0.87 |

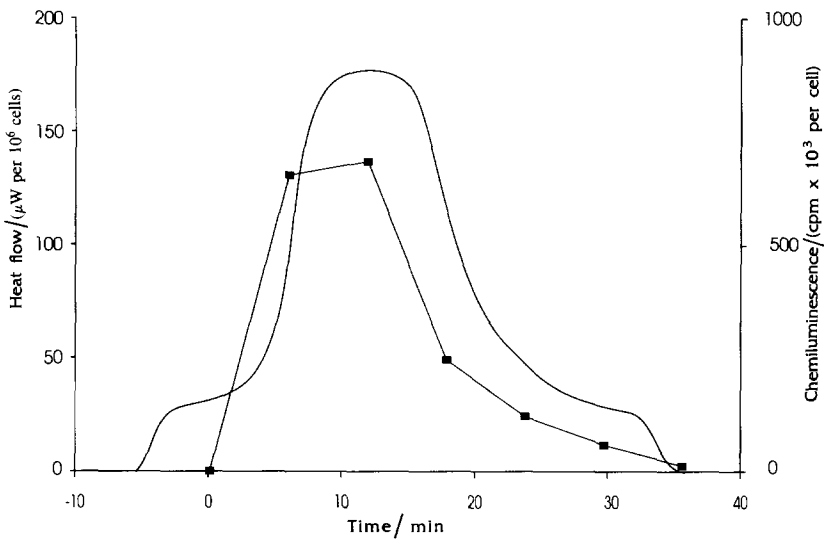


Fig. 1. Respiratory burst of activated 2C11-12 mouse macrophage hybridoma cells triggered with phorbol-12-myristate-12-acetate (PMA). The increased metabolic activity with time is represented as (i) heat flow ($\phi = dQ/dt$ in μW per $10^6$ cells) and (ii) chemiluminescence (■–■) enhanced with luminol (cpm × $10^3$ per $10^6$ cells).

The nearly-doubled oxygen consumption (see Table 2) was accompanied by a modest increase (38%) in glucose consumption but a truly-doubled production of carbon dioxide gave a higher gas exchange ratio. The increase in aerobic processes was associated with a 44% decrease in lactate production, emphasizing the switch in glucose catabolism from being primarily an anaerobic process in activated cells to

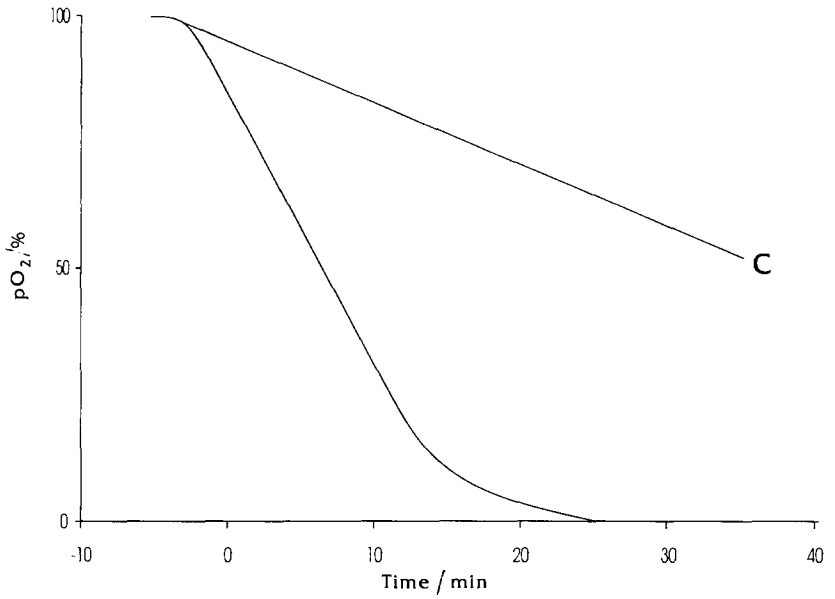


Fig. 2. Increased oxygen consumption by triggered mouse macrophage hybridoma cells when compared with uptake by their activated counterparts (C) before treatment with PMA.

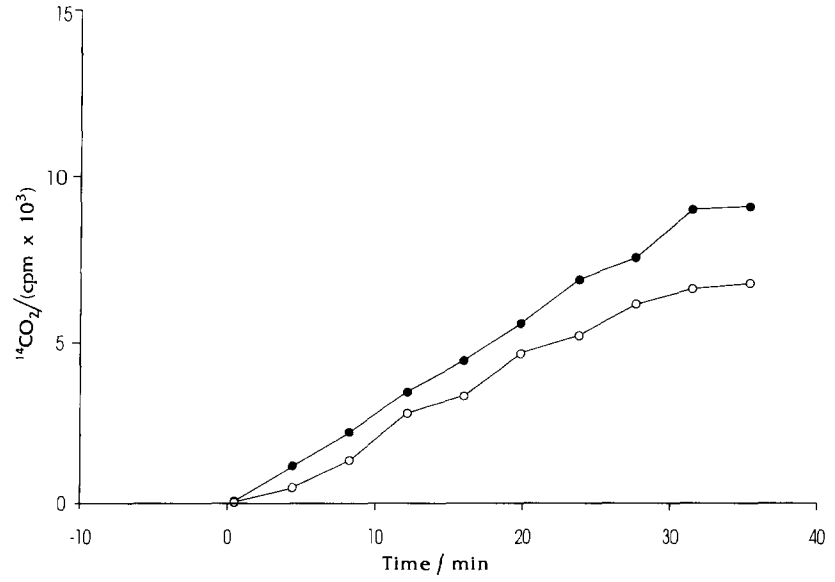


Fig. 3. Production of $^{14}CO_2$ by triggered mouse macrophage hybridoma cells from (1-$^{14}$C) glucose (●–●) and (6-$^{14}$C) glucose (○–○).

being an aerobic one in triggered cells. At the same time, glutamine was used less and this was reflected in the lower production of ammonia by deamination and amino acids by transamination.

The raised metabolic activity was quantified as a 70% increase in the heat flux averaged over the 30 min period. The changed emphasis in glucose metabolism was the reason for the less negative CR ratio, the numerical value for which could be explained by the relatively small flux of the anaerobic processes in the presence of the predominant aerobic pathways ($\Delta_k H_{O_2(ox+Lac)} = -499$ kJ per mol $O_2$). Judging by the carbon C1/C6 ratio of $CO_2$ production, one of the pathways of increased activity was that of pentose phosphate.

Stoichiometric calculations showed that complete oxidation of the glutamine available after transamination could not have produced sufficient enthalpy change to match cellular heat flux (Table 4). This conclusion was supported by the fact that more oxygen was consumed than could oxidize all the available glutamine. The ammonium balance revealed that, actually, not all the glutamine was utilized, because only 63% of the available ammonium ions were liberated from the amino acid. Radioisotope studies (Table 5) led to calculations showing that the large majority (87%) of the heat flux from triggered cells were derived from the oxidation of glucose to $CO_2$ (Table 6). This compared with less than 50% in pretriggered cells and emphasized the total commitment to the respiratory burst. It was associated with an increase in the small amount of pyruvate produced by the cells and to a general decrease in lactate production by both glycolysis and glutaminolysis.

## 4. Discussion

In 1959 Eagle [30] demonstrated that glutamine was more extensively metabolized by tissue cells in culture than other amino acids. Since then it has been shown in many studies that glutamine is an important energy source and biosynthetic precursor [14,31], the relative importance between the two functions seeming to vary with the origin of the cells. Despite the growing evidence to the contrary, some still regard glucose as the primary energy source with glutamine only as a biosynthetic precursor for growth [32]. Of course, the distinction is arbitrary because energy as ATP is still produced when glycolysis and glutaminolysis are favoured pathways by cells growing under fully aerobic conditions. The cells would not use these two pathways, however, primarily for energy production except in conditions of oxygen stress or low capacity tricarboxylic acid cycle/oxidative phosphorylation. Since both pathways terminate with lactate, it would appear reasonable that its flux be regarded as a measure of the production of biosynthetic precursors in cells growing in normoxic conditions which, of course, would allow complete oxidation of the two substrates. The experiments in which cells were suspended in Percoll and gave the same results as cells pumped from the culture vessel supports the contention that the macrophages were cultured under normoxic conditions.

The device used to explore this question was $^{14}C$-radioisotope trace studies. The problem of the interconnections between catabolic pathways is recognized as a

constraint on the validity of this approach. Nevertheless, the data do have qualitative information of value to the analysis of this work. The relevant results in Tables 2 and 5 were used to calculate the enthalpy flux for each pathway or series of reactions (Table 6). The values from Table 2 showed that if some of the glucose had been converted to all of the lactate, the contribution to the heat flux would have been 4.7 μW per $10^6$ cells. Only 6.5 pmol $s^{-1}$ ($37-(61/2)$ pmol $s^{-1}$ per $10^6$ cells) of the glucose would have been available for complete oxidation to give 19.6 μW per $10^6$ cells (glucose catabolism $(4.7+19.6)=24.3$ μW, but only a small amount of glutamine could have been oxidized and none to lactate).

Enthalpy fluxes calculated from the information in Table 5 are indicative of the importance of aerobic and anaerobic processes in the glucose metabolism of activated cells (Table 6). They accounted for $(11.6+4.2)=15.8$ μW per $10^6$ cells and gave an enthalpy recovery of 2.2. Part of the "missing" metabolism (18.2 μW) involved glutamine but there were discrepancies revealed by the stoichiometric calculations. In activated cells after deamination (Table 4), 16.3 pmol $s^{-1}$ of glutamine per $10^6$ cells was consumed catabolically but only 9 pmol $s^{-1}$ of lactate and 10 pmol $s^{-1}$ carbon dioxide were produced (Table 5), when the ratio of lactate:$CO_2$ should be 1:2 for the partial oxidation by glutaminolysis [13]. If the lactate value was accurate and the $CO_2$ figure an underestimate, then the calculated enthalpy flux would be 6.3 μW per $10^6$ cells. Unless the $CO_2$ value was an extraordinary underestimate, there would be no scope at all for complete oxidation of glutamine. If $CO_2$ production had been accurately assessed and lactate excretion was overestimated, then partial oxidation gave an enthalpy flux of 3.5 μW per $10^6$ cells. Glutaminolysis would account for between 5 and 9 pmol $s^{-1}$ per $10^6$ cells and not the 16.3 $pmol^{-1}$ of glutamine available. Complete oxidation to $CO_2$ and $NH_4^+$ could given an enthalpy flux of up to 4.6 μW per $10^6$ cells depending on the degree of overestimation of lactate production, but this would reduce the estimate for partial oxidation. This study has recovered between 19.3 and 22.1 μW of the heat flux by activated cells (1.76 to 1.54 recovery). It is suspected that fatty acids bound to BSA will be found to be responsible for the difference.

The data in Table 5 indicate that glucose was the primary measured source of energy in activated 2C11-12 mouse macrophage hybridomas. Its concentration in the medium (5.5 mmol $dm^{-3}$) was higher than that of glutamine (2.0 mmol $dm^{-3}$) which may have influenced the result because the two substrates can undertake a reciprocal Crabtree effect [14]. In any case, the majority of both substrates was utilized in the production of lactate (Table 4) by glycolysis and glutaminolysis and so provided biosynthetic precursors for growth. Only a minority (25%) of the substrate C moles were consumed in respiration but they produced up to 60% of the heat.

The major end products of glutamine metabolism in activated cells were glutamate, asparate, alanine and ammonia. Of the glutamine utilized by the cells, ammonia accounted for nearly 50% of glutamine-nitrogen which, because the glutaminase reaction produces ammonia, suggests that glutamate (Tables 1–5) produced from glutamine was metabolized via a transaminase reaction rather than glutamate dehydrogenase. For the glutamine metabolized beyond glutamate, cells

usually produce large quantities of either aspartate or alanine by the action of the appropriate aminotransferase [33]. The 2C11-12 cells processed only 18% of glutamine as aspartate and 6% as alanine. The majority went to the formation of lactate.

On the basis of finding certain metabolic end products of metabolism (Table 2) and of some enzymes with high activities (Table 5), it is suggested that the pathway for glutamine conversion to lactate is glutaminolysis [13,31,33]. This would result initially in the production of pyruvate, most of which appears to be converted to lactate. The reaction is required in anaerobic conditions in order to regenerate the $NAD^+$ needed for the oxidation of glyceraldehyde-3-phosphate. The only reasons for its formation under aerobic conditions would be a high LDH/PDH ratio causing a bias against the formation of acetyl CoA, and a low capacity in the aspartate/malate shunt. Of the glutamine utilized by the cells, 87% can be brought into account as glutamate, alanine, aspartate and lactate (Table 2). This suggests that 13% (3 pmol $s^{-1}$ per $10^6$ cells) was oxidized via the Krebs' cycle but the radioisotope studies do not allow sufficient $CO_2$ for this pathway. Perhaps the value is too high, due to experimental error. Better account can be made of the partial oxidation of glutamine to produce $2CO_2$ by decarboxylation reactions, using oxoglutarate dehydrogenase and phosphoenopyruvate carboxykinase [13,31]. The activities of these enzymes are quite high in macrophages (Table 7).

The evidence is that activated macrophage hybridomas produce aspartate and alanine by glutaminolysis. Analysis of the end products (Table 1 and 2) also shows that serine is produced by activated cells. This amino acid is synthesized from 3-phosphoglycerate and is a probable reason why glucose is metabolized in the glycolytic pathway. Besides being required, like alanine and aspartate, for protein synthesis, serine is a precursor in phosphoglyceride production. Glutamine, either directly or indirectly via aspartate, is a precursor for purines and pyrimidines. Thus there is a clear understanding why glucose and glutamine are important to growing cells as providers of precursors for cellular macromolecules. This is additional to the requirements for energy provision, and introduces an interesting problem in flux control in which synthesis and oxidation can be regarded as separate branches of the glycolytic and glutaminolytic pathways [33].

When activated cells were triggered by PMA to produce a respiratory burst, there was a rapid rise in heat production (Fig. 1). It was shadowed by increases in enhanced chemiluminescence and oxygen consumption (Fig. 2), the latter rapidly exhausting the available oxygen in the Connop flask and resulting in a return of heat production to below the level of activated cells. The heat flow increased by an average of 70% over the 30 min period, though the peak was five times greater (175 μW per $10^6$ cells) than the plateau for activated cells. The CR ratio [6,7,34] was less exothermic (Table 2) and the Cl/C6 ratio for $CO_2$ evolution increased because the rate of 6-$^{14}CO_2$ production decreased after the cells had been in the presence of PMA for 20 min. Analysis of the data in Tables 2 and 5 showed that the great majority of the heat production was from the complete oxidation of glucose (Table 6). This gave an enthalpy recovery of 1.28 (78%) compared to 2.93 (34%) for cells prior to the trigger. In absolute terms, the enthalpy fluxes for glycolysis and

glutaminolysis were less than in activated cells and metabolism was totally geared to the respiratory burst [1]. The cells require NADPH for the formation of oxygen metabolites and this is produced in the cytosol by the pentose phosphate pathway during the oxidation of glucose-6-phosphate to ribose-5-phosphate (R5P). Depending on cellular requirements, different proportional amounts of R5P and NADPH can be formed from most of the former and little of the latter to the opposite when $CO_2$ or pyruvate and $CO_2$ are formed instead of R5P, with much NADPH. The exact balance in a cell depends on demand for the various biosynthetic processes, ribose in nucleic acid synthesis and NADPH in the reductive biosynthesis of fatty acids. Oxidation of glucose by the cytosolic pentose phosphate pathway to form ribose can be distinguished from oxidation to $CO_2$ by mitochondrial pathways by the ratio of C1 to C6 $CO_2$, but such detection is not possible if only NADPH and $CO_2$ are formed by the transketolase route. The radioisotope method also does not work if NADPH and pyruvate instead of R5P are formed and the pyruvate is subsequently oxidized to $CO_2$ in mitochondria. Some cell types undertaking the respiratory burst have few mitochondria; for instance, neutrophils. Calorimetric studies on these cells showed that a transient peak in heat production when triggered was accompanied by a high C1/C6 ratio for $CO_2$ [9]. This indicated that the pentose phosphate pathway in neutrophils produced ribose and/or pyruvate together with NADPH from the reduction of glucose.

In the activated mouse macrophage hybridomas, the C1/C6 ratio for $CO_2$ was 1.18, signifying that there was some activity of the pentose phosphate pathway to produce NADPH plus ribose and/or pyruvate (Table 2). Because the activated cells were growing, it is reasonable to suppose that the NADPH might be required in the synthesis of fatty acids and ribose in the construction of nucleic acids. When the cells were triggered, much more glucose was oxidized to $CO_2$ and there was a small rise in pyruvate production (Table 2). Even so, the C1/C6 ($CO_2$) ratio increased only slightly, indicating that any greater flux in NADPH formation was associated mostly with a rise in $CO_2$ production alone, and not ribose. The increase in the level of pyruvate would indicate that, in the formation of more NADPH, at least some of the R5P was converted by the oxidative branch of the pentose phosphate pathway to pyruvate. This was not then converted to lactate; indeed, the lactate flux was less in triggered cells than in activated ones. The rise in glucose oxidation may have been due, therefore, to the cytosolic pentose phosphate pathway for using NADPH rather than to mitochondrial pathways, but there was no apparent reason for the accumulation of pyruvate.

The enthalpy balance approach has enabled considerable progress to be made in defining the catabolic pathways in activated 2C11-12 mouse macrophage hybridoma cells prior to and after triggering with PMA. Problems remain, however, in distinguishing between partial and complete oxidation of glutamine in the presence of the glycolytic conversion of glucose. It may well be that oxidation of fatty acids bound to BSA will account for the deficits in enthalpy balance but, because that will require the difficult extraction and identification of those acids, a better approach may be to introduce to BSA free of fatty acids, known quantities of metabolically-important acids.

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