

ADAPTATIONS OF ENERGY METABOLISM IN THE CULTIVATED MACROPHAGE*

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The *in vitro* differentiation of the monocyte into the larger, structurally more complex, functionally more active macrophage is accompanied by an increase in glycolytic activity as well as an increased dependence on aerobic metabolism (1-3). There is little information regarding the factors that regulate these changing patterns of energy metabolism during differentiation. In view of previous observations that environmental constituents have a profound effect on endocytosis (4, 5), one of the principal functional activities of the macrophage, it was of interest to determine if such factors exert a similar influence on the sources of energy provision. Pyruvate kinase activity provides an accurate index to glycolytic capacity (6-8) and cytochrome oxidase an index to mitochondrial oxygen utilization (9, 10). The effects of serum and hypoxia on pyruvate kinase and cytochrome oxidase activities of the cultivated macrophage are examined in the present report.

Materials and Methods

Animals.—Male mice (25-30 g) of the NCS/PA strain were used in all experiments. The newly designated strain was derived from the NCS (pathogen-free) mice of The Rockefeller University and has been maintained since 1970 as an outbred colony at the Veterans Administration Hospital, Palo Alto, Calif.

In Vitro Cultivation of Mononuclear Phagocytes.—Cells were harvested from the peritoneal cavity of unstimulated mice in heparinized, phosphate-buffered saline pH 7.4 by techniques described previously (1, 5). A 10 ml sample of cells (3.0×10^6 /ml) in Medium 199 (Schwarz/Mann, Division of Becton, Dickinson and Co., Orangeburg, N.Y.) containing 20% newborn calf serum (NBCS)¹ (Grand Island Biological Co., Berkeley, Calif.) was dispensed to each 30 cm² T flask, incubated for 60 min at 37°C, and washed twice in Medium 199 as previously described (5). Cells were reincubated in fresh Medium 199 containing either 2 or 30% NBCS.

At the time of harvest, tissue culture media was removed and monolayers were rinsed three

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¹ Abbreviation used in this paper: NBCS, newborn calf serum.

times with 5-ml portions of physiological saline at room temperature. All subsequent procedures were carried out at 0–4°C. Cells were removed by scraping, resuspended in 3.0 ml of saline, and centrifuged at 700 *g* for 5 min. Cell pellets were washed twice in 5-ml portions of Ringer's solution, resuspended to a final volume of 2.0 ml in 35 mM Tris buffer (pH 7.4), and disrupted by sonication (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 30 s at a power output of 70 W.

Ambient Oxygen Tension.—Ambient oxygen tension within culture vessels could be regulated by flushing vessels with defined gas mixtures and maintained at stable levels for time periods up to 72 h by tightly stoppering the flasks. Aerobic culture conditions, achieved by flushing with a 95% air-5% CO₂ mixture, resulted in a media PO₂ of 140 mmHg and anaerobic conditions, achieved by similar treatment with a 95% N₂-5% CO₂ mixture, resulted in a PO₂ of 10–15 mmHg.

Pyruvate Kinase Assay.—Pyruvate kinase activity was assayed by a modification of the spectrophotometric method of Valentine (11). An 0.05 ml sample of cell sonicate was added to a 2.95 ml reaction mixture containing: 0.5 ml of 50 mM triethanolamine-HCl buffer (pH 7.5), 1.45 ml of distilled water, 0.1 ml of 2.25 M KCl, 0.1 ml of 240 mM MgSO₄, 0.2 ml of 6 mM adenosine diphosphate, 0.1 ml of lactic dehydrogenase (18 enzyme units), 0.4 ml of 1.4 mM nicotinamide adenine dinucleotide (NADH), and 0.1 ml of 45 mM phosphoenolpyruvic acid (trisodium salt). NADH oxidation at 25°C was followed by measuring decrease in optical density at 340 nm in a model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Enzyme activity was calculated during a period of zero order kinetics using a molar extinction coefficient of 6.2×10^3 for NADH. Enzyme activity has been expressed as micromoles phosphoenolpyruvate converted to pyruvate \times minutes⁻¹.

Cytochrome Oxidase.—Cytochrome oxidase was measured spectrophotometrically by techniques previously described (9, 12). An 0.1 ml sample of cell sonicate was added to 2.9 ml of reaction mixture containing: 0.3 ml of 10% sodium deoxycholate, 2.5 ml of 13 mM phosphate buffer (pH 7.4), and 0.1 ml of 3% reduced cytochrome *c* (Nutritional Biochemicals Corp., Cleveland, Ohio). Oxidation of cytochrome *c* at 25°C was followed by measuring decrease in optical density at 550 nm in a model DB spectrophotometer. Enzyme activity was calculated using a molar extinction coefficient of 19.1×10^3 for cytochrome *c*. Enzyme activity has been expressed as micromoles cytochrome *c* oxidized \times minutes⁻¹.

Protein.—Protein content was determined by the method of Lowry (13) using crystalline human albumin (Warner-Chilcott Laboratories, Div. Warner-Lambert Co., Morris Plains, N.J.) as the standard.

Protein Synthesis.—The incorporation of [³H]leucine into trichloroacetic acid (TCA)-precipitable protein by macrophage monolayers was used to assay protein synthesis. Cell monolayers were rinsed twice and incubated in 4.0 ml of leucine-free Medium 199 containing 2 or 30% dialyzed NBS for 60 min at 37°C. The media was decanted and fresh media containing 5 μ Ci of L-[4,5-³H]leucine (sp act 270 mCi/mg) (Amersham/Searle, Arlington Heights, Ill.) was added and flasks were incubated at 37°C. Duplicate flasks were harvested 15, 30, 60, and 90 min after the addition of isotope and assayed for radioactivity as follows: tissue culture media was aspirated, monolayers were rinsed three times with saline at 0°C, and 2.0 ml of cold 5% TCA was added to each flask. Monolayers were scraped from the flask with a rubber policeman, quantitatively transferred to conical centrifuge tubes, and washed twice with cold 5% TCA. Precipitates were prepared for liquid scintillation counting by incubation with 0.5 ml of NaOH (0.5 N) for 18 h before the addition of liquid scintillation fluid containing 40 ml of Liquifluor (New England Nuclear, Boston, Mass.) per liter of toluene. A Nuclear Chicago Mark II liquid scintillation counter was used to assay for radioactivity. The incorporation of [³H]leucine into TCA-precipitable protein was linear for 90 min under the assay conditions employed.

Aliquots of macrophage homogenates were assayed for protein. Total leucine concentration of tissue culture media was assayed with a Beckman/Spinco (Beckman Instruments Inc.,

Spinco Div., Palo Alto, Calif.) model 120C amino acid analyzer (14, 15). Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was used as an inhibitor of protein synthesis.

Data Analysis.—Each data point depicted in the illustrations represents the mean of duplicate values from six or more experiments. Statistical inferences were derived from data analysis using the Student's *t* test for unpaired samples.

RESULTS

Pyruvate Kinase.—In the initial experiments pyruvate kinase activity of cells cultivated in 30% NBCS was examined (Fig. 1). A continuous increase in enzyme specific activity (activity per protein) was observed during in vitro differ-

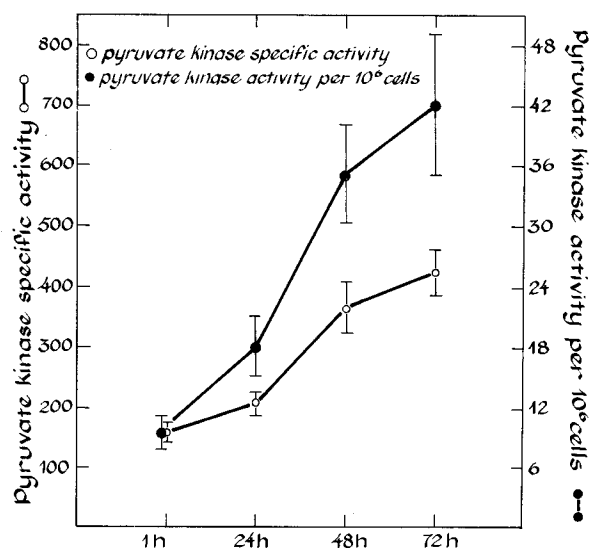


FIG. 1. Temporal changes in pyruvate kinase activity of macrophages cultivated under aerobic conditions in 30% NBCS. Enzyme activity (micromoles phosphoenolpyruvate converted to pyruvate \times minute⁻¹) \pm SEM is normalized for: protein content (enzyme activity \times milligrams protein⁻¹) (○), and cell count (enzyme activity \times 10⁻⁶ cells) (●).

entiation. Activity values for cells cultivated 48 and 72 h were significantly greater ($P < 0.001$) than those for freshly explanted cell monolayers. Cultivation of cells for time periods up to 72 h resulted in peak enzyme activity values two to threefold greater than those for cells cultivated for 1 h.

Under the culture conditions employed, peritoneal macrophages are not capable of multiplication and cell numbers remain nearly constant (1). However, cells maintained in vitro in 30% NBCS exhibit an increase in cell size and protein content. Specific activity determinations for pyruvate kinase underestimate increases in cellular enzyme activity due to the rising baseline in monolayer protein content. For this reason pyruvate kinase activity per cell was calculated. During cultivation there was a continuous and nearly linear increase in

pyruvate kinase activity per cell reaching values at 72 h that were five to seven-fold greater than those for freshly explanted cells. Values for pyruvate kinase activity per cell at 24, 48, and 72 h were significantly greater ($P < 0.001$) than those for cells cultured for 1 h.

Prior studies have demonstrated that environmental constituents, including the concentration of serum in the media, have a marked effect on morphological and biochemical properties of the cultivated macrophage (4, 5). Therefore, the effect of altering serum concentration on the level of pyruvate kinase activity was examined.

As shown in Table I the concentration of serum employed in the culture media had a significant effect on pyruvate kinase activity. Increases in activity for cells cultivated in 2% NBCS were substantially less than those for cells cul-

TABLE I
Pyruvate Kinase and Cytochrome Oxidase† Specific Activities for Mouse Macrophages Cultivated under Aerobic Conditions*

| Enzyme | Serum concentration | Incubation time | | | |
|--------------------|---------------------|-----------------|------------|------------|------------|
| | | 1 | 24 | 48 | 72 |
| | % | | | | |
| Pyruvate kinase | 30 | 158 ± 15§ | 204 ± 60 | 366 ± 41 | 426 ± 35 |
| | 2 | 172 ± 9 | 202 ± 17 | 207 ± 24 | 290 ± 29 |
| Cytochrome oxidase | 30 | 10.8 ± 0.5 | 11.2 ± 0.6 | 13.5 ± 1.3 | 14.4 ± 0.3 |
| | 2 | 11.4 ± 0.6 | 12.1 ± 0.5 | 10.9 ± 0.6 | 11.0 ± 0.8 |

* Expressed as micromoles phosphoenolpyruvate converted to pyruvate \times minute⁻¹ \times milligrams protein⁻¹.

† Expressed as micromoles cytochrome *c* oxidized \times minute⁻¹ \times milligrams protein⁻¹.

§ Mean \pm SEM for six to nine experiments.

vated in 30% NBCS. Values at 24 and 48 h were not significantly different from those for cells harvested after 1 h of cultivation. By 72 h pyruvate kinase specific activity had increased by 40% from values at 1 h, increases that were less than one-third those observed for cells cultivated in 30% NBCS. Histograms depicting the effect of serum concentrations on pyruvate kinase activity are shown in Fig. 2.

Cytochrome Oxidase.—Additional studies were performed in which duration of in vitro cultivation and concentration of serum were examined for their effects on cytochrome oxidase activity. As shown in Table I, cultivation of cells for 72 h in 30% NBCS resulted in a small but significant ($P < 0.01$) increment in cytochrome oxidase activity. Values at 24 and 48 h, however, were not significantly different from those at 1 h.

The magnitude of the increase in specific activities normalized for protein content or cell count can be appreciated from inspection of Fig. 3. During 72 h of cultivation, values for cytochrome oxidase activity per protein increased by

40% whereas activity per cell increased by 90%. Unlike the findings for pyruvate kinase activity, cytochrome oxidase activity for cells cultivated in 2% NBCS showed no change during 72 h of cultivation.

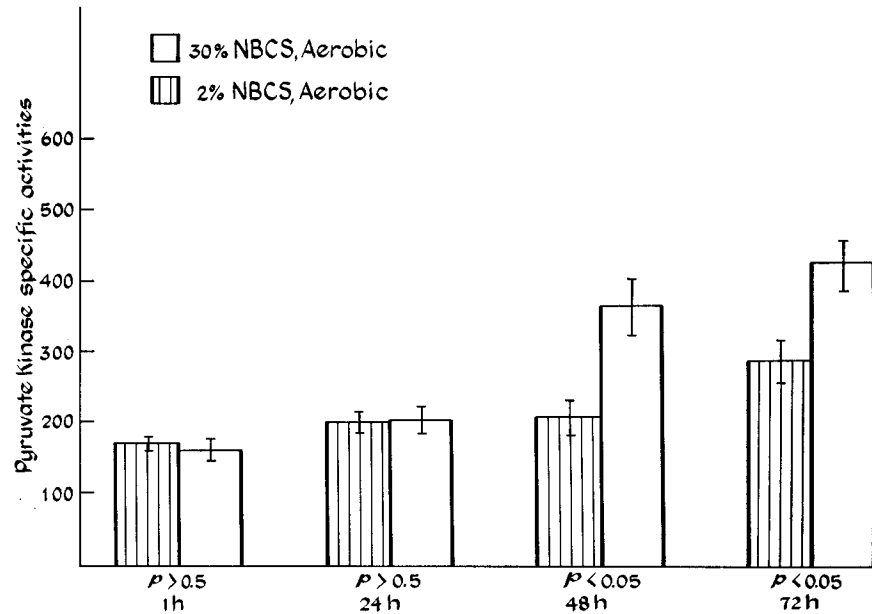


FIG. 2. Effect of serum concentration (NBCS) on pyruvate kinase activity of macrophages cultivated under aerobic conditions.

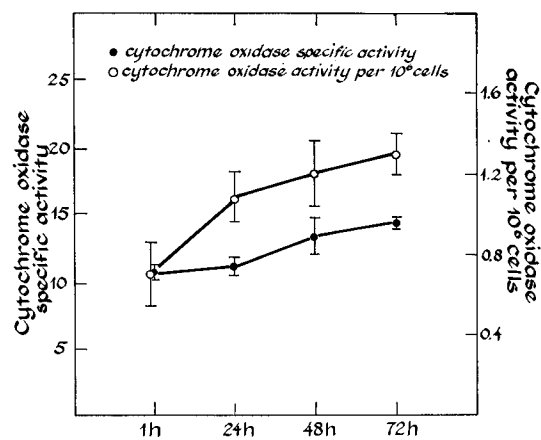


FIG. 3. Temporal changes in cytochrome oxidase activity of macrophages cultivated under aerobic conditions in 30% NBCS. Enzyme activity (micromoles cytochrome *c* oxidized \times minute⁻¹) \pm SEM is normalized for: protein content (enzyme activity \times milligrams protein⁻¹) (○); and cell count (enzyme activity $\times 10^{-6}$ cells) (●).

Effect of Anaerobiosis on Pyruvate Kinase and Cytochrome Oxidase Activity.—Pyruvate kinase specific activities for cells cultivated under anaerobic conditions in 30% NBCS are shown in Fig. 4. The rate as well as magnitude of the increase in pyruvate kinase activity were greater than those observed for cells cultivated under aerobic conditions. By 48 h peak levels five-fold higher than those of freshly explanted macrophages were achieved.

Similar to the findings for the aerobic experiments, cells cultivated anaerobically in 2% NBCS exhibited a lesser increase in pyruvate kinase activity than those cultured in 30% NBCS (Table II). However, peak levels were achieved sooner. Cells maintained anaerobically in 2% NBCS reached peak pyruvate

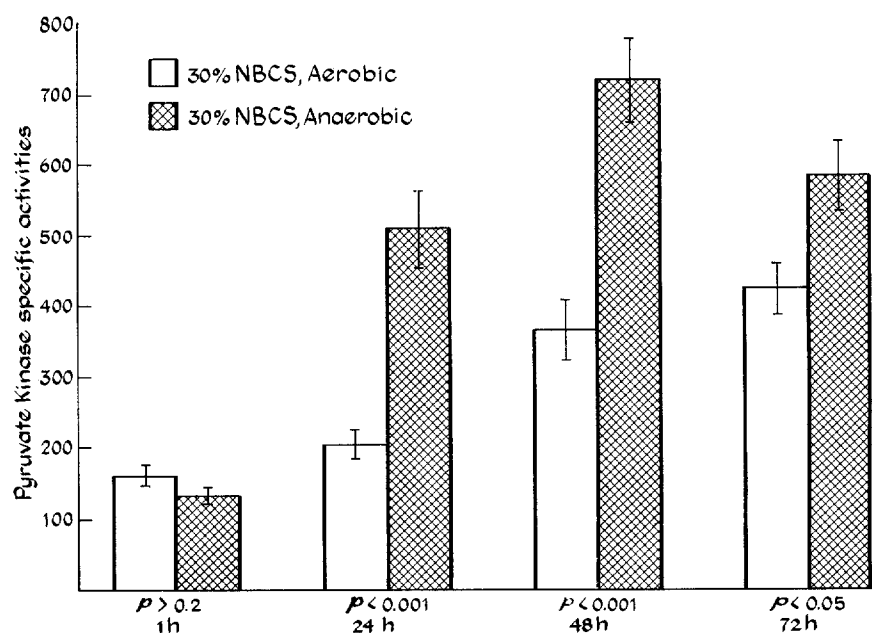


FIG. 4. Effect of aerobic or anaerobic culture conditions on pyruvate kinase activity of macrophages cultivated in the presence of 30% NBCS.

kinase activity values after 24 h of cultivation, whereas similar levels for cells grown aerobically were not achieved before 72 h of cultivation.

Temporal changes in cytochrome oxidase specific activities for anaerobically cultivated cells are shown in Table II. Cells cultivated in either 30 or 2% NBCS exhibited a decrease in cytochrome oxidase activity. This pattern of cytochrome oxidase response for anaerobically grown cells differed markedly from that for aerobically cultured cells. As shown in Fig. 5, cytochrome oxidase activity for cells grown in 30% NBCS increased by 40% under aerobic culture conditions and dropped by 40% under anaerobic conditions. Similar but less marked changes were observed for cells cultivated in 2% NBCS.

TABLE II
Pyruvate Kinase and Cytochrome Oxidase† Specific Activities for Mouse Macrophages Cultivated under Anaerobic Conditions*

| Enzyme | Serum concentration | Incubation time | | | |
|--------------------|---------------------|-----------------|------------|------------|-----------|
| | | 1 | 24 | 48 | 72 |
| | % | | | | |
| Pyruvate kinase | 30 | 133 ± 11§ | 509 ± 56 | 721 ± 61 | 585 ± 51 |
| | 2 | 133 ± 20 | 346 ± 38 | 331 ± 24 | 307 ± 30 |
| Cytochrome oxidase | 30 | 12.0 ± 0.6 | 11.7 ± 0.9 | 10.3 ± 1.0 | 7.7 ± 0.8 |
| | 2 | 11.6 ± 0.4 | 11.1 ± 0.4 | 9.9 ± 0.6 | 7.8 ± 1.0 |

* Expressed as micromoles phosphoenolpyruvate converted to pyruvate × minute⁻¹ × milligrams protein⁻¹.

† Expressed as micromoles cytochrome *c* oxidized × minute⁻¹ × milligrams protein⁻¹.

§ Mean ± SEM for six to eight experiments.

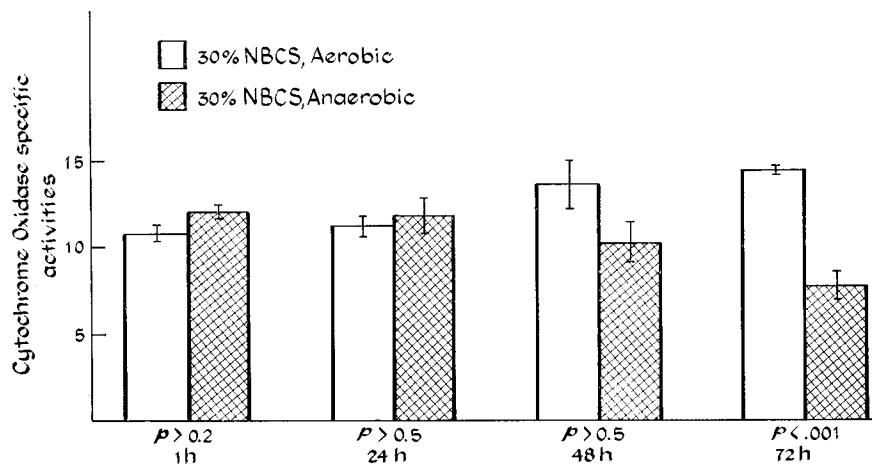


FIG. 5. Effect of aerobic or anaerobic culture conditions on cytochrome oxidase activity of macrophages cultivated in the presence of 30% NBCS.

Effects of Environmental Stimuli on Cellular Protein Content and Protein Synthesis.—The effects of environmental alteration on total cellular protein were examined. The results of such studies are shown in Table III. Cells cultivated in 30% NBCS under aerobic conditions exhibited a 70% increase in cellular protein during the first 48 h of in vitro cultivation. A lesser increase was noted for cells grown aerobically in 2% NBCS. The effects of aerobicity, concentration of serum, and duration of time in culture can be more easily appreciated by inspection of the relative values for cellular protein (Fig. 6).

Influences of serum and anaerobiosis on overall protein synthesis by the mac-

TABLE III
*Protein Per 10⁶ Cells of Mouse Macrophages Cultured under
 Aerobic and Anaerobic In Vitro Conditions*

| | Serum con- centration | Incubation time | | | | |
|-----------|--------------------------|-----------------------|---------------------|----------------------|----------------------|----|
| | | 1 | 24 | h | 48 | 72 |
| | % | | | | | |
| Aerobic | 30 | 58.3 ± 7.0* (100)‡ | 82.5 ± 8.3 (142) | 101.1 ± 6.7 (173) | 93.1 ± 8.9 (160) | |
| | 2 | 66.5 ± 6.2 (100) | 60.6 ± 5.0 (91) | 86.6 ± 5.0 (130) | 81.9 ± 6.6 (123) | |
| Anaerobic | 30 | 51.6 ± 12.9 (100) | 68.5 ± 7.3 (133) | 64.3 ± 7.8 (125) | 58.1 ± 10.6 (113) | |
| | 2 | 58.5 ± 5.3 (100) | 61.9 ± 4.0 (106) | 60.0 ± 5.5 (103) | 60.2 ± 5.1 (103) | |

* Expressed as micrograms protein per 10⁶ cells; mean ± SEM.

‡ Relative protein content expressed as percentage of 1 h mean value.

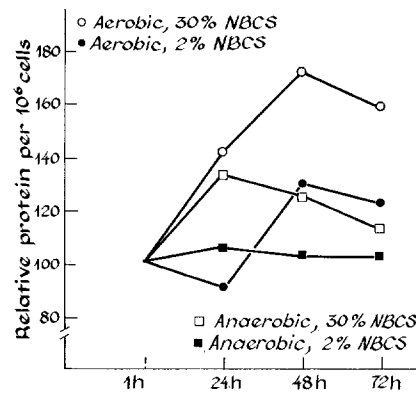


FIG. 6. Changes in cellular protein content of macrophages cultivated under aerobic or anaerobic conditions in the presence high (30%) or low (2%) concentrations of NBCS.

rophage were examined (Fig. 7). Highest values for protein synthesis were observed in cells cultured aerobically in 30% NBCS. Despite a broad range among individual values, synthesis rates for cells maintained in 30% NBCS were consistently 10–20 times greater than those observed in cells maintained in 2% NBCS. In the presence of 30% NBCS protein synthesis remained nearly constant for 48–72 h despite a 70% increment in cellular protein content.

Maintenance of cells under anaerobic conditions resulted in a prompt reduction in overall protein synthesis. Synthesis rates for freshly explanted macrophages as well as those maintained anaerobically for 24–72 h were 50–70% lower than those for cells cultured aerobically.

Cycloheximide and Pyruvate Kinase.—Environmental stimuli were shown to exert a profound effect on the activity levels of pyruvate kinase. To assist in distinguishing among the possible mechanisms responsible for these changes it was of interest to determine if they were dependent on continued protein synthesis. To examine this possibility, the effect of cycloheximide on pyruvate kinase activity of cells cultured under aerobic and anaerobic conditions was examined. As shown in Fig. 8 cycloheximide-treated cells maintained in 30% NBCS under

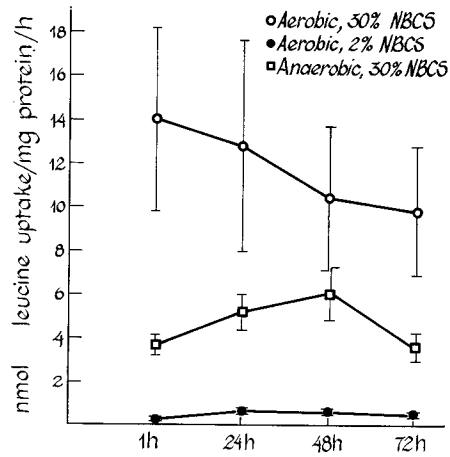


FIG. 7. Protein synthesis by cultivated macrophages. Influences of NBCS concentration and ambient oxygen tension on linear rates of incorporation of L-[³H]leucine into TCA-insoluble fraction during 90 min pulse with the isotope.

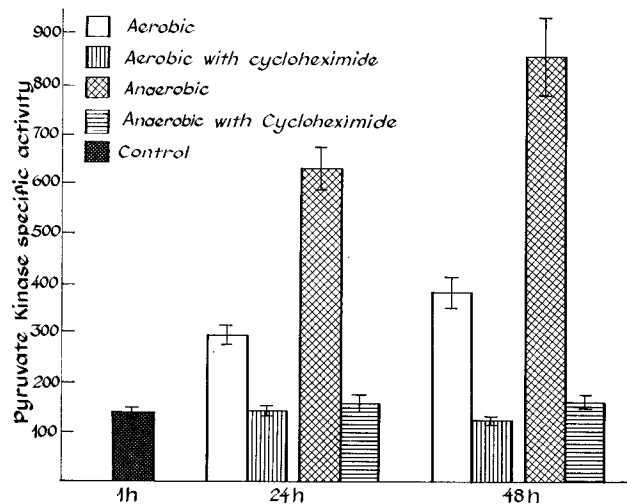


FIG. 8. Effects of cycloheximide (1.8×10^{-4} M) on pyruvate kinase specific activity for cells cultivated under aerobic or anaerobic conditions in 30% NBCS.

aerobic conditions exhibited no increase in pyruvate kinase activity during 48 h of cultivation. Similarly, cycloheximide treatment inhibited by 95% the fivefold increase in pyruvate kinase activity characteristic of cells cultured under anaerobic conditions. Treatment of cells with cycloheximide at doses of 1.8×10^{-4} M resulted in an overall reduction in protein synthesis by 90% but did not reduce cell numbers.

DISCUSSION

It has been demonstrated previously in experiments employing various tissues and cell types that pyruvate kinase (6-8) and cytochrome oxidase (9, 10) provide indices for glycolytic capacity and mitochondrial oxygen utilization, respectively. In the present study adaptive changes in these energy-regulating enzymes were shown to accompany the *in vitro* differentiation of cultivated mouse peritoneal macrophages. Both pyruvate kinase and cytochrome oxidase activities increased in cells maintained under aerobic conditions.

The increased pyruvate kinase activity is consistent with previous observations that monocytes exhibit increased rates of glucose utilization and lactate production during cultivation (2). The finding that cytochrome oxidase activity of macrophages increased during cultivation under aerobic conditions confirms previous observations by Bennett investigating the *in vitro* differentiation of horse blood monocytes into macrophages (2). Together, these quantitative data are in keeping with observations based on morphologic studies that mitochondrial mass increases during differentiation of mononuclear phagocytic cells (1).

The cultivated macrophage was shown to be profoundly influenced by environmental stimuli. More specifically, it was shown that there was a direct relationship between serum concentration in the culture media and activities of pyruvate kinase and cytochrome oxidase. Cells cultivated aerobically in 30% NBCS for 72 h exhibited a 300-400% increase in pyruvate kinase activity per cell, whereas cells cultivated in 2% NBCS exhibited a 65% increase.

The basis for an explanation of the serum effect may be found in a previous report of functional correlates of macrophage differentiation. A direct relationship between serum concentration and pinocytotic activity was noted (3, 4). Macrophages cultivated in 50% NBCS exhibited a 10-fold increment in pinocytotic activity over cells cultivated in 1% NBCS. Our observations of a similar relationship between serum concentration and activities of pyruvate kinase and cytochrome oxidase suggest the possibility that demand for glycolytic activity and oxygen consumption required for increased endocytic activity could provide a stimulus for changes in pyruvate kinase and cytochrome oxidase activities. In this regard it should be noted that endocytosis is an energy-requiring process involving both glycolysis and oxidative metabolism (3, 16).

Whatever role endocytosis may play in pyruvate kinase and cytochrome oxidase regulation it alone is not sufficient to account for all of the changes observed. Cultivation of cells under anaerobic conditions that inhibit pinocytosis

led to increases in pyruvate kinase activity that were nearly twice those found in cells cultivated under aerobic conditions. Cytochrome oxidase activity, on the other hand, decreased by nearly 40% in response to cultivation under anaerobic conditions. During anaerobiosis the energy required for all aspects of cellular physiology must be derived from glycolysis, and augmented pyruvate kinase activities may reflect an enhanced capacity for glycolytic energy provision in a cell with decreased electron transport chain activity. This hypothesis is in keeping with earlier observations that pyruvate kinase activity varied in a direct and linear fashion with tissue glycolytic capacity as measured by lactate production (8). Similarly the decreased cytochrome oxidase activity of cells cultivated anaerobically is in keeping with studies showing a direct relationship between cytochrome oxidase activity and tissue O₂ consumption (12, 17-19).

The results of inhibitor studies employing cycloheximide suggest that increases in pyruvate kinase activity resulting from alterations in serum concentration or anaerobiosis are dependent on continued protein synthesis. It seems likely that the increased enzyme activity represents increased enzyme synthesis, but the possibility that such increases result from alteration in enzyme activation, inactivation, or degradation are not ruled out by the use of inhibitors alone.

Environmental constituents that stimulate endocytic activity led to increases in cellular protein content, presumably through increased protein biosynthesis. However, anaerobically cultured cells exhibited enhanced pyruvate kinase activity despite a decrease in overall rate of protein synthesis. Priority for enhanced glycolytic enzyme activity and possibly enzyme synthesis may be considered a successful adaptation in maintaining cell viability despite limited oxygen availability. These observations suggest the existence of at least two independent mechanisms that regulate protein biosynthesis, (*a*) those involved in equipping cells for enhanced functional activity, and (*b*) those involved in providing energy for cell work during anaerobiosis.

SUMMARY

Adaptive changes in energy metabolism, as reflected by pyruvate kinase and cytochrome oxidase activities, were examined during *in vitro* differentiation of the cultivated macrophage. Serum concentrations of tissue culture media, which directly influence endocytic activity, and ambient oxygen tension were both shown to influence pyruvate kinase and cytochrome oxidase activities. Cells maintained in high serum concentrations (30% newborn calf serum [NBCS]) exhibited a 300-400% increase in pyruvate kinase activity and a 40% increase in cytochrome oxidase activity, whereas cells maintained in low serum concentrations (2% NBCS) exhibited a lesser increase (65%) in pyruvate kinase activity and no change in cytochrome oxidase activity. Anaerobiosis resulted in additional alterations in pyruvate kinase and cytochrome oxidase activities. Cells maintained for 48-72 h under anaerobic conditions exhibited a 500-600%

increase in pyruvate kinase activity and a 40% decrease in cytochrome oxidase activity. Increased pyruvate kinase activity was dependent on continued protein synthesis. Enzyme increases occurred in anaerobically cultured cells despite an overall reduction in cell protein synthesis. It is suggested that adaptive changes in pyruvate kinase and cytochrome oxidase activity resulting from alterations in either serum concentration or ambient oxygen tension are regulated by two independent mechanisms. One mechanism is aimed at providing energy for endocytic activity and the other in compensating for impaired oxidative metabolism during anaerobiosis.

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