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Metabolic Effects of Hyperthermia and Ascorbic Acid in Ehrlich Ascites Tumor Cells[1] (40391)

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The ability of hyperthermic treatment to arrest or destroy cancerous tissue, either separately or combined with ionizing radiation or chemotherapy, has stimulated considerable interest at the research and clinical levels. Substantial evidence exists that hyperthermia can delay tumor growth or lead to complete regression (1–3). Partial or total regression of human neoplasms are reported following severe fevers resulting from infectious disease or administration of bacterial toxins (4–7).

In general, normal cells may be more resistant to heat than tumor cells (8–10). However, this selective effect of hyperthermia is reduced or lost at temperatures above 43° (11). Many studies (8, 9) have shown that hyperthermia is capable of irreversibly inhibiting cellular respiration. Altered cellular respiration may represent a relatively late alteration in the tumor cell and is probably highly dependent on cellular death.

Ascorbic acid is a toxic agent under specific conditions to Ehrlich Ascites Tumor Cells (EATC) *in vitro* (12). Low or depleted tissue ascorbate concentrations are also associated with the malignant state (13). Ascorbic acid can inhibit the enzyme, phosphodiesterase, and thus potentiate cAMP levels (14). Since cellular growth is known to be inhibited by increasing cAMP (15), ascorbic acid may be a useful agent in the control of oncogenic metabolism and division.

These studies examined the interaction of hyperthermia and ascorbic acid upon the metabolism and viability of EATC. Experimental hyperthermia studies were conducted at 42.5 ± 0.1° for up to 3 hr so that the influence of cellular death would be minimized. In experiment 1, the effect of hyperthermia (37.5 vs. 42.5°) and ascorbic acid (0–1 m*M*) were examined on cell viability as a function of time. Experiment 2 was designed to determine the influence of varying concentrations of ascorbic acid on glucose metabolism of EATC. In experiment 3, the effect of duration of incubation was examined in cells maintained at 37.5 or 42.5° with or without supplemental ascorbic acid. Experiment 4 examined the effects of hyperthermia and ascorbic acid on the metabolism of citrate and pyruvate. Kidney slices and minced spleen served as a source of nontumorous, highly differentiated cells for comparison to EATC in experiment 5.

Materials and methods. Ehrlich ascites tumor cells[3] (EATC) were serially transplanted intraperitoneally at regular intervals in male swiss mice.[4] Cells were harvested 6–14 days after transplantation. The effects of experimental hyperthermia and ascorbic acid supplementation were examined simultaneously. The protein content or the number of cells per flask was determined in each experiment. Proteins were determined by a biuret method using bovine serum albumin as the standard (16). Cell counts were determined with a hemocytometer. Cell viability data were determined by the dye exclusion technique, using 0.1% trypan blue-saline solution (17).

Metabolic studies were performed in 25 ml stoppered siliconized glass Erlenmeyer flasks with specially constructed center wells for $^{14}CO_2$ collection. All incubations were performed in KRP without calcium (18). The components of KRP were: 0.085M Na_2HPO_4,

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[3] The original cells were a gift of Dr. E. Racker, Cornell University.

[4] ICR strain, Harlan Industries, Indianapolis, Indiana.

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0.016 *M* NaCl, 0.005 *M* KCl, 0.0013 *M* $MgSO_4 \cdot 7H_2O$, and adjusted to a final pH of 7.4.

Labelled [U-^{14}C]-glucose (NEC-042) and [1-^{14}C]-pyruvate (NEC-255) were obtained from New England Nuclear (Boston, MA). [1,5-^{14}C]-Citrate (CFA·263) was obtained from Amersham Corporation (Arlington Hts, IL). Tracers were diluted with their respective cold substrate before addition to the incubation flasks. Depending on the experiment, each flask contained 0.25 to 0.42 μCi of label in 20 μmoles cold substrate. The final volume in each Erlenmeyer flask was 4 ml. The concentration of ascorbic acid was 0 or 1 m*M* in the flasks unless otherwise noted. Each flask was capped with a rubber serum stopper and placed in a Dubnoff metabolic shaker bath. A proportional temperature controller (Yellow Springs Instruments Model 72) was used to control shaker bath water temperature (± 0.1°).

The $^{14}CO_2$ collection technique has been described (18). Briefly, fluted filter paper (Whatman #1, 2.7 × 33 cm) was inserted into glass shell vials (Kimble 60930-L) and treated with 0.2 ml hyamine hydroxide (New England Nuclear). The vial with paper was placed in the Erlenmeyer flask's center well for $^{14}CO_2$ collection.

Following the incubation period, the reactions were stopped by injecting 1 ml of 2 *N* $HClO_4$ through the serum caps. The flasks were then shaken an additional hour to recover $^{14}CO_2$. Blanks were prepared by adding PCA to the flask before the cell suspension. After shaking with PCA, the paper and vial were removed and placed in a scintillation vial containing 15 ml of cocktail (4 g PPO, 0.05g POPOP, diluted to 1 L with toluene). Samples were held at least 5 hr at room temperature before counting in a Packard Tri-Carb scintillation spectrometer. Quench correction was done using the channels ratio method.

Kidney slices from male Sprague-Dawley rats[5] and male Swiss mice were used for normal cell metabolism data. Minced spleen from mice also served as a control for normal tissue for these respiration studies. Tissue slices were prepared on a Stadie-Riggs microtome. Slices were approximately 100 μm thick. Approximately 200 mg of kidney slices or minced spleen were added to each incubation flask. Kidney and spleen protein content was determined by the biuret method (16).

Hyperthermia is defined in our experiments as incubation of cells at 42.5°. Criteria for significance was $P < 0.05$. All data were analyzed by analysis of variance and LSD applied to test significance.

Results. The effects of hyperthermia, ascorbic acid, and incubation time on cell viability are listed in Table I. Clearly, incubations at 37.5°C had no substantial effect on cell viability as determined by dye exclusion over a 3-hr incubation period. At 42.5°, cell viability was significantly affected at 2- and 3-hr periods. However, a 1 hr incubation at 42.5° had no significant effect on cell viability. Ascorbic acid (1mM) had no effect upon EATC viability at 37.5°. Limitations in assessing cell viabilities by dye exclusion are well recognized (12). Other techniques such as *in vitro* plating efficiencies or reinoculation to determine viabilities may supply more information on the long term effects of hyperthermia and ascorbic acid treatments. Ascorbic acid seemed to reduce cellular death at 42.5° (Table I). The maximum pH shift following a 1 hour incubation period irrespective of treatment was 0.15 units.

Data on metabolism of [U-^{14}C] glucose with different concentrations of vitamin C are shown in Table II. Glucose metabolism was significantly increased as a function of increased concentrations of vitamin C. Ascorbic acid (10 m*M*) caused an approximate 2.5-fold increase in glucose metabolism.

The effects of hyperthermia and of vitamin C on EATC [U-^{14}C] glucose metabolism

TABLE I. EFFECT OF HYPERTHERMIA AND ASCORBIC ACID ON EATC VIABILITY (%).*

Time (hr)	Treatment			
	37.5°	42.5°	37.5° (+C)	42.5° (+C)
1	95 ± 2^a	96 ± 2^a	97 ± 2^a	98 ± 0^a
2	97 ± 1^a	87 ± 3^b	97 ± 1^a	96 ± 1^a
3	97 ± 2^a	74 ± 3^b	97 ± 2^a	81 ± 8^{a,b}

* The initial viability was 97 ± 1%. Means ± SE at the same time period with an unlike superscript differ ($P < 0.05$, LSD test). Ascorbic acid (+C) was added at a final concentration of 1 m*M*.

[5] Harlan Industries, Indianapolis, Indiana.

are shown in Fig. 1. Data are normalized per mg protein and per 10^6 cells. At 37.5° and at 42.5° ascorbic acid (vitamin C) significantly increased the metabolism of uniformly labelled glucose. There is no statistically significant effect of hyperthermia in the presence or absence of vitamin C for 1, 2, or 3-hr incubation periods. Increasing release of $^{14}CO_2$ with time suggest that the experimental treatments had little effect on cellular mortality.

Ascorbic acid significantly increased citrate metabolism at 37.5° and at 42.5° (Table III). Hyperthermia without vitamin C had no significant effect. However, hyperthermia in the presence of vitamin C increased citrate metabolism significantly. The effects of hyperthermia and of vitamin C on EATC metabolism of pyruvate are similar to those obtained with citrate. However, hyperthermia in the presence or in the absence of ascorbic acid has no significant effect upon pyruvate metabolism (Table III).

The effects of hyperthermia and vitamin C on glucose metabolism of a normal tissue preparation of rat and mouse kidney slices are shown in Table IV. Neither hyperthermia nor ascorbic acid significantly effected the metabolism of this substrate in these tissues. The metabolism of glucose in minced spleen samples was also not effected by ascorbic acid supplementation at 37.5°.

TABLE II. EFFECT OF ASCORBIC ACID ON METABOLISM OF [U-^{14}C] GLUCOSE BY EATC.*

Ascorbic acid (m*M*)	dpm/mg protein	dpm/10^6 cells
0.0	130 ± 4^a	57 ± 2^a
0.05	140 ± 9^a	61 ± 4^a
1.0	212 ± 3^b	93 ± 1^b
10.0	307 ± 18^c	135 ± 8^c

* EATC incubated 1 hr at 42.5° with 0.28 μCi [U-^{14}C] glucose and 20 μmoles cold glucose. Means ± SE with an unlike superscript differ ($P < 0.05$, LSD test), three replicates per datum at 0.05 m*M*, all others two replicates.

TABLE III. EFFECTS OF HYPERTHERMIA AND ASCORBIC ACID ON METABOLISM OF [1,5-^{14}C] CITRATE AND [1-^{14}C] PYRUVATE BY EATC.*

Treatment	dpm/mg protein from citrate	dpm/mg protein from pyruvate
37.5°	80 ± 9^a	580 ± 18^a
37.5° + Ascorbate**	149 ± 15^b	997 ± 57^b
42.5°	$103 \pm 6^{a,b}$	531 ± 44^a
42.5° + Ascorbate**	289 ± 33^c	855 ± 70^b

* EATC incubated 1 hr with 0.42 μCi [1,5-^{14}C] citrate or 0.02 μCi [1-^{14}C] pyruvate and 20 μmoles cold respective substrate. Means ± SE with an unlike superscript differ ($P < 0.05$). Vitamin C (+C) concentration was 1 m*M*.

** Ascorbic acid was added at a final concentration of 1 m*M*.

Discussion. Consistent with previous reports hyperthermia did decrease the percentage of viable tumor cells in our experiments as determined by dye exclusion techniques. Ascorbic acid did not appear to alter EATC viability when incubated for 1 hr with glucose, citrate, or pyruvate as substrates. Ascorbic acid has been shown to be lethal to EATC in the absence of glucose (12). However, ascorbic acid had no effect on EATC viability in the presence of glucose (12).

The data presented in this paper demonstrates that hyperthermic treatment alone does not significantly affect EATC metabo-

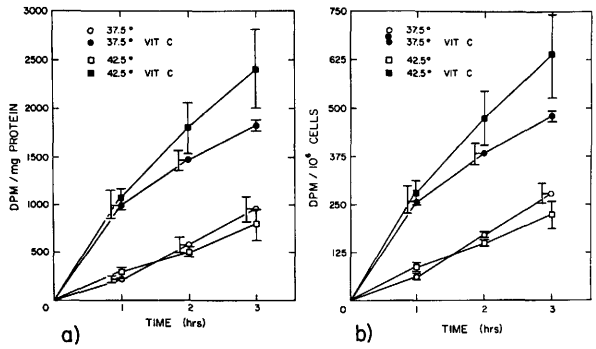

FIG. 1. Effect of hyperthermia and ascorbic acid (1 m*M*) on [U-^{14}C]-glucose metabolism by EATC as a function of time.

TABLE IV. EFFECTS OF HYPERTHERMIA AND ASCORIBC ACID ON THE METABOLISM OF [U-^{14}C] GLUCOSE BY KIDNEY SLICES AND MINCED SPLEEN.*

Treatment	Rat kidney slices dpm/mg protein	Mouse kidney slices dpm/mg protein	Minced spleen
37.5°	288 ± 57[a]	492 ± 43[a]	109 ± 7[a]
37.5° + Ascorbate**	296 ± 48[a]	509 ± 53[a]	100 ± 2[a]
42.5°	243 ± 46[a]	438 ± 50[a]	—
42.5° + Ascorbate**	286 ± 73[a]	435 ± 26[a]	—

* Kidney slices and minced spleen were incubated 1 hr with 0.25 μCi [U-^{14}C] glucose and 20 μmoles cold glucose. Means ± SE for six replicates per datum at 37.5° and four replicated per datum at 42.5° for rat kidney slices. Five replicates were used per incubation temperature for mouse kidney slices. Data are for three replicates of minced spleen at 37.5°.

** Ascorbic acid was added at a final concentration of 1 m*M*.

lism of glucose, pyruvate, or citrate. Although large changes in metabolism are reported in the literature for incubation periods beyond 3 hours, interpretation of these results is complicated by a probable increased percentage of nonviable cells (8, 9). Consistent with this study, Cavaliere *et al.* (8) and Mondovi (9) have not observed any significant alterations of O_2 consumption with short term hyperthermic exposure. Many hyperthermic effects are reported to be sensitive to small temperature changes. Schulman and Hall (19) reported that the surviving fraction of cultured mammalian cells declines more than one order of magnitude for 2 hr incubation at 43° instead of 42°. Overgaard and Overgaard (20) heated mouse mammary carcinoma 1 hr *in vivo* and observed a cure rate of 6% at 42° and 25% at 43°.

Ascorbic acid stimulated the metabolism of all three labelled substrates by EATC. Increasing the concentration of vitamin C to 10.0 m*M* further stimulates glucose metabolism linearly ($r = 0.93$). Ascorbic acid did not significantly affect glucose metabolism in kidney tissue while it markedly stimulated glucose metabolism in EATC. Previous reports demonstrated that ascorbate increases the oxygen consumption of embryonic chick tibias and of guinea pig polymorphonuclear leukocytes (24, 25). Both of these normal tissues have characteristics that are similar to oncogenic cells. In particular, polymorphonuclear leukocytes have a high rate of lactate production as do cancer cells (25, 26). Malignant cells are also characterized by some degree of dedifferentiation and may have metabolic characteristics similar to undifferentiated normal tissues (27).

Hyperthermia in the presence of vitamin C did not affect EATC glucose or pyruvate metabolism, but did significantly increase metabolism of [1,5-^{14}C] citrate. Since citrate enters the tricarboxylic acid cycle directly, the mitochondria may be the locale of hyperthermia and ascorbate interaction. Hyperthermia may have increased the permeability of citrate through the cell and mitochondrial membrane (21). Ascorbate may have also affected metabolism by acting as a reducing agent in the electron transport chain or, by inhibiting the enzyme phosphodiesterase, thereby potentiating cAMP concentrations (22, 23). Temperature independent reactions may similarly occur in the glycolytic pathway since hyperthermia with ascorbic acid had no effect on glucose or pyruvate metabolism.

Although the lack of an ascorbate effect on kidney slices and minced spleen may be due to the particular system chose, further research may produce information concerning bioenergetic differences between normal and malignant tissues. If ascorbic acid does differentially affect neoplastic cell metabolism it could become a useful research and perhaps therapeutic tool.

Summary. The effects of hyperthermia and ascorbic acid upon the metabolism of [U-^{14}C] glucose, [1-^{14}C] pyruvate, and [1,5-^{14}C] citrate by EATC were examined. All experiments were conducted at 37.5° and 42.5° in the presence or absence of ascorbic acid. Hyperthermia treatment for 2 and 3 hr significantly decreased cell viability as determined by trypan blue exclusion. Ascorbic acid did not affect cell viability. Hyperthermia did not significantly alter EATC metabolism of glucose, pyruvate, or citrate. However, ascorbic acid (1 m*M*) significantly increased the metabolism of all three compounds by EATC. Neither hyperthermia or ascorbic acid had any significant effect upon the metabolism of [U-^{14}C] glucose by kidney slices or minced spleen.

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