The Effects of Microwave Radiation, Hyperthermia, and *L*-Ascorbic Acid on Ehrlich Ascites Carcinoma Cell Metabolism

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Abstract—**The effects of microwave radiation, hyperthermia, and *L*-ascorbic acid on metabolism of (U-^{14}C) glucose, (2-^{14}C) glucose, and (1,5-^{14}C) citrate in Ehrlich ascites tumor cells (EATC) were studied. Microwave irradiated and water bath incubated cells were maintained at the same temperature for comparison. Data were obtained at 37.5°C and 42.5°C in the presence or absence of *L*-ascorbic acid. Ascorbic acid increased the rate of metabolism of both glucose and citrate. No significant differences in glucose and citrate metabolism were found between irradiated and nonirradiated cells at the same temperature and ascorbic acid concentration. A comparison of glucose metabolism in a normal tissue preparation and EATC was made. Vitamin C did not increase metabolism in the normal cell preparation.**

I. Introduction

THE SUPPOSITION that heat may be therapeutically beneficial as a cancer treatment probably developed from observations of cures or considerable improvements in cancer patients who simultaneously had febrile reactions caused by infection or administration of bacterial toxins [1]–[4]. Substantial evidence exists that hyperthermia can delay tumor growth, or lead to its complete regression [5]–[7]. Hyperthermia used in conjunction with ionizing radiation or chemotherapeutic agents may affect tumor growth synergistically and thus become a workable treatment regimen [8]–[10].

The heating of tumors at the clinical level may involve the use of microwave radiation [11], ultrasonic radiation [12], or radio-frequency currents [13]. Therefore, it is important to determine whether the use of a particular heating technology produces biological effects (beneficial or otherwise) attributable to mechanisms distinct from heating.

Ascorbic acid (vitamin C) is a dietary essential for man. Several reports have pointed to low or depleted concentrations of ascorbate in malignant activity [14]. Cellular growth can be inhibited by increasing cyclic adenosine monophosphate (cAMP) [15]. Ascorbic acid is known to inhibit phosphodiesterase activities thereby increasing cAMP concentrations [16]. Ascorbic acid, therefore, becomes of interest as a potential agent in controlling oncogenic metabolism and division.

We studied the metabolic response of Ehrlich ascites carcinoma cells (EATC) *in vitro* at 37.5°C and 42.5°C using two distinct heating modalities. Conventional hot water bath heating and a microwave irradiation heating scheme were employed. The effects of hyperthermia (microwave versus water bath) on metabolism in the presence of vitamin C were determined. Experiments on normal tissues (rat kidney slices) were performed to determine if our results were specific for neoplastic tissue. The effects of microwave radiation, water bath heating, and ascorbic acid on the metabolism of normal and malignant cells were compared.

II. Methods

A. Biological Techniques

EATC were serially transplanted intraperitoneally at regular intervals in male Swiss mice (ICR strain, Harlan Industries, Indianapolis, IN). Cells used were harvested 6–12 days after transplantation.

Incubations were performed in 25-ml siliconized glass Erlenmeyer flasks with specially constructed center wells for $^{14}CO_2$ collection. All incubations were performed in Krebs–Ringer Phosphate buffer (KRP) without calcium [17]. The buffer's composition was: 0.085M Na_2HPO_4, 0.016M NaCl, 0.005M KCl, 0.0013M $MgSO_4 \cdot 7H_2O$, and at a final pH of 7.4. A stock solution of *L*-ascorbic acid (vitamin C, Sigma Chemical Company), 20-mM diluted with KRP, was prepared fresh before each experiment.

Labelled (U-^{14}C) glucose (NEC-042) and (2-^{14}C) glucose (NEC-044) were obtained from New England Nuclear (Boston, MA) while (1,5-^{14}C) citrate (CFA·263) was obtained from Amersham Corporation (Arlington Hts, IL). Tracers were diluted with the respective "cold" substrates. Depending on the experiment, each flask contained 0.25–0.42 μcuries of label with 20 μmoles of "cold" substrate.

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Harvested cells were heparinized and diluted with KRP to a final cell concentration of approximately 40×10^6 cells/cc. Undiluted ascites suspension contain approximately 200×10^6 cells/cc. The protein content and the number of cells per flask were determined in each experiment. Protein was determined by a biuret method using bovine serum albumin as a standard [18]. Cell counts were determined with a hemocytometer. Cell viability was determined by the dye exclusion technique, using 0.1-percent trypan blue-saline solution [19].

Each Erlenmeyer flask contained KRP (3.0 or 3.2 ml), vitamin C (0.2 or 0.0 ml), tracer and "cold" substrate (0.2 ml), KRP diluted cells (0.6 ml), and $^{14}CO_2$ collection vial. The final volume in every flask was 4 ml. The final vitamin C concentration in the flask was 0 or 1 mM. The flasks were capped with rubber serum stoppers and placed in either a Dubnoff metabolic shaker bath (Lab Line #3575) or a Plexiglas jig for the microwave irradiation.

The water bath and circulating saline in the microwave exposure system (discussed below) were maintained at the same temperature (37.5°C or 42.5°C) for any given experiment. These temperatures were regulated within ±0.1°C as verified with a digital thermometer (Bailey BAT-8C).

All incubations were one hour duration. Previous water bath incubations for 1, 2, and 3 h intervals indicated a linear increase in $^{14}CO_2$ versus incubation time. Following a one hour incubation, the reactions were stopped by injecting 1 ml of 2N $HClO_4$ (PCA) through the serum caps. The microwave irradiated flasks were transferred to the water bath. All flasks were shaken an additional hour to collect $^{14}CO_2$. Blanks were prepared by adding PCA before addition of the cell suspension. Collection of $^{14}CO_2$ was performed using previously reported techniques [20]. Briefly, fluted filter paper (Whatman #1, 2.7×3.3 cm) was inserted into shell vials (Kimble 60930-L) and treated with 0.2-ml hyamine hydroxide (New England Nuclear). After shaking with PCA, the shell vial and paper were removed and placed in liquid scintillation vials containing 15 ml of cocktail (4 g PPO, 0.05 g POPOP, diluted to 1.0 l with toluene). Samples were held at least 5 h at room temperature before counting in a Packard Tri-Carb scintillation spectrometer. Quench correction was done using the channels ratio method. Data after quench correction are expressed in disintegrations per minute (DPM).

Kidney slices from male Sprague–Dawley rats (Harlan Industries, Indianapolis) were used for normal cell metabolism data. All slices were approximately 100 μm thick. Approximately 200 mg of kidney slices were placed into each incubation flask. Protein content was determined by a biuret method on kidney tissue homogenates [18].

The student's t test was used in statistical analysis of the data. Criteria for significance was $p \leqslant 0.02$.

B. Microwave Techniques

A temperature controlled Dubnoff shaker bath (Lab Line #3575) was used to incubate the nonirradiated control flasks. Irradiated flasks were placed in an Eccosorb lined microwave chamber 45 cm below the aperture of 15×20-cm horn antenna. Both irradiated and nonirradiated cells were constantly agitated by the shaker bath platform at 75 cycles/min. The thin Plexiglas flask holder in the microwave chamber was mechanically linked to the shaker bath platform by a wood dowel passing through the chamber wall (Fig. 1). The platform moved sinusoidally with a 3.8-cm peak to peak amplitude.

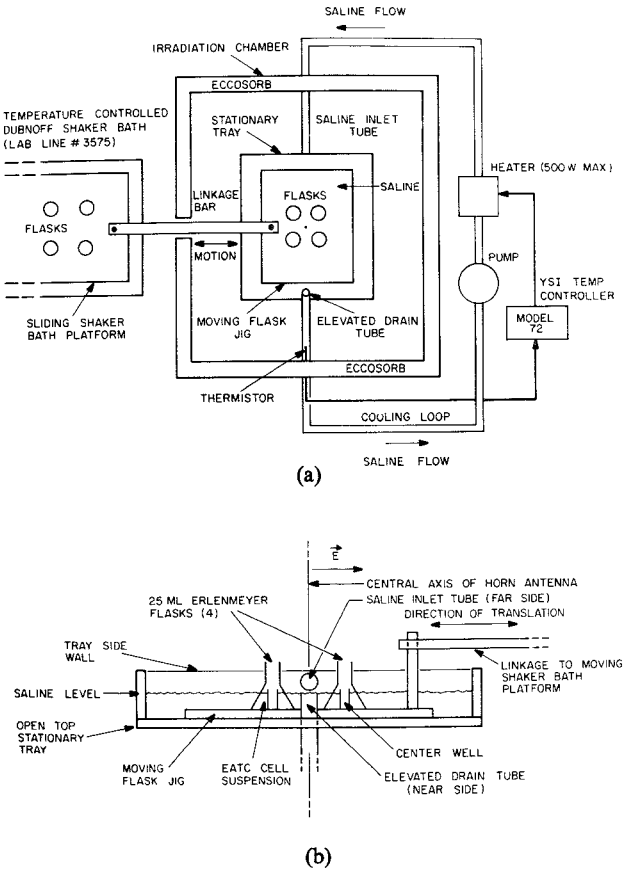

Fig. 1. Microwave exposure system. (a) Irradiation and temperature control system (top view). (b) Tray and flask holder (side view).

Physiological saline was circulated through a tray constructed from thin Plexiglas. The tray supported the moving flask holder. With a platform 75-cycle/min movement and a 2.0 l/min flow rate of temperature controlled saline, the internal flask temperature was maintained within ±0.1°C of the desired temperature. This was verified by periodic temperature measurements using a digital thermometer (Bailey BAT 8-C) with thermocouple probes. All temperature measurements were made with the microwave source off.

The flask holder and temperature control system are shown in Fig. 1. Four flasks were irradiated simultaneously. The flasks were placed symmetrically about the central axis of the horn antenna. Distance between the centers of the flasks was 4.5 cm. The depth of saline surrounding the flasks without agitation was 0.75 cm. The cell suspension inside the Erlenmeyer flask was at the same level as the surrounding saline bath (see Fig. 1(b)).

With this exposure system, the irradiated and water bath cell suspensions were mechanically translated at exactly the same rate. Agitation of the microwave irradiated flasks served to minimize temperature gradients within the cell suspension. Since the dielectric properties of the cell suspensions and the surrounding saline solution were similar, fringing of the electromagnetic field was also minimized.

Specific absorption rate (SAR) in the cell suspension was 53 ± 4 W/kg. SAR was estimated from a series of temperature rise measurements made after 10 and 15 s irradiation intervals. The initial temperature was 25°C. The flasks were irradiated and moved sinusoidally, but with the circulating pump off. Final temperature measurements were made within 3 s following the irradiation period with the digital thermometer and thermocouple probe.

Both water bath and irradiated cell suspensions reached within 3-min temperature equilibration with the surrounding temperature controlled fluids. The differences in equilibration times between irradiated and water bath samples were less than 1 min.

Incident power density in the absence of the tray and flask holder was 200 mW/cm^2 as measured by a Narda 8110 EM Monitor. The microwave source was a 2.45-GHz continuous wave Litton 3510 "Microtron."

III. Results

Data on EATC metabolism of (U-^{14}C) glucose are listed in Table I. At 37.5°C and at 42.5°C vitamin C significantly increased metabolism of this substrate. The stimulatory effect of vitamin C was observed in the water bath and microwave treated EATC. Statistical analysis of the respective water bath and microwave data in groups with the same ascorbic acid concentration at 37.5°C and at 42.5°C revealed no significant differences between these two heating methods. In addition, there was no significant effect of changing temperature from 37.5°C to 42.5°C on EATC (U-^{14}C) glucose metabolism in groups with the same ascorbic acid concentration.

Data obtained from experiments on EATC metabolism of (2-^{14}C) glucose are presented in Table II. At both 37.5°C and 42.5°C metabolism of this substrate increased significantly in the presence of vitamin C. This result applied to water bath and microwave heated cells. There were no statistically significant differences between respective water bath and microwave data at both temperatures. EATC (2-^{14}C) glucose metabolism data obtained at 37.5°C versus 42.5°C were not significantly different in groups with the same ascorbic acid concentration.

The metabolic response data from EATC with (1,5-^{14}C) citrate as the substrate are shown in Table III. Vitamin C affected metabolism significantly at 37.5°C and at 42.5°C. The corresponding data from water bath and microwave heated samples are not different at 37.5°C and at 42.5°C. Hyperthermia (37.5°C–42.5°C) without vitamin C for the water bath and the microwave data produced no significant differences. However, raising the temperature from 37.5°C to 42.5°C in the presence of vitamin C increased citrate metabolism significantly for both water bath and microwave irradiated cell suspensions.

Kidney tissue slices were used as an example of normal cell metabolism. The metabolic response of kidney slices using (U-^{14}C) glucose is given in Table IV. There was no significant effect of vitamin C at 37.5°C or at 42.5°C for either water bath or microwave heated samples. In addi-

TABLE I
METABOLISM OF (U-^{14}C) GLUCOSE BY EATC FOR ONE HOUR INCUBATION[1]

	$^{14}CO_2$	
	37.5°C	42.5°C
BATH	154.9 ± 31.6 (56.7 ± 11.8)	143.9 ± 14.4 (55.5 ± 4.5)
BATH (+C)	274.5 ± 36.0 (100.5 ± 14.6)	352.8 ± 96.7 (134.7 ± 30.4)
MICROWAVE	171.0 ± 26.8 (63.4 ± 9.9)	165.5 ± 20.9 (60.9 ± 7.7)
MICROWAVE (+C)	315.6 ± 45.2 (116.9 ± 16.7)	335.5 ± 10.9 (123.5 ± 4.0)

[1]Each flask contained 0.25-μCi (U-^{14}C) glucose and 20 μmoles cold glucose in 4-ml total volume. Data are average ± standard deviation (7 replicates per datum for bath sets, 4 replicates per datum for microwave sets). Data expressed as DPM/mg protein (DPM/10^6 cells).

TABLE II
METABOLISM OF (2-^{14}C) GLUCOSE BY EATC FOR ONE HOUR INCUBATION[2]

	$^{14}CO_2$	
	37.5°C	42.5°C
BATH	78.9 ± 23.5 (26.8 ± 8.0)	86.0 ± 5.2 (26.9 ± 1.6)
BATH (+C)	151.4 ± 20.8 (51.3 ± 7.1)	141.1 ± 16.0 (44.1 ± 5.0)
MICROWAVE	65.7 ± 8.8 (22.3 ± 3.0)	77.8 ± 3.9 (24.3 ± 1.2)
MICROWAVE (+C)	137.5 ± 14.5 (46.7 ± 4.9)	135.1 ± 16.1 (42.3 ± 5.0)

[2]Each flask contained 0.25-μCi (2-^{14}C) glucose and 20 μmoles cold glucose in 4-ml total volume. Data are average ± standard deviation (3 replicates per datum for bath 37.5°C, all other sets had 4 replicates per datum). Data expressed as DPM/mg protein (DPM/10^6 cells).

TABLE III
METABOLISM OF (1,5-^{14}C) CITRATE BY EATC FOR ONE HOUR INCUBATION[3]

	$^{14}CO_2$	
	37.5°C	42.5°C
BATH	79.8 ± 17.3 (32.8 ± 7.1)	103.4 ± 11.2 (44.8 ± 4.9)
BATH (+C)	148.8 ± 29.6 (61.1 ± 12.2)	289.2 ± 66.8 (125.2 ± 28.9)
MICROWAVE	99.2 ± 26.4 (40.8 ± 10.9)	118.1 ± 26.8 (51.2 ± 11.6)
MICROWAVE (+C)	162.0 ± 25.0 (66.6 ± 10.3)	319.6 ± 29.7 (138.4 ± 12.9)

[3]Each flask contained 0.42-μCi (1,5-^{14}C) citrate and 20 μmoles cold citrate in 4-ml total volume. Data are average ± standard deviation (4 replicates per datum). Data expressed as DPM/mg protein (DPM/10^5 cells).

TABLE IV
METABOLISM OF (U-^{14}C) GLUCOSE BY RAT KIDNEY SLICES FOR ONE HOUR INCUBATION[4]

	$^{14}CO_2$	
	37.5°C	42.5°C
BATH	227.1 ± 60.5	270.4 ± 101.2
BATH (+C)	266.0 ± 74.0	317.6 ± 162.5
MICROWAVE	358.9 ± 78.4	309.4 ± 99.0
MICROWAVE (+C)	333.0 ± 146.7	339.3 ± 160.1

[4]Each flask contained 0.25-μCi (U-^{14}C) glucose and 20 μmoles cold glucose in approximately 4-ml total volume. Data are average ± standard deviation (4 replicates per datum). Data expressed as DPM/mg protein.

tion, there were no significant metabolic changes resulting from incubation at 42.5°C versus 37.5°C.

IV. DISCUSSION

Relatively few studies have been made on the effects of nonionizing electromagnetic radiation on tissue metabolism. A decrease in brain succinate dehydrogenase activity in animals irradiated at 3.0 GHz has been reported [21]. Uncoupled oxidative phosphorylation has been reported in animals irradiated with pulsed microwaves [22] and pulsed 7-kHz electromagnetic radiation [23]. Continuous exposure of mice for 160 h to radiation at 3.105 GHz (2 mW/cm^2) resulted in large increases in protein synthesis in the liver, thymus, and spleen [24]. Prolonged exposure of rats to UHF radiation at low intensities (0.06–10 μW/cm^2) was reported to cause a reduction in liver glycogen due to increased phosphorylase activity. At the same time, changes in oxidative coupling and phosphorylation processes in rat liver mitochodria were detected [25]. Observed effects in animals can also be elicited from stress induced by heat [26], surgery [27], or even handling [27]. Therefore, extreme care should be given to the design of *in vivo* experiments and to the interpretation of results. Certainly, more work is needed to characterize the metabolic effects of microwave radiation upon laboratory animals.

In contrast to these studies, 2.45-, 3.0-, and 3.4-GHz irradiation of rat liver mitochondria *in vitro* at an SAR of 41 W/kg caused no change in respiratory acitvity [28]. Our results are consistent with this study.

Ascorbic acid is known to inhibit phosphodiesterase activities thereby increasing cyclic adenosine monophosphate (cAMP) concentration [16]. Increased cAMP can inhibit cellular growth [15]. Ascorbic acid has been effective in blocking *in vitro* formation of *N*-nitroso compounds from nitrate and amines or amides [29]. In rats given ascorbate, liver tumors induced by morpholine and nitrate showed a longer induction period, a lower incidence, and an abscence of lung metastasis. This indicates that ascorbate had inhibited *in vivo* formation of nitrosomorpholine [30].

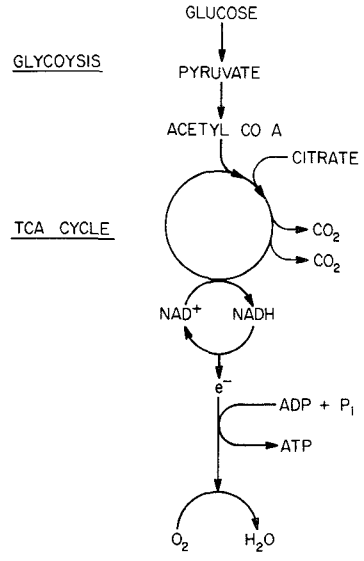

Fig. 2. Schematic showing pathway of glucose catabolism in chemotrophic cells. Notice that citrate enters the TCA cycle directly and that intermediates of glucose catabolism enter the TCA cycle only after traversing the glycolytic pathway.

TABLE V
DESCRIPTION OF $^{14}CO_2$ RELEASE FROM THE LABELLED SUBSTRATES USED[5]

Substrate	Point of Release
(U - ^{14}C) Glucose	nonspecific, $^{14}CO_2$ may be released in any oxidative step.
(2 - ^{14}C) Glucose	incorporated as C - 2 of pyruvate which beomes C - 5 of citrate, half of the C - 5 citrate becomes C - 6 of citrate following one revolution of the TCA cycle.[1]
(1,5 - ^{14}C) Citrate	$^{14}CO_2$ from 1 - ^{14}C citrate is released in the ketoglutarate dehydrogenase reaction (TCA cycle). Half of 5 - ^{14}C citrate becomes C-6 of citrate following one revolution of the TCA cycle.[1]

[5]C-6 of citrate is released as $^{14}CO_2$ in the isocitrate dehydrogenase reaction (TCA cycle).

Ascorbic acid has been shown to be an effective lethal agent in EATC [31]. Some of the ascorbate induced toxicity was attributed to the accumulation of hydrogen peroxide (H_2O_2).

Fig. 2 is a highly simplified schematic representation of cellular metabolism showing glycolysis, tricarboxylic acid (TCA) cycle, and electron transport and oxidative phosphorylation. The points of $^{14}CO_2$ release in the metabolic pathways from (U-^{14}C) glucose, (2-^{14}C) glucose, and (1,5-^{14}C) citrate are described in Table V. Intermediates of (U-^{14}C) glucose release $^{14}CO_2$ in the glycolytic pathway and the TCA cycle. $^{14}CO_2$ is released from (2-^{14}C) glucose only in the TCA cycle. (1,5-^{14}C) Citrate enters the TCA cycle directly. Hence, by studying the effects of various treatments on metabolism of these different tracers, information may be obtained concerning loci of interaction.

The results given in Tables I, II, and III clearly demonstrate that ascorbic acid markedly stimulates the metabolism of all three compounds in EATC. This stimulation of

metabolism by ascorbic acid was observed at both temperatures (37.5°C and 42.5°C) in the microwave irradiated and water bath samples. Ascorbate may stimulate metabolism by acting as a reducing agent in the electron transport chain facilitating electron transfer [32], or by stimulating cyclic AMP [33].

Hyperthermia, in the absence of ascorbic acid, did not significantly alter metabolism of the substrates used for one hour incubation periods (Tables I, II, and III). This was true for microwave irradiated and water bath samples. These results generally agree with previous reports in that glycolysis and respiration are only moderately affected by one hour hyperthermic treatment [34], [35].

Hyperthermia, in the presence of ascorbic acid, significantly altered cirtate metabolism but not glucose metabolism. These results apply to microwave irradiated and water bath samples. Since citrate enters the TCA cycle directly without traversing the glycolytic pathway, the interaction site of ascorbic acid and hyperthermia upon citrate metabolism could be in the mitochondria. Because hyperthermia in the presence of ascorbic acid did not alter glucose metabolism, it is possible that rate limiting temperature independent reactions occur in the glycolytic pathway. Further research will be necessary to elucidate exact mechanisms responsible for the synergistic effect of hyperthermia and vitamin C on citrate metabolism.

Metabolism in microwave irradiated samples versus water bath samples was not statistically different. This observation applies to results obtained using (U-^{14}C) glucose, (2-^{14}C) glucose, and (1,5-^{14}C) citrate at 37.5°C or at 42.5°C, with ascorbic acid or without ascorbic acid.

Data on metabolism of (U-^{14}C) glucose by rat kidney slices are given in Table IV. Ascorbic acid did not significantly affect glucose metabolism in this normal tissue while it markedly stimulated glucose metabolism in EATC. It has been reported that ascorbate increases the oxygen consumption of embryonic chick tibias [36] and of guinea pig polymorphonuclear leukocytes [37]. Both of these normal tissues have certain characteristics which are similar to oncogenic cells. In particular, polymorphonuclear leukocytes have a high rate of lactate production [37] as do cancer cells [38], [39]. Malignant cells are characterized by some degree of dedifferentiation [40], [41] and may have metabolic characteristics similar to undifferentiated embryonic tissues.

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

The data reported are applicable to one hour incubation periods. Trypan blue viability results indicate virtually 100-percent live cells at 37.5°C and 42.5°C for one hour incubation. Cell viability for two hour incubations at 37.5°C was typically 100 percent; at 42.5°C viability decreased to approximately 85 percent.

It appears that one hour hyperthermic treatment produces rather small changes in tumor cell metabolism. This conclusion is supported by O_2 consumption studies in the literature. Large changes in metabolism are also reported for longer incubation periods [34], [35], but interpretation of these results is complicated by the increasing percentage of nonviable cells.

V. Summary

Metabolism of (U-^{14}C) glucose, (2-^{14}C) glucose, and (1,5-^{14}C) citrate in Ehrlich ascites tumor cells was studied. These data were compared to (U-^{14}C) glucose metabolism in rat kidney slices. Microwave irradiated and nonirradiated control (water bath) samples were incubated at 37.5°C and 42.5°C in the presence or absence of vitamin C. The metabolic responses of EATC and kidney slices were not statistically different between microwave irradiated and nonirradiated samples.

Vitamin C significantly increased metabolism of all three labelled substrates in EATC. This effect of ascorbic acid was found at 37.5°C and 42.5°C in both irradiated and nonirradiated control groups. One hour hyperthermic treatment alone did not significantly affect metabolism in EATC. Hyperthermia, in the presence of ascorbic acid, significantly increased EATC citrate metabolism but not glucose metabolism. This result held for both irradiated and control samples.

No significant alterations were observed in (U-^{14}C) glucose metabolism by kidney slices with or without vitamin C. Metabolism in irradiated and nonirradiated kidney samples was not statistically different. There was no significant effect of one hour hyperthermic treatment on kidney glucose metabolism.

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