

PET Measurements of Hyperthermia-Induced Suppression of Protein Synthesis in Tumors in Relation to Effects on Tumor Growth

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Hyperthermia-induced metabolic changes in tumor tissue have been monitored by PET. Uptake of L-[1-¹¹C]tyrosine in rhabdomyosarcoma tissue of Wag/Rij rats was dose-dependently reduced after local hyperthermia treatment at 42, 45, or 47°C. Tumor blood flow, as measured by PET with ¹³NH₃, appeared to be unchanged. The L-[1-¹¹C]tyrosine uptake data were compared to uptake data of L-[1-¹⁴C]tyrosine and with data on the incorporation of L-[1-¹⁴C]tyrosine into tumor proteins. After intravenous injection, the ¹⁴C data were obtained from dissected tumor tissue. Heat-induced inhibition of the incorporation of L-[1-¹⁴C]tyrosine into tumor proteins tallied with the L-[1-¹¹C]tyrosine uptake data. Heat-induced inhibition of amino acid uptake in the tumor correlated well with regression of tumor growth. It is concluded that PET using L-[1-¹¹C]tyrosine is eligible for monitoring the effect of hyperthermia on tumor growth.

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Since positron emission tomography (PET) is a noninvasive tool able to monitor quantitatively metabolic activities of tissues, the effects of therapy on tumor tissues can be investigated with this technique.

Relatively few investigators have applied positron-emitting tracers in experiments with tumor-bearing animals subjected to therapy. In the majority of these studies, uptake of tracer was determined in dissected tumor tissue before and after treatment. The effects of chemotherapeutics on tumor tissue have been investigated in terms of amino acid transport using L-¹¹C- α -isobutyric acid (1). Radiotherapeutic effects on glycolysis in tumor tissue have mainly been assessed by applying 2-³H-deoxyglucose (2) and ¹⁸F-2-fluoro-2-deoxyglucose (3). Recently, the response of the amino acid transport system of tumor to ionizing radiation has been investigated with L-[methyl-¹¹C]methionine (4). Using a pinhole-collimated gamma camera, Knapp and co-workers (5,6) investigated the up-

take of ¹³N-glutamate in relation to tumor blood flow in the Walker 256 carcinosarcoma before and after chemotherapy and radiotherapy.

Application of heat to tissues induces many changes on cellular structures and on metabolic activities (7). From the literature, it is known that protein synthesis is inhibited by hyperthermia in a heat dose dependent way (8-10). Especially the rate of recovery of protein synthesis seems to correlate with the extent of cell survival after hyperthermia. Heat-induced inhibition of tumor growth has been shown in a number of animal studies (11). Studies on the in vivo inhibition of protein synthesis in tumors, parallel with the measurement of tumor growth during hyperthermia, are important in order to explore to what extent PET is a prognostic tool for tumor growth by measuring alterations in protein synthesis. For this purpose, L-[1-¹¹C]tyrosine (¹¹C-tyr) is available as a tracer (12,13).

In tumor tissue, hyperthermia may change the tumor blood flow which might affect the uptake of radiolabeled compounds and the removal of heat (14-16). These changes can be investigated with ¹³NH₃ (17).

The aims of the current study are:

1. To investigate the effects of different thermal doses on protein synthesis in tumors using L-[1-¹⁴C]tyrosine (¹⁴C-tyr) in rhabdomyosarcoma (RMS) bearing rats, and to correlate these effects with the corresponding effects on ¹¹C-tyr uptake as measured by PET.
2. To investigate the effects of the thermal doses on tumor growth and to correlate these with the ¹¹C-tyr data obtained by PET.

MATERIALS AND METHODS

Chemicals

Nitrogen-13-ammonia was prepared according to Vaalburg et al. (18). Briefly, during irradiation of distilled water with 18 MeV protons, ¹³N-activity was generated, no-carrier-added, in the form of ¹³N-nitrites and ¹³N-nitrates. These were reduced to ¹³NH₃, using Devarda's alloy in strong alkaline solution. L-[1-¹¹C]tyrosine was prepared according to Bolster et al. (19). After carbox-

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ylation with 7.4 GBq (200 mCi) ^{11}C CO₂ of the lithiated p-methoxyphenylethyl-isocyanide, 110–140 MBq (3–4 mCi) enantiomerically pure L-[1- ^{11}C]tyrosine were obtained with a specific activity of at least 3.7 GBq/ μmol (100 Ci/mmol).

Animals

The rhabdomyosarcoma tumor in the Wag/Rij rat was used as a tumor model (20). Cubic pieces (100 mg) of solid, homogeneous, rhabdomyosarcoma tissue were grafted into the left flanks of 2-mo-old female Wag/Rij rats that weighed 140 g. A volume of between 4 and 5 ml was measured 18 days after transplantation. At this stage, the ellipsoid-shaped tumors were free of necrotic parts, and therefore histologically homogeneous. Animals were fed on a standard diet and given water ad libitum.

L-[1- ^{14}C]tyrosine Uptake and Incorporation

Tumor-bearing rats were anesthetized with a dose of 3 mg sodium pentobarbital (3 g/100 ml saline) per 100 g body weight intraperitoneally. A supplement of 1 mg anaesthetic was given every 2 hr. The tumor was locally heated for 15 min at 42, 45, or 47°C. Rats were given an intravenous dose of 93 kBq (2.5 μCi) L-[1- ^{14}C]tyrosine (Amersham International, Buckinghamshire, UK) dissolved in 0.2 ml saline intravenously with a specific activity of 2 GBq/mmol (56 mCi/mmol) at intervals of 10, 30, 60, or 120 min after treatment. Five animals were used per thermal dose and per point in time. Rats were killed by a heart puncture 60 min after the ^{14}C -tyr injection. Tumors were excised, and seven tumor tissue samples with a weight of 50–100 mg each were randomly selected. Five of these tumor samples were used for the measurement of the total tissue activity. These samples were weighed and dissolved in Protosol® (Dupont, Boston, MA). After addition of 10 ml Plasmasol® scintillation liquid (Packard Instruments, Downers Grove, IL), the total ^{14}C radioactivity was measured by liquid scintillation counting. The tumor uptake was expressed as differential absorption ratio (DAR); i.e. (counts/g tissue) \times (g body weight/total injected counts). The values of the five samples were averaged. To determine the protein synthesizing activity of the tumor tissue at different times after hyperthermia, the incorporation of ^{14}C -tyr into proteins was measured. Therefore, the two remaining tumor samples were homogenized and treated with trichloro acetic acid (TCA) at 0°C. Subsequently, the TCA-insoluble fraction was determined. From the total ^{14}C uptake and the TCA-insoluble fraction, the accumulated ^{14}C radioactivity incorporated into protein was calculated and expressed as DAR. For 10 untreated rats, the uptake and incorporation of ^{14}C -tyr was also determined; these animals served as controls.

Hyperthermia

A modified, commercially available Curadar 2000 (Enraf Nonius, Delft, The Netherlands) was used as a hyperthermia device. This device emitted 2.45 GHz microwaves, which were locally applied on the tumor by means of a home-built 4 \times 4 cm² applicator. Three thermocouples were placed into the tumor; one at the core and two at the periphery. The desired temperature was attained within 2 min. The electric feedback from the thermocouples to the microwave generator, in combination with a predefined temperature threshold, regulated the amount of energy released from the microwave generator on the tumor. The tumor temperature was kept constant with an accuracy of 0.3°C. Marked differences in temperature, registered at the three different positions of the thermocouples, were not observed. A fourth rectal thermocouple monitored whole body temperature. Notable ele-

vation of the body temperature was not registered during the hyperthermia treatment.

PET Experiments with L-[1- ^{11}C]tyrosine and $^{13}\text{NH}_3$

Animals were anesthetized with sodium pentobarbital, as described above. During experimentation, body temperature was measured rectally. It is known that anaesthesia causes a drop in temperature of the body. To prevent this, body temperature was maintained above 37°C by the use of infra red lamps. A catheter with a low volume (<0.05 ml) was inserted into a tail vein. Complete administration of tracer was achieved by flushing the catheter with 0.05 ml of saline after each injection. The rat was dorsally positioned in a longitudinal positron camera (21). The PET data were acquired as described previously by Daemen et al. (22,23). A dose of 0.37 MBq (10 μCi) $^{13}\text{NH}_3$ in a volume of 0.1 ml saline was administered to the untreated rat via the catheter as a fast bolus. Ten minutes after the injection, the uptake of the ^{13}N radioactivity into the RMS tumor was measured by PET. Subsequently, after physical decay of the ^{13}N activity to a negligible background value (<0.5%), an injection of 1.1 MBq (30 μCi) ^{11}C -tyr in 0.2 ml phosphate buffer (pH 4.6) was administered to the same animal. Sixty minutes after this injection, the uptake of ^{11}C -tyr into tumor tissue was measured. The rat was removed from the camera and the tumor was given a local thermal dose. The treated rat was then repositioned in the positron camera and was given a second injection of 0.37 MBq (10 μCi) $^{13}\text{NH}_3$ 10 min after hyperthermia. In analogy to the pre-treatment measurement, the ^{13}N -uptake in tumor was determined 10 min after injection. Thirty minutes after hyperthermia, the rat was given a second injection of 1.1 MBq (30 μCi) ^{11}C -tyr. The ^{11}C -uptake into the tumor tissue was measured 60 min after injection. The time interval between the $^{13}\text{NH}_3$ injection and the ^{11}C -measurement was 80 min. The contribution of the ^{13}N -background in the second L-[1- ^{11}C]tyrosine measurement is less than 0.4% and therefore considered to be negligible. The PET data were corrected for physical decay and nonuniform response of the system. The uptake in tissue was calculated as DAR(PET); i.e., (Counts tumor/Volume tumor) \times (Weight rat/Counts rat).

Volume Measurements and Growth Delay

The three principal diameters of the tumor, growing in situ, were measured with a vernier calliper. The volume of the tumor was calculated by the formula: $V = \frac{1}{6} \pi \times (\text{product of 3 orthogonal diameters})$ (24). Data obtained from the tumor volume measurements in time course were used to construct the growth curves. From the growth curves, the tumor doubling times (time to double a certain volume, TD) were calculated. The growth delay (GD) was determined for the treated animals. GD is defined as the number of doubling times saved by therapy and is calculated by the formula: $\text{GD} = (\text{TD}_t - \text{TD}_c)/\text{TD}_c$. The TD_t is the doubling time of the treated tumors. These values were determined for 19 animals (7 at 42°C, 6 at 45°C, and 6 at 47°C). The TD_c is the doubling time of a second control group which consisted of nine untreated animals. The statistical significance of the growth delays were analyzed with Fisher's distribution-free sign test (25).

RESULTS

L-[1- ^{14}C]tyrosine Experiments

Data on the total uptake of ^{14}C -tyr into the tumor and its incorporation into tumor proteins are presented in

TABLE 1
Total ^{14}C Uptake of L-[1- ^{14}C]tyrosine and Incorporation of This Amino Acid into Proteins of Rhabdomyosarcoma Tissue at Different Time Points After Hyperthermia

Thermal dose		Time after hyperthermia			
		70 min	90 min	120 min	180 min
15 min 42°C	Total ^{14}C	1.55 ± 0.20	1.37 ± 0.08	1.17 ± 0.06*	1.44 ± 0.19
	Protein ^{14}C	1.12 ± 0.15	1.05 ± 0.10	0.66 ± 0.11*	1.18 ± 0.19
15 min 45°C	Total ^{14}C	0.73 ± 0.15*	0.86 ± 0.11*	1.12 ± 0.05*	1.58 ± 0.12
	Protein ^{14}C	0.30 ± 0.07*	0.28 ± 0.09*	0.53 ± 0.16*	1.19 ± 0.15
15 min 47°C	Total ^{14}C	0.89 ± 0.03*	0.71 ± 0.08*	0.74 ± 0.10*	1.09 ± 0.18* [‡]
	Protein ^{14}C	0.50 ± 0.09*	0.33 ± 0.08*	0.44 ± 0.08*	0.64 ± 0.22* [‡]
Untreated	Total ^{14}C	1.58 ± 0.05 [†]			
	Protein ^{14}C	1.30 ± 0.12 [†]			

Total uptake of L-[1- ^{14}C]tyrosine and incorporation into protein are expressed as DAR (see Materials and Methods); errors are s.e.m.

* P values of 0.05 or less from Student's t-test for difference with untreated.

n = 5 per point of time.

[†] n = 10.

[‡] n = 4.

Table 1. The tumor tissue has been exposed to ^{14}C -tyr during a time span of 60 min (time between injection and dissection). The measured endpoint values are to be considered as a resultant of multiple metabolic processes and redistribution phenomena.

Table 1 shows that treatment of the tumor at 42°C for 15 min does not have a pronounced influence on the uptake of ^{14}C -tyr. A significant reduction in uptake of ^{14}C -tyr was only observed at 120 min after hyperthermia. Hyperthermia at 45°C and 47°C gave rise to statistically significant reductions of the ^{14}C -tyr uptake already measured 70 min after treatment. In the 45°C experiments, this reduced uptake was restored to the control level after 180 min. In the 47°C experiments, the reduction was more prolonged than in the 45°C experiments.

From the total uptake values (Table 1) and the percentages incorporated into proteins, the amount of accumulated ^{14}C -tyr that was converted to protein was calculated, representing the protein synthesizing activity of the tumor tissue. This parameter is presented in Table 1. This table shows that at 120 min after the 42°C treatment, the amount of incorporated ^{14}C -tyr is reduced by a factor of 2, as compared to the untreated tumors. Even larger reductions were measured in the 45°C and the 47°C experiments, where four-fold reductions were found 90 min after treatment.

PET Experiments with L-[1- ^{11}C]tyrosine and $^{13}\text{NH}_3$

As can be seen in Figure 1A (arrow), PET studies with ^{11}C -tyr in the rhabdomyosarcoma-bearing rat revealed good visualization of the tumors. In Figure 2A, a curve of the kinetics of the uptake of ^{11}C -radioactivity into the untreated rhabdomyosarcoma tissue is shown. A typical biphasic pattern is observed. The first phase, between 0 and 5 min after injection, is observed as a rapid uptake of ^{11}C radioactivity into the tumor. Between 5 and 60 min, a

second phase is observed as a slow, linear increase of ^{11}C radioactivity. The statistical error in the measurement of the ^{11}C activity in the tumor, measured 60 min after injection, is less than 1% for the untreated situation. For PET data acquisition, a time span between 30–90 min after hyperthermia was chosen. The heat dose-dependency on the reduction of ^{14}C -tyr uptake was best reflected in this period (see Table 1).

In Table 2A, ^{11}C -tyr PET data are shown obtained prior to and after administration of different thermal doses to the tumor. Uptake of ^{11}C -tyr into the tumor tissue was calculated as DAR. In Table 2A, it is shown that uptake of ^{11}C -tyr in the tumor after hyperthermia is reduced as compared to the untreated situation. These statistically significant reductions amounted to 21%, 28%, and 35% after hyperthermia at 42°C, 45°C, and 47°C, respectively. The statistical significance of these reductions (p values), as obtained with paired Student's t-tests, increased with the rise in thermal dose.

Studies with $^{13}\text{NH}_3$ in the RMS-bearing rat revealed that

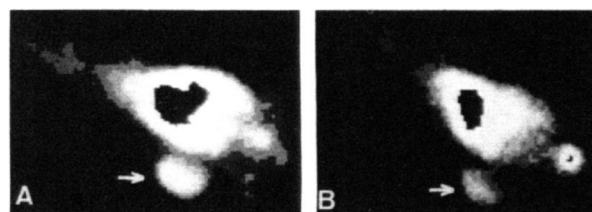
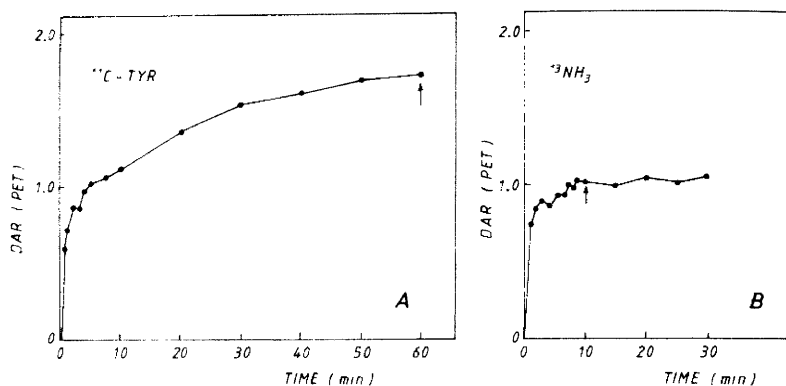


FIGURE 1. (A) Distribution of ^{11}C -tyr, measured by PET, 60 min after intravenous injection into a rat bearing a RMS tumor of 5 ml. (B) Distribution of $^{13}\text{NH}_3$, 10 min after injection into the same animal. Arrows indicate the position of the tumor. Black areas in the images [e.g., liver (^{11}C -tyr, $^{13}\text{NH}_3$) and bladder ($^{13}\text{NH}_3$)] are caused by windowing of the PET images, in order to obtain clearer visualization of the tumor.

FIGURE 2. Uptake curve of ^{11}C -activity into untreated rhabdomyosarcoma tissue 60 min after injection of ^{11}C -tyr. (B) Uptake curve of ^{13}N -activity into the tumor tissue 30 min after injection of $^{13}\text{NH}_3$. Uptake of radioactivity is expressed as DAR(PET). The arrows indicate the moments of radioactivity measurements for the hyperthermia experiments.



^{13}N activity was rapidly taken up by the tumor tissue (Fig. 2B), which resulted in a good visualization of the tumor on the PET image (see Fig. 2B). Between 5 and 10 min after injection, the level of ^{13}N -activity, which was corrected to the time of injection, reached a plateau value (Fig. 3B). This plateau value was observed between 10 and 30 min after injection. The plateau value, which was measured at 10 min after injection of $^{13}\text{NH}_3$, was taken as control parameter for tumor blood flow.

In Table 2B, the $^{13}\text{NH}_3$ uptake values of the RMS tumor prior to and after hyperthermia are presented. No statistically significant differences in uptake of ^{13}N activity was observed at each of the three different heat doses. The profile of the $^{13}\text{NH}_3$ uptake curves did not change after hyperthermia treatment (not shown).

A comparison between the uptake value of ^{11}C -tyr and ^{14}C -tyr in the RMS at 30–90 min after hyperthermia is given in Figure 3. It was observed that 42°C and 45°C treatments, no statistically significant differences between the PET and the dissection experiments were found. With the 47°C treatments, the reduction in uptake in the dissection experiments with ^{14}C -tyr was more pronounced than the reduction as measured by PET using ^{11}C -tyr.

Growth Delay

Tumors with volumes between 4 and 5 ml were heated and subsequently growth curves were measured. In Table

3, the effects of the different hyperthermia treatments on tumor growth are summarized. After the 42°C treatments, no significant growth delays were observed. Growth delays of 0.17 ($p < 0.011$) and 0.18 ($p < 0.016$), respectively, were measured at the 45°C and 47°C treatments.

DISCUSSION

In this report, the possible use of PET to monitor the effect of hyperthermia on tumor growth is described in experiments with rhabdomyosarcoma-bearing Wag/Rij rats.

In these experiments, carboxylic-labeled tyrosine was used to measure amino acid uptake and incorporation into protein of tumor tissue. In the current studies, ^{11}C -tyr was preferred above the more widely used L-[methyl- ^{11}C]methionine. The advantages of ^{11}C -tyr are a higher incorporation into proteins and a lower amount of metabolites (13,26). Moreover, the predominant metabolite $^{11}\text{CO}_2$ is washed rapidly from the tissue into the plasma bicarbonate pool and consequently does not contribute to the amount of ^{11}C -radioactivity measured in the tumor tissue.

From in vitro studies, it is known that hyperthermia reduces the incorporation of amino acids into proteins (8, 9). In our studies with the RMS tumor, this inhibition was also observed. The suppressed incorporation of ^{14}C -tyr was accompanied by a decreased uptake of total ^{14}C radioac-

TABLE 2
Uptake of L-[1- ^{11}C]tyrosine (A) and $^{13}\text{NH}_3$ (B) into Rhabdomyosarcoma Tissue Before and After Hyperthermia as Measured by PET

Thermal dose	Before	(%)	After	(%)	p value	Number
A. 15 min 42°C	1.72 ± 0.11	(100)	1.35 ± 0.12	(79 ± 5)	0.05	n = 6
15 min 45°C	1.63 ± 0.12	(100)	1.17 ± 0.06	(72 ± 3)	0.01	n = 5
15 min 47°C	1.75 ± 0.10	(100)	1.14 ± 0.09	(65 ± 4)	0.002	n = 6
B. 15 min 42°C	1.04 ± 0.11	(100)	1.18 ± 0.14	(104 ± 7)	n.s.	n = 6
15 min 45°C	1.10 ± 0.15	(100)	1.13 ± 0.15	(110 ± 16)	n.s.	n = 5
15 min 47°C	0.93 ± 0.10	(100)	0.99 ± 0.07	(112 ± 12)	n.s.	n = 6

Uptake values expressed as DAR(PET).

Values are mean ± s.e.m.

Percentual amounts between brackets.

Probability values from paired Student's t-test; n.s. = not significant.

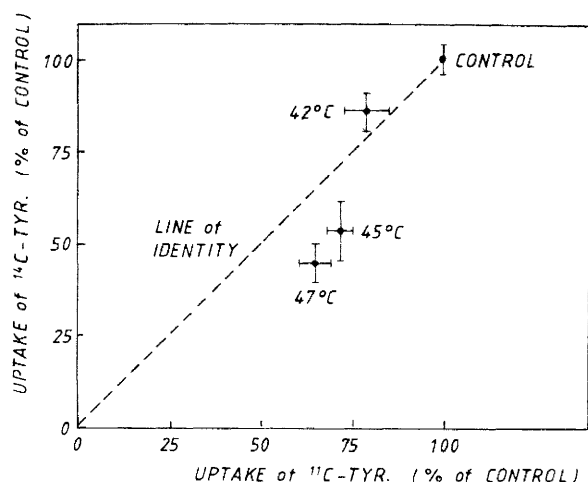


FIGURE 3. Comparison between the PET method (^{11}C -tyr) and the dissection method (^{14}C -tyr) for measuring differences in uptake of radioactivity in tumor tissue as a result of hyperthermic treatment. In both methods, radioactivity was measured in the same period (30–90 min) after hyperthermia. The uptake values after hyperthermia were normalized to the untreated situation (100%).

tivity. In cultured cells, Magun (27) also observed such a decreased amino acid uptake, which could be ascribed to a suppressed demand of amino acid caused by inhibition of protein synthesis and not to a hyperthermia-induced effect on amino acid transport.

The absorbed dose of heat by the tumor is a function of the elevation of tumor temperature and of the duration of this elevation. Panniers and Henshaw (10) observed that immediately after temperature elevation in Ehrlich ascites cells, inhibition of protein synthesis increased steeply over a range of elevated temperatures. Henle and Leeper (28) found a similar result with Chinese Hamster ovary cells. In their study, an increase of the treatment time at the same temperature was accompanied with an augmented reduction in incorporation of amino acid into tumor protein shortly after hyperthermia. These findings are in agreement with our observation that in the 30–90 min period after hyperthermia, the decreased uptake correlated posi-

tively with the increase of temperature and consequently with the thermal dose.

The inhibition of protein synthesis caused by treatment is observed to be reversible. The restoration of protein synthesis in the experiments with ^{14}C -tyr has a time lag which appeared to be dose dependent (Table 1). Studies with cultured cells (10,28) also indicated that the higher thermal dose the longer the period required for restoration of protein synthesis.

In the corresponding PET studies, reduced uptake of ^{11}C -tyr was observed after application of different thermal doses, as was expected from the ^{14}C -tyr studies. The reduction of ^{11}C -tyr uptake was dose-dependent up to a maximum of 35% in the 47°C experiments.

When compared, the PET method and the dissection method gave similar reductions in uptake of radiolabeled tyrosine after hyperthermia at 42°C and 45°C. At the 47°C treatments, the reduction in uptake of ^{11}C -tyr was smaller than the reduction in uptake of ^{14}C -tyr after dissection. One explanation might be that only after hyperthermia at 47°C the RMS became edematous. This hyperthermic effect was also observed by Mooibroek and co-workers (29) who found edema after heating the RMS tumor at 43°C for 120 min. Edema, due to enlargement of the tumor volume, leads to the lowering of the concentration of radioactivity in the tumor tissue. In the ^{11}C -tyr studies, unlike the ^{14}C -tyr studies, this effect was not accounted for. Therefore, the uptake values of ^{11}C -tyr are overestimated in the 47°C experiments, yielding an underestimation of the hyperthermia-induced reduction of amino acid uptake.

In a tumor, after first pass extraction, $^{13}\text{NH}_3$ is trapped via binding to amino acids such as glutamic acid. Within 10 min after injection, the level of ^{13}N -radioactivity in the RMS tumor rapidly reaches a plateau level. This plateau was also found by Schelstraete (17) in a number of tumors of human origin. The effect of hyperthermia on blood flow was extensively reviewed by Reinhold and Endrich (16) who pointed out that hyperthermia, especially above 42°C, might decrease the blood flow in tumors. But not all blood vessels in tumors respond to hyperthermia in the same manner. Using labeled microspheres, after a 60-min treatment at 45°C, Song and co-workers (30) found no alterations of flow in the Walker 256 carcinosarcoma. In our studies with the RMS tumor, hyperthermia did not change tumor blood flow at temperatures between 42°C and 47°C as monitored during 10–20 min after hyperthermia. From these results, it is concluded that shortly after treatment, the hyperthermia-induced reductions in uptake of ^{11}C -tyr are not caused by a change in blood flow.

Reductions on tumor growth were observed after 15 min treatments at 45°C and 47°C, but not at 42°C. These data (Table 3) correspond with results of Zywiets (31) obtained after treatment of a RMS tumor at 43°C for 30 min, where similar delays in tumor growth were observed.

In the 45°C and 47°C treatments, a reduced uptake of

TABLE 3

Effects of Different Thermal Doses on Tumor Growth of the Rhabdomyosarcoma Tumor Represented as Growth Delay Values

Treatment	Growth delay (GD)	p value	Number
15 min at 42°C	0.07 ± 0.14	n.s.	n = 7
15 min at 45°C	0.17 ± 0.04	0.11	n = 6
15 min at 47°C	0.18 ± 0.03	0.016	n = 6

Values are mean \pm s.e.m. For definition of GD see Materials and Methods.

Probability values from Fisher's distribution-free sign test.

n.s. = not significant.

^{11}C -tyr was accompanied by a delay in tumor growth. Therefore, we conclude that the hyperthermia-induced suppression of protein synthesis, as measured by PET, is an appropriate prognostic indicator for the effect of heat on tumor growth.

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