

# Effect of Cancer Cachexia and Amiloride Treatment on the Intracellular Sodium Content in Tissue Cells<sup>1</sup>

Ivan L. Cameron and Keithley E. Hunter

Department of Anatomy, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

## ABSTRACT

This study was designed to investigate the effects of a growing H6 hepatoma on the intracellular element content in three distinctly different tissue cell populations of the mouse host (hepatocytes, fibroblasts, and cryptal enterocytes). X-ray microanalysis measurements of the intranuclear concentrations of several elements (sodium, magnesium, phosphorus, sulfur, chlorine, and potassium) were made. Briefly, the tumor presence significantly increased intranuclear sodium concentration but not the concentration of magnesium, phosphorus, sulfur, chlorine, or potassium in three tissue cell types of mice that were anorectic and cachectic. A second aim of the study was to see if injections of the diuretic amiloride, a drug reported to block passive influx of sodium into mammalian cells, would counteract the effect of the tumor presence and lower the intranuclear concentration of sodium towards that of a non-tumor-bearing host. Amiloride did significantly lower the intranuclear level of sodium in the host tissues to that of non-tumor-bearing mice. The amiloride-caused decrease on intracellular sodium was correlated to a decreased cell proliferation activity in the tumor cells and duodenal enterocytes. A possible relationship between the intracellular concentration of sodium in tissue cells and cancer cachexia is discussed.

## INTRODUCTION

The metabolic characterizations of a tumor-caused wasting of body mass, referred to as cancer cachexia, are incomplete and poorly understood. The TBH<sup>2</sup> often shows a number of paraneoplastic changes which may be involved in cancer cachexia. Such changes include nutritional imbalance, immune system responses, protein metabolism, endocrine functions, water structure, and glucose homeostasis (1, 2, 5, 9-11, 15-17). Such systemic effects are often said to be caused by circulating tumor products, although there appears to be no direct experimental evidence to confirm this theory.

The present investigation was done to test the hypothesis that a tumor might also perturb the intracellular concentration of ions or elements in host tissue cells. This study appears to be the first to investigate the effects of a tumor on the intracellular element content in tissue cell populations from a TBH as compared to a non-tumor-bearing animal. Briefly, the tumor significantly increased intranuclear content of sodium but not of potassium, chlorine, magnesium, or other elements measured in host tissue cells of cachectic and anorectic mice. A second aim of this study was to see if injections of the diuretic

amiloride (a drug reported to block passive influx sodium into a number of mammalian cells) into such TBH mice might counteract the effect of the tumor and change the intranuclear level of sodium towards that of the non-tumor-bearing animal. In brief, amiloride did lower intranuclear sodium in host tissue cells.

## MATERIALS AND METHODS

Male A/J mice were used as tumor hosts and were obtained from The Jackson Laboratory (Bar Harbor, Maine). The H6 mouse hepatoma was routinely propagated by the s.c. injection of a 0.1- to 0.2-ml suspension of cells in lactated Ringer's solution on the right flank. The transplanted hepatoma was not found to metastasize. Amiloride was kindly supplied by Dr. E. Cragoe of Merck Sharp and Dohme (West Point, Pa.) Solutions of the drug were prepared fresh for i.p. injections and were given every 8 hr. To determine how amiloride treatment influenced cell proliferation activity as well as the intranuclear content of sodium and several other elements, the following experiment was run with mice harboring a 1-cm-diameter H6 hepatoma 13 days after tumor cell inoculation. These mice were randomly placed into groups. The tumor-bearing mice in one group were given 3 injections of amiloride made up in lactated Ringer's solution at a concentration of 1.0  $\mu\text{g/g}$  body weight, and each injection was spaced 8 hr apart. The other group of tumor-bearing mice were given the same series of injections of lactated Ringer's solution without amiloride. A third group of non-tumor-bearing mice were also given a similar series of 3 injections of lactated Ringer's solution without amiloride. All 3 groups of mice were also given an injection of tritiated thymidine (specific activity, 6.0 Ci/mmol) at a dose of 1  $\mu\text{Ci/g}$  body weight 3 hr after the last injection of amiloride or Ringer's solution, and all mice were killed by decapitation 1 hr later at noon.

Segments of the liver and the duodenum (taken 1 cm below the pyloric valve) were then processed either for electron probe X-ray microanalysis or for autoradiography.

In preparation for electron probe X-ray microanalysis, tissue was removed and attached to a cylindrical hollow brass tube 3 mm in diameter. The specimen was dissected and frozen by immersion in liquid propane cooled in a liquid nitrogen bath in less than 1 min. The specimen was then transferred to a cryostat (Harris Manufacturing Co., Inc., North Billerica, Mass.) maintained at  $-40^\circ$  (2). Two- $\mu\text{m}$ -thick sections were cut with a Minot custom microtome (Damon/IEC Div., Damon Corp., Needham Heights, Mass.).

Sections 2  $\mu\text{m}$  thick were positioned on a film of Formvar (0.9% in dioxane) spanning a 1.5-mm hole in a 3-mm carbon grid. To minimize curling or movement of the sections, a cover was laid over the sections. The cover consists of a circular piece of heavy aluminum with a carbon-coated Formvar film stretched across a hole in the center. Thus, the section was sandwiched between 2 Formvar films. The sandwiched specimen was dried for 16 hr at  $-40^\circ$  in a custom-made cryoabsorption apparatus, warmed to room temperature, vented with nitrogen gas, and stored in a desiccator. At the time of analysis, the aluminum ring was teased away, leaving a flat dried section sandwiched between 2 layers of Formvar film. The sandwiched sections were examined at 25 kV in a JEOL-35 scanning electron microscope equipped with an Si(Li) X-ray detector (Nuclear Semiconductor, Inc., Menlo Park, Calif.) Spec-

<sup>1</sup> Supported by Grant PCM80484 from the National Science Foundation and Grant CA30956 from the National Cancer Institute.

<sup>2</sup> The abbreviations used are: TBH, tumor-bearing host; ANOVA, analysis of variance; PVP, polyvinyl pyrrolidone.

Received July 26, 1982; accepted November 30, 1982.

imen-to-detector distance was 1.5 cm, and the takeoff angle was 40°. The data were collected, stored, and processed by an NS-880 pulse-height analysis system (Tracor Northern, Middleton, Wis.). Morphology of the specimens was recorded by photographing the transmitted image from the recording cathode ray tube. For data collection, a rapid scan at  $20 \times 10^3$  to  $90 \times 10^3 \times$  was made for 100 sec. The specimen area rastered was 0.27 to 5.50 sq  $\mu\text{m}$ . Specimen current as measured on the beryllium planchet was 0.15 na. Our microanalysis quantitation was based upon the Hall mass fraction technique.

Nuclei of hepatocytes in the liver, duodenal enterocytes in the proliferative zone of the crypts, and fibroblasts in the lamina propria of the duodenum were analyzed for element content. X-ray pulse-height distribution was measured in the energy range of 0 to 10.22 keV with a resolution of 20 eV/channel. The Tracor Northern Super ML (multiple least-squares) program was used to deconvolute the spectra and to calculate elemental peak/continuum ratios for each element in the spectrum. Continuum was arbitrarily designated as 4.50 to 5.00 keV, an energy interval in which no characteristic peaks were generated from the samples. For each of the detectable elements of biological relevance, the 3 groups of data were subjected to the ANOVA statistical test.

Quantification of data was performed with a series of standards prepared like those of Cameron *et al.* (3). Known amounts of dried salts were added to aqueous solutions of PVP (special grade; Aldrich Chemical Co., Milwaukee, Wis.). The standards were prepared to a composition of approximately 20% dry solids, frozen, sectioned to 2  $\mu\text{m}$ , and freeze-dried in the usual procedure of this laboratory. By adjusting the relative amounts of the various salts, a series of standards were prepared in which the total weight of electrolytes was kept nearly constant in all of the PVP standards. Microanalysis was done on PVP sections of 4 different concentrations for a given element and on 20% PVP without added salts. A least-squares linear regression analysis was used to obtain a constant of proportionality value for each element from the peak to continuum data. This constant of proportionality was used to convert peak to continuum values to concentration (mmol/kg dry weight) for each element.

For autoradiography examination, pieces of tissue were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4  $\mu\text{m}$ . The sections were then mounted on glass slides for autoradiography.

To determine the effects of amiloride on proliferation of cells in the duodenal crypts, autoradiographs were prepared from deparaffinized mounted histological sections after washing in methanol:glacial acetic acid (3:1, v/v) followed by washing in distilled water. The slides were processed for autoradiography using NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.). Autoradiographic exposure time was 2 months, and then the slides were developed in Microdol X (Kodak). The developed autoradiographs were stained through the emulsion with hematoxylin and eosin. The number of cells with a labeled nucleus per crypt were scored. Crypts, the lumen of which was visible from the base to the mouth, were scored. A total of 20 such crypts were used to obtain the average per mouse.

## RESULTS

Table 1 summarizes the effects of the rapidly growing H6 tumor on body weight and on food intake. During the 5 days in which they were monitored, the mice without a tumor gained an average of 8.4% and consumed an average of 22.0 g of food. During the same 5 days, the groups of mice with a tumor demonstrated body weight changes ranging from a 1.6% gain to a 7.8% weight loss. Food intake in these same groups of mice ranged from an average of 18.2 to 12.4 g/mouse. A least-squares linear regression analysis of the average food intake over weight change for the 4 groups of mice listed in Table 1 gives a positive slope indicating a 1.51% increase in weight for each additional g of food intake. The goodness of fit

Table 1  
Effect of tumor presence on food intake and body weight changes during the 5 days before amiloride treatment

	Cage	Av. % wt change/ mouse	Av. food intake/ (g)
Mice without tumors	1	8.4 (4) <sup>a</sup>	22.0
Mice with tumors	2	1.6 (4)	18.2
	3	0.6 (4)	14.8
	4	-7.8 (4)	12.4

<sup>a</sup> Numbers in parentheses, number of mice measured to obtain the average.

of these data to the linear model is shown by a 0.95 correlation coefficient. These data show that the mice with tumors have a reduced voluntary food intake (anorexia) which directly related to a percentage of decrease in body weight (cachexia).

Statistical analyses of the data obtained from determining how the tumor and how amiloride treatment influenced intranuclear content of sodium and several other elements in the 3 different tissue cell populations are summarized in Table 2. Table 2 gives the results of a 2-way analysis of variance for each of the 6 elements measured: sodium, potassium, chlorine, magnesium, sulfur, and phosphorus. Two independent experimental variables were arranged in a 3  $\times$  3 factorial design. The first independent variable, type of tissue, consisted of (a) hepatocytes in the liver, (b) duodenal enterocytes in the proliferative zone of the crypts, and (c) fibroblasts in the lamina propria of the duodenum. The second independent variable, type of treatment, consisted of (a) mice without tumors, (b) mice with tumors, and (c) mice with tumors that were given 3 injections of amiloride over the last 24 hr prior to being sacrificed.

The 2-way analysis of variance was used to sort out significant effects in element content due to (a) tissue cell type, (b) treatments (tumor presence or absence and amiloride treatment), and (c) possible interactions between the 2 independent variables (tissue cell type and the treatments).

The analysis revealed that there was a significant main effect of tissue cell type for the concentration of sodium, potassium, chlorine, and phosphorus. Neither magnesium nor sulfur concentrations showed a significant main effect due to tissue cell type. Briefly, this shows that there exist significant differences in sodium, potassium, chlorine, and phosphorus concentration in the 3 tissue cell types measured. Since the main reason for the study was not concerned with differences between tissue cell types, this significant main effect was not pursued further.

The 2-way ANOVA further revealed that there was a significant main effect of treatment without interaction only in the case of intranuclear sodium concentration.

In the case of sodium, in which a significant main effect of treatment was revealed without any interaction, a multiple comparison test procedure is appropriate to determine which treatments (tumor absence or presence or tumor presence plus amiloride treatment) were significantly different from one another across the 3 tissue cell types. Thus, the Student-Newman-Keuls multiple comparison test was applied to the sodium data and revealed that the intranuclear sodium concentration was significantly higher in the tissue cell types of the tumor-bearing mice ( $p < 0.05$ ) than in the tissue cell types of the tumor-bearing mice treated with amiloride and in the non-tumor-bearing mice. The latter 2 groups of mice did not show significant differences in sodium concentration (Chart 1).

Table 2

Effect of 3 injections of amiloride at 1.0 µg/g body weight each on intranuclear element content of 3 tissue cell types in vivo  
 There were 4 mice/treatment group, and 10 cells from each tissue in each mouse were measured to obtain a mean value. These mean values were then subjected to statistical analysis.

	Intranuclear element content (mmol/kg dry wt)			Results of 2-way ANOVA					
	Without tumor	With tumor	With tumor + amiloride	Tissue		Treatment		T <sup>a</sup> × TR	
				F	p	F	p	F	p
<b>Sodium</b>									
Liver	121 ± 15 <sup>b</sup>	204 ± 11	116 ± 16	3.456	<0.05	4.071	<0.05	1.059	NS
Duodenum	174 ± 11	173 ± 18	160 ± 28						
Fibroblast	189 ± 21	223 ± 23	178 ± 28						
Treatment means	154	201	152						
<b>Magnesium</b>				1.680	NS	0.132	NS	0.940	NS
Liver	44 ± 4	49 ± 7	58 ± 7						
Duodenum	60 ± 3	59 ± 3	58 ± 3						
Fibroblast	56 ± 9	56 ± 2	50 ± 3						
Treatment means	52	55	55						
<b>Potassium</b>				12.000	<0.001	1.100	NS	1.240	NS
Liver	375 ± 42	359 ± 50	407 ± 22						
Duodenum	570 ± 16	556 ± 25	485 ± 23						
Fibroblast	518 ± 80	581 ± 33	456 ± 7						
Treatment means	476	499	449						
<b>Sulfur</b>				0.86	NS	1.23	NS	3.67	<0.05
Liver	210 ± 26	215 ± 25	263 ± 7						
Duodenum	234 ± 19	217 ± 4	176 ± 10						
Fibroblast	221 ± 25	251 ± 16	170 ± 5						
Treatment means	220	228	203						
<b>Chlorine</b>				22.820	<0.001	2.010	NS	0.347	NS
Liver	89 ± 11	107 ± 10	74 ± 7						
Duodenum	152 ± 5	153 ± 13	139 ± 10						
Fibroblast	169 ± 24	210 ± 13	174 ± 23						
Treatment means	131	157	129						
<b>Phosphorus</b>				11.760	<0.001	0.62	NS	0.813	NS
Liver	491 ± 49	462 ± 51	528 ± 25						
Duodenum	721 ± 28	648 ± 29	603 ± 24						
Fibroblast	704 ± 120	717 ± 23	642 ± 12						
Treatment means	686	652	591						

<sup>a</sup> T, tissue; TR, treatment; NS, not significant.  
<sup>b</sup> Mean ± S.E.

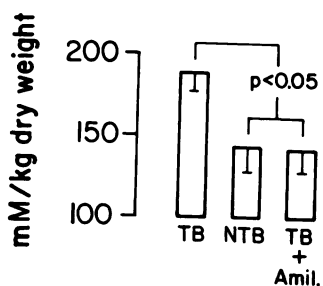


Chart 1. Mean concentration of sodium in 3 tissue cell types of (a) mice with tumors (TB), (b) mice without tumors (NTB), and (c) mice with tumors that were given 3 injections of amiloride (TB + amil.; 1.0 µg/g body weight given every 8 hr). Each value is the mean of 4 mice (10 nuclei measured/mouse). Bars, S.E.

Thus, the tumor caused a significant increase in intranuclear sodium concentration over that of non-tumor-bearing mice, while amiloride treatment of tumor-bearing mice counteracted the effects of the tumor and reduced the intranuclear sodium levels in the tissue cell types to that of the non-tumor-bearing mice.

For completeness, it seemed necessary to investigate the significant interaction between tissue cell type and treatment

which occurred in the 2-way ANOVA of intranuclear sulfur content. This analysis involved comparing each of the 3 tissue cell types for each of the 3 separate treatments. This analysis showed that fibroblasts in mice with tumors responded significantly to amiloride treatment by reducing intranuclear sulfur concentration [*i.e.*, 241 ± 15 (S.E.) versus 163 ± 5 mmol/kg dry weight].

Analysis of cell proliferation activity in the duodenal crypts as measured by tritiated thymidine autoradiography of the number of labeled cells per crypt revealed the following: (a) mice without tumors had 12.05 ± 0.60 labeled cells per crypt section, (b) mice with tumors had 12.52 ± 0.85 labeled cells per crypt section, and (c) mice with tumors that were treated with amiloride had 8.40 ± 0.81 labeled cells per crypt section. One-way ANOVA showed significant differences to exist between the groups of mice, and the Student-Newman-Keuls multiple comparison test showed that the mice with tumors were not significantly different from mice without tumors but that both were significantly different (*p* < 0.02) from the mice with tumors that were treated with amiloride. Thus, amiloride lowered the proliferative activity in the duodenal crypts of the mice with tumors.

## DISCUSSION

The hypothesis that the tumor might perturb the concentration of intracellular ions or elements in host tissue cells is supported by the finding of increased intranuclear sodium in the 3 tissue cell types measured. That sodium alone but no other element measured showed a significant change in concentration implicates sodium as being involved in a perturbed electrochemical state in the 3 host tissue cell types measured. That all 3 tissue cell types measured show the same perturbation of their intranuclear sodium concentration suggests that the tumor had a systemic effect on intracellular sodium levels.

How might the tumor cause an increase in intranuclear sodium in the host tissues? Some authors have put forth the idea that a product of the tumor might in some way cause an alteration of host tissue metabolism. Hypothetically, a tumor product could cause tissue cells to increase their intracellular sodium perhaps by causing a leaky membrane or by changes in the adsorption of sodium to macromolecules within the cell.

Is there evidence that the increased concentration of sodium in tissue cell types has perturbed normal cellular activity? The following discussion indicates that there is some support for this concept. The intracellular and intranuclear concentration of sodium has been implicated as playing a role in both mitogenesis and oncogenesis (4, 12-14). Specifically, it has been shown that the high level of sodium in tumor cells is in some way related to the proliferative activity in tumor cells *in vivo*. We recently found that injections given to tumorous mice with the diuretic amiloride (which is reported to inhibit sodium influx in rapidly dividing mammalian cells) lowered the intranuclear sodium concentration but not the concentration of magnesium, phosphorus, chlorine, or potassium and also lowered the proliferation rate in tumor cells *in vivo* (14). That amiloride acts solely to block the passive influx of sodium into cells has been challenged by indications that it has a direct inhibitory effect on protein synthesis in a reticulocyte lysate system (8) and may also have direct actions to inhibit enzymes such as renal kallikrein (10).

The present study extends the correlative observation between intranuclear sodium and cell proliferation rate to a rapidly dividing normal cell population (duodenal crypt enterocytes). That sodium alone changed in parallel with cell proliferation activity implicates sodium in the ionic regulation of cell proliferation. These observations therefore provide some evidence that the changes in intracellular sodium concentration can cause changes in the normal cellular activity, as exemplified by the correlation between sodium concentration and cell proliferation activity in the duodenal crypts.

Among the possible changes in the TBH are increased energy utilization by the growing tumor and increased energy demands on the host tissues caused by the presence of a tumor. We therefore asked if there is evidence that the tumor has caused significant changes in host metabolism requiring increased expenditures of energy. Burt *et al.* (2) have provided data in a sarcoma-bearing rat model which supports the idea that the increased glucose utilization by the growing tumor forces the host to increase production of glucose to maintain a homeostatic blood glucose level. The increased production of glucose was shown to be both by way of increased gluconeogenesis and by way of increased rate of glucose recycling (1). The gluconeogenesis involved the use of the amino acid

alanine as a precursor to glucose production. The source of the alanine used for gluconeogenesis must be either from food intake or from mobilization of protein reserves in the host.

Norton *et al.* and Stein *et al.* (11, 15-17) have provided evidence to show that the TBH dramatically increases both breakdown and synthesis of whole-body proteins. As Stein points out, protein turnover normally accounts for as much as 40% of the total resting energy expenditure. Thus, the increase in turnover of whole-body proteins as seen in a TBH might help account for the increased energy demands of the TBH. While synthesis of proteins is expected to increase in the growing tumor, the host liver often increases in size, perhaps because of the stimulation of the liver to increase glucose output. The protein turnover in skeletal muscle favors increased catabolism, which in turn provides amino acids like alanine needed for gluconeogenesis to produce glucose for tumor glycolysis (5).

One area of possible energy expenditure which does not appear to have received consideration involves the question of the energy requirements needed to maintain an electrochemical gradient between the intracellular and extracellular environment on the part of host tissue cells. Although there are 2 opposing concepts of the mechanism for maintaining the electrochemical gradients of sodium and potassium and other elements, the more popular theories involve use of the membrane pump to move sodium out of the cell and to move potassium into the cell. On the other hand, adsorption theories require adsorption of electrolytes to cellular macromolecules in various degrees. Both types of theories require energy expenditure. Estimates of the energy requirements needed to operate the pump(s) are not yet agreed upon, but we have seen no estimates of less than 10 to 30% of the total resting energy (6, 7, 18).

Therefore, it is clear that the maintenance of electrochemical gradients of ions in tissue cells requires a considerable expenditure of energy. The possibility that a tumor-caused increase in intracellular sodium in tissue cells of the TBH is in some way related to increased energy demands of the TBH and to the phenomenon of cancer cachexia is at this time purely conjectural. Evidence that the intracellular concentration of an electrolyte like sodium is perturbed in the TBH should not be used as direct evidence that the energy expenditure of the tissue cells in the TBH is modified. Further studies are now needed to test for possible intracellular sodium content and energy expenditure in the cells of the TBH.

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