SHORT NOTE.

FURTHER STUDIES ON THE ROLE OF PARAMAGNETIC ION CONTENTS ON THE NMR RELAXATION TIME, T_1 OF NORMAL TISSUES AND CANCER CELLS

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In 1983 I presented evidence (Ling, 1983) that lower paramagnetic-ion contents significantly contribute to the longer NMR relaxation times, T_1 and T_2, of water protons in malignant cancer cells than in normal tissues (Damadian, 1971, Iijima and Fujii, 1972, Hazelwood et al, 1972, Damadian et al, 1973).

In my 1983 paper, 7 types of normal mouse tissues and 6 strains of pure, maximally deviated cancer cells were dried and then incinerated in a muffle furnace at 600°C. The ashes obtained were dissolved in a volume of 0.15 N HCl equal to 20% of the original weight of the tissues or cells. To this extract was then added enough 17.3% paramagnetic-ion-free ovalbumin solution so that the total volume equaled that of the full volume of the original wet tissue and the final concentration of ovalbumin equaled 10.4%. T_1 and T_2 of each sample were then measured with standard pulsed NMR methods.

The T_1 results from the earlier studies are reproduced here under Column II of Table I. Note that like the T_1's of their normal, unincinerated counterparts (shown here under Column I of Table I), the T_1's of the ash-extracts of cancer cells are also longer than those of the normal tissues.

Incineration removes all organic materials from the cells, leaving ashes made up of heat-resistant ions. Of those ions that are known to be present in living cells and that also have strong influence on the NMR relaxations times of water protons are the paramagnetic ions, notably manganese, copper and iron.

It is well known that part of the iron in the living cells exists in a complexed form. A familiar example is hemoglobin, which makes up 97% of the total intracellular proteins of the mature human erythrocyte. The four iron atoms in a hemoglobin molecule are so well shielded that they have no influence on the NMR relaxation times of the water protons in the surrounding medium. Yet after incineration and dissolution, the shielded iron is liberated as free ferric and ferrous ions, with the full power of causing relaxation of water molecules. For this reason, it would be more reassuring to use a different, perhaps less drastic, way of extracting (only) the free iron and other free paramagnetic ions from the cell and tissues, leaving behind in the tissue residues, shielded paramagnetic ions complexed to proteins.

Preliminary studies led me to adopt two alternative extractives:

Extractive procedure A: Thorough grinding of the isolated tissues or cells in 3 volumes of 10% trichloracetic acid (TCA) with a motor-driven all glass homogenizer, was followed by heating in a boiling water bath for 20 minutes. Evaporation loss was made up with ion-free distilled water. After cooling and closing tightly the open top with parafilm, the homogenizer tube (which was constructed from a heavy walled 15 ml graduated

centrifuge tube) and its content were centrifuged at about 1000 g for 5 minutes. Aliquots of the clear supernatant were then transferred to NMR tubes for T_1 measurements.

Extractive procedure B: essentially the same as Extractive procedure A except that the final concentration of TCA in the extractive-tissue mixture is higher (ca. 1.26 M) and it also contains ethylenediaminetetracetic acid (EDTA) at a final concentration of 68 mM. In preliminary trials, Extractive procedure B extracted more NMR-active materials from certain tissues such as the liver. However, the liver contains a variety of complexed forms of paramagnetic ions. Until we know a great deal more about the concentration, nature and responses to the extractive procedures of each one of these complexed paramagnetic ions, whether or not this apparent greater effectiveness in Extraction procedure B is truly an advantage is open to questions.

Nonetheless, I did show that Extractive procedure B does not liberate a significant amount of free iron from commercially available bovine hemoglobin (ca. 75% methemoglobin and 25% oxyhemoglobin), myoglobin and cytochrome C. As Table II shows, extraction of these proteins with Extractive procedure B liberates no more than 0.1% of the iron in the protein-bound iron, as judged from the shortening of T_1 of the extracts (in comparison with the extractive alone) ob-

TABLE I. T_1's of water protons in fresh normal mouse tissues and fresh cancer cells, in comparison with T_1's of water protons in aqueous extracts of similar tissues and cells obtained by ashing and by TCA-EDTA extraction. Water proton NMR relaxation times, T_1's were measured with a coherent CPS-2 NMR pulse spectrophotometer (Spin Lock, Ltd., Port Credit, Canada) operating at a resonance frequency of 17.1 MHz. T_1 was determined with $180° - \tau - 90°$ pulse sequence. All the cancer cells studied were obtained in the pure ascites form, (carried mostly in ICR mice) after centrifugal separation from the ascites fluids. Data on fresh normal mouse tissues and fresh cancer cells under Column I were partly from Ling and Tucker (1980) and partly from unpublished data. Data on extracts obtained by dry ashing were taken from Ling (1983). Data of T_1 of TCA-EDTA extracts are new and obtained by procedures described in the text. Each average was obtained from at least 4 independent determinations.

		I In Vivo	II Ashes in 10.4% Ovalbumin Solution	III TCA-EDTA Extract
Normal Tissue	Brain	693 ± 12.5	441 ± 7	1773 ± 16.9
	Heart	632 ± 30	93 ± 3	1130 ± 29.4
	Intestine	487 ± 20		
	Kidney	524 ± 7.5	176 ± 2	1310 ± 11.8
	Liver	396 ± 12	109 ± 3	420 ± 10.2
	Lung	607 ± 27	91 ± 3	1477 ± 12.2
	Muscle	552 ± 16	550 ± 4	1770 ± 18
	Spleen	641 ± 39	49.5 ± 1.0	540 ± 5.9
Cancer Cells	Ehrlich	815 ± 7.07	910 ± 17	2024 ± 23.8
	Hepatoma 134		853 ± 18	
	LSA		710 ± 17	2090 ± 33.4
	Meth A	805 ± 10.4	938 ± 10	2063 ± 20.3
	P-815		950 ± 10	
	Sarcoma 180	803 ± 15.5	970 ± 20	2120 ± 14.4
r			+0.77 (I, II)	+0.75 (I/III)

served. This amount of artificially generated free paramagnetic iron will not affect the results.

Assuming that the TCA-EDTA mixture does not liberate significant amounts of shielded paramagnetic ions, I applied the B extractive procedure to the extraction of 7 normal mouse tissues and 4 maximally deviated mouse cancer cells. The T_1 of their extracts observed are listed under Column **III** of Table I.

As in the case of the T_1 obtained by the ashing procedure, the T_1's of all the cancer cell extracts are also consistently longer than those from all the normal tissues studied. This observation is in full harmony with my earlier results from ashed tissues and cells. It is also in agreement with an earlier contention that lower paramagnetic ion contents of cancer cells is one of the major factors that produces the longer T_1 seen in maximally deviated cancer cells than in normal tissues (Ling and Tucker, 1980).

In this and the preceding paper (Ling, 1983), emphasis has been placed on the comparison of the NMR relaxation times of normal tissues and cancer cells each as a group and on the fact that the normal-tissue group has shorter T_1 (and T_2) than in the group of cancer cells. Now, we want to take another look at the two sets of data and see what kind of correlation exists between the T_1 of the normal tissues and cancer cells and their counterparts either ashed or extracted in TCA-EDTA.

To achieve this objective, I compared the normal in-vivo T_1 data (Table I, Column I) with the ashing produced data (Table I, Column II) and obtained a linear correlation coefficient, r, equal to $+0.77$. A similar comparison of the normal in-vivo data (Column I) with the TCA-EDTA produced data (Table I, Column III) yielded a linear correlation coefficient, r, of $+0.75$.

These positive linear correlation coefficients clearly point out that variation in paramagnetic-ion content is definitely a highly significant factor that determines not only the differences in the NMR relaxation times of normal versus cancer cells, it also determines the differences in the NMR relaxation times of water protons in different normal tissues.

Finally, one may roughly estimate the quantitative contribution of the paramagnetic ion content in the T_1 differences observed among normal as well as cancer cells.

It is well known that the square of the linear correlation coefficients, r^2, may be described approximately as the estimated proportion of the variable (T_1) that can be attributed to its linear regression on the second variable (i.e., paramagnetic ion contents) (see Snedecor and Cochran, 1980, p.

TABLE II. Percentage of iron liberated from shielded iron in three iron-containing proteins: hemoglobin, myoglobin and cytochrome C, after a 20-minute extraction in a mixture of TCA (final concentration, 1.26 M) and EDTA (final concentration, 68 mM) kept in a boiling water bath. Maximum Fe liberated was estimated from the T_1 measures of the extractives and a standard curve constructed from various TCA-EDTA extractives containing known concentrations of free Fe.

	Concentration of Protein (%)	Concentration of Total Fe (mM)	Estimated T_1 of Extracts (msec)	Maximum Fe in Extracts (mM)
hemoglobin	9.12	1.16	1937 ± 38	$< .001$
myoglobin	3.04	0.39	2030 ± 31	$< .001$
cytochrome C	2.77	0.46	1987 ± 25	$< .001$

181). Therefore as high as $(0.75)^2$ = 56% to $(0.77)^2$ = 59%, or more than half of the T_1 variation seen can be attributed to the variation of the paramagnetic-ion content.

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