were used for assays of total enzyme activity. To solubilize NAD-dependent aldehyde dehydrogenase from mitochondria, sonication, freezing and thawing, and treatment with detergent (sodium deoxycholate) were found to be equally effective. Sodium deoxycholate was chosen by us because it gave clear solutions for spectrophotometric assay.
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Phosphorus-31 as a Nuclear Probe for Malignant Tumors

Abstract. *Phosphorus-31 may prove useful as an additional nucleus for detecting malignancy by nuclear magnetic resonance. The spin-lattice relaxation times for phosphorus-31 determined by a saturation technique employing a 90°-τ-90°-τ-90° . . . pulse sequence were significantly higher for two rat malignancies, Novikoff hepatoma and Walker sarcoma, and the Crocker sarcoma of mice than for normal liver, muscle, brain, kidney, and intestine tissues. No individual measurement of malignant tissues overlapped any of the measurements of normal tissues, and the probabilities of insignificance ranged from .029 for Crocker sarcoma to .000184 for Novikoff hepatoma. The phosphorus-31 nucleus, because of its strategic placement in the nucleic acid molecule, may be useful as a new probe for exploring the mechanism of carcinogenesis. The results call attention to another nucleus that may prove valuable for nuclear magnetic resonance equipment aimed at the detection of internal malignancies in humans.*

Damadian and co-workers (*1*) suggested that nuclear magnetic resonance (NMR) might prove useful in the detection of internal cancers. Some experiments with animals provided evidence that it might be possible to make a noninvasive discrimination between normal and malignant tissue by this method. These authors also suggested that NMR might be used to diagnose and even to measure the extent of malignancy in surgical biopsy specimens. The initial observation relied mainly on the altered water signal of malignant tissue, and the technique has since been confirmed and refined at many laboratories, where new insights have been contributed (*2, 3*).

It seemed that both objectives, detection of internal tumors and tissue diagnosis, could best be served by the addition of other magnetic nuclei for diagnosing malignancy. Damadian and Cope (*4*) studied ^{39}K resonance in normal and malignant tissues, but did not find any significant differences in spin-lattice relaxation time, T_1. However, they did observe an oscillation in the T_1 plot for malignant tissue and for normal intestinal tissue; such oscillations had not previously been seen with biological tissue. In this report we discuss an investigation of ^{31}P as another possible nuclear probe of malignancy.

Because of its strategic placement in the nucleic acid molecule, ^{31}P seemed a particularly useful nucleus with which to probe malignancy. Boyd (*5*), in his *Textbook of Pathology*, has written that "the chief characteristics of the neoplastic cell as revealed by the light microscope are nuclear and chromosomal aberrations." Tumor cells characteristically have polyploid nuclei and elevated mitotic rates, and these nuclear changes permit the microscopic diagnosis of malignancy. We considered that the DNA changes implicit in these chromosomal aberrations might be reflected in the ^{31}P relaxations of the malignant cell.

Phosphorus-31 resonance has been used in determining the structure of relatively pure compounds that can be isolated in significant quantity (*6*). For NMR studies of biological molecules it has the advantages of large chemical shifts and narrow linewidths. The reduced NMR sensitivity of phosphorus compared to hydrogen and the poor solubility of many organic phosphorus compounds has limited its use. The use of ^{31}P in the NMR analysis of a biological tissue was reported by Moon and Richards (*7*) for red blood cells, where it was introduced as a new technique for monitoring intracellular *p*H. Kornberg and McConnell (*8*) and Berden *et al.* (*9*) used ^{31}P NMR to study phospholipid structure in membrane vesicles. To the best of our knowledge, it has not been applied to organ tissues where its placement in the nucleic acid molecule could prove useful.

Sprague-Dawley rats were used as the source of all normal and malignant tissue, with the exception of the Crocker sarcoma 180 of mice. Rats were killed by cervical dislocation; the desired tissue was then quickly dissected out of the animal, blotted, and placed in a test tube on ice. After 15 minutes at ice temperature, the chilled tissue was cut into small pieces and packed into NMR tubes (outer diameter, 5 mm), which were stored in crushed ice.

The NMR probe was maintained at a temperature of 7° ± 1°C to minimize tissue degeneration during the analysis. This was accomplished by circulating a stream of cold nitrogen gas through the probe at a rate of 12 cubic feet per hour (0.34 m^3/hour). The temperature of the probe was monitored continually by two thermistors, one over the probe body and one at the nitrogen outflow port of the probe. Temperatures of both thermistors were maintained within 0.5°C of each other.

All relaxation measurements were made with a Nuclear Magnetic Resonance Specialties Corporation PS-60 AW pulse spectrometer operating at 100 Mhz, a high Q probe at this frequency (SEIMCO, New Kensington, Pa.), and a Westinghouse superconducting magnet operating at 58,000 gauss. Because of the low concentrations of phosphorus in tissue samples, it was necessary to make use of a computer of average transients (Fabri-Tek Instruments, model 1072) for signal enhancement.

Spin-lattice relaxation times (T_1) were measured by the method of progressive saturation (*10*), in which the incident radiation consists of a train of 90° pulses at the resonant frequency. The pulses have the same amplitude and duration and are adjusted so that the height of the free induction decay (FID) obtained is maximal and corresponds to a rotation of the net magnetization from the Z-axis into the X-Y plane. Once the magnetization has been rotated, it tends to return to its equilibrium along the Z-axis at a rate proportional to T_1, in general taking approximately $5T_1$ to return to equilibrium. A second pulse at this time will produce the same height of the FID. If the time between pulses, τ, is less than T_1, the height of the FID, h, will be less than maximal and will diminish to a constant value in the course of the saturation train in accordance with the relation

$$h = h_0(1 - e^{-\tau/T_1})$$

where h_0 is the height of the FID for $\tau \geq 5T_1$. Therefore, when $\ln[(h_0 - h)/h_0]$ is plotted against τ, a straight line with a slope of $-1/T_1$ is obtained.

This method is more efficient for measuring T_1, particularly for samples where many repetitions of the experiment are required before a usable signal is obtained. In the more conventional methods using pulse sequences of 180°-τ-90° or 90°-τ-90°, it is necessary to wait for a time longer than $5T_1$ (10 to 25 seconds for tissue phosphorus) before a second pulse can be given. In addition, prior knowledge of T_1 is not required in the progressive saturation method, since τ is simply increased until h reaches a maximum, thereby specifying h_0.

We chose values of τ in the range of $0.1T_1$ to T_1—that is, 0.26 to 4.2 seconds for normal tissues and 0.524 to 8.4 seconds for malignant tissues. From 2^9 to 2^{12} repetitions were required in the signal averager

Table 1. Spin-lattice relaxation times for normal and malignant tissues. For each type of tissue the mean ± standard error of the mean is given. Probability P_A is a test of significance in the means between the malignant tissue and the most appropriate normal tissue for comparison; P_B is a test of significance between the malignant tissue and the normal tissue of highest T_1.

T_1 (seconds)							
Normal tissues					Tumors		
Liver	Muscle	Brain	Kidney	Intestine	Novikoff hepatoma	Walker sarcoma 256	Sarcoma 180
2.05	2.09	1.23	1.03	2.14	8.35	5.23	3.23
2.73	2.13	1.04	1.38	1.72	4.29	7.93	9.39
2.54	2.16	1.20	1.58	1.97	4.35	4.82	3.78
2.37	2.40	1.04	1.74	2.33	5.40	5.04	4.35
1.97	2.17			1.69	6.07	3.90	
					5.94		
					7.43		
2.33	2.19	1.13	1.43	1.97	5.98	5.38	5.19
±0.14	±0.05	±0.05	±0.15	±0.12	±0.57	±0.68	±1.42
	Comparison normal mean				2.33 ±0.14	2.19 ±0.05	
				P_A	.000184	.000762	
				P_B	.000184	.00112	.0286

for a given τ to produce an FID with a sufficient signal-to-noise ratio to be usable. The value of h_0 was obtained from the height of the FID for $\tau \geq 4T_1$ for normal tissues and $\geq 3T_1$ for malignant tissue. This observed h_0 is 98 percent of the true h_0 for normal tissue and 95 percent of the true h_0 for tumor tissue. A small error in the T_1 values is introduced by this tendency for T_1 to appear shorter than it is, but the error is greater in malignant than in normal tissues; that is, tumor T_1 is shortened more by this technique than normal tissue T_1. Two tissues, brain and Walker sarcoma, did not have a single exponential curve, but a curve that was better approximated by two exponentials. In these cases we assumed that two observable nonexchanging fractions with different T_1's were present and compared the T_1's of the major fractions of tumor and normal tissue.

The data, summarized in Table 1, show that the T_1's for Novikoff hepatoma (5.98 ± 0.57 seconds), Walker sarcoma (5.38 ± 0.68 seconds), and sarcoma 180 of the mouse (5.19 ± 1.42 seconds) are significantly longer than for any of the normal tissues measured. No T_1 for any individual tumor was measured that overlapped any of the individual T_1 determinations for normal tissue. Most importantly, the values for the tumors were significantly different from those of the respective normal tissues—for example, liver (2.33 ± 0.14 seconds) compared to Novikoff hepatoma (5.98 ± 0.57 seconds) and muscle (2.19 ± 0.05 seconds) as compared to the Walker sarcoma (5.38 ± 0.68 seconds) (muscle is the closest normal tissue to the Walker sarcoma, for which an exact comparison tissue does not exist). These differences correspond to probabilities of insignificance between the means for normal and malignant tissues of .000183 for Novikoff hepatoma and .000762 for Walker sarcoma. A more rigorous comparison of the Walker sarcoma would match its T_1 against the highest T_1 available from normal tissue. It was more than twice as long.

When each tumor group was compared with the normal tissue of longest T_1 (liver) the probabilities of insignificance were .000184, .00112, and .0286 for Novikoff hepatoma, Walker sarcoma, and sarcoma 180, respectively. The distinction between these normal and malignant tissues appears to be more pronounced with ^{31}P NMR than with proton (*1–3*) or ^{39}K NMR (*4*).

We have considered the possibility that the differences in ^{31}P relaxation in tumor and normal tissue may reflect alterations in cellular DNA or nucleic acid in general. The loss of oxidative respiration in malignant tissue was pointed out by Warburg more than 40 years ago (*11*). The role of phosphorus in biological oxidation and reduction reactions is well known, and significant impairment of oxidative phosphorylation may also be reflected by our ^{31}P NMR measurements. Cancer cells also have surface peculiarities, such as loss of contact inhibition. Perhaps alterations in membrane phospholipids contribute to the changes in ^{31}P relaxation. Finally, differences in ^{31}P relaxation may be simply the result of significant differences in the ^{31}P concentrations of malignant and normal tissues. Chemical determinations of ^{31}P in the three tumors and the normal tissues of this study indicate that the phosphorus content per gram of dried tissue is significantly elevated above normal in tumors, while the phosphorus concentration is not materially altered.

From our data, it appears that the phosphorus nucleus may prove to be a sensitive probe of malignancy, perhaps more sensitive than either protons or potassium. It could be useful in the future for the diagnosis of malignancy in surgical specimens and in the NMR detection of internal neoplasms. Moreover, ^{31}P and ^{39}K resonance data could be used to resolve ambiguous diagnoses based on proton resonance data alone. Ambiguities would be minimized by use of a multinuclear combined malignancy index (CMI) of the form

$$\mathrm{CMI} = T_1(^1\mathrm{H}) \times OC(^{39}\mathrm{K}) \times T_1(^{31}\mathrm{P}) \times T_1(i)$$

where OC is the oscillation coefficient for ^{39}K (*2*) and i indicates other nuclei that may prove useful. The commercial availability of NMR instruments with variable frequency (SEIMCO) makes this a practical objective. Finally, the presence of phosphorus in a number of strategic molecular locations within the cell suggests its use as a new probe for exploring the mechanism of carcinogenesis.

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Tail Pinch Induces Eating in Sated Rats Which Appears to Depend on Nigrostriatal Dopamine

Abstract. *Mild tail pinch reliably and rapidly induced eating, gnawing, or licking behavior in all animals tested. Eating was by far the predominant response. Pharmacological analysis of the involvement of the brain catecholamines in tail-pinch behavior suggests that it is critically dependent on the nigrostriatal dopamine system.*

Studies in recent years have demonstrated that peripherally applied stressful stimulation, for example, electric shock to various parts of a rat's body, can reliably induce aggression or copulatory behavior, or both, depending on stimulus conditions (*1*). We report here that a similar stimulus, such as tail pinch, induces eating in sated rats when food pellets are present. Gnawing and licking are also observed, but these behaviors occur much less frequently than does eating.

Eating induced by mild tail pinch typically appears to be identical to normal eating. Immediately after pressure is applied to the tail, the animal begins to sniff and explore its environment for a few seconds. A food pellet is then picked up and held between the forepaws, and the animal begins to bite the pellet and chew. During a sustained pinch, animals pause and swallow quite normally between bites, and eating behavior is almost invariably maintained for the duration of the pinch. Relatively little spillage of food is seen in the tail pinch situation as the animals typically show unhurried consumption of a single pellet. In some animals, however, the pinch appears to represent a more stressful stimulus (as indicated by vocalization), and these animals often move from pellet to pellet and may spill or shred some of the food.

Tail pinch–induced consummatory behavior is an exceptionally reliable phenomenon, having been rapidly and repeatedly demonstrated in every animal in this study. This behavior does not appear to be causally dependent on the activation of pain mechanisms, as it can be reliably induced by applying a pinch of minimal intensity which does not produce vocalization. Since the brain catecholamines, norepinephrine (NE), and dopamine (DA) have frequently been linked to the control of eating (*2*), we examined their respective roles relative to tail pinch–induced eating. Our findings indicate that eating, as well as the other consummatory behaviors that we observed during tail pinch, is critically dependent on brain DA.

Male albino rats (weighing 250 to 350 g) were purchased from a number of suppliers (Marland Farms, Blue Spruce, Holtzman, and Zivic-Miller) to ensure that the tail-pinch phenomenon was not restricted to specific breeding conditions or populations. Rats were housed in pairs and maintained on a natural day-night cycle with food and water freely available. Testing was done during the daytime in shallow bowls 34.3 to 44.5 cm in diameter; each bowl contained six to ten pellets of Purina rat chow. A surgical hemostat, 25 cm long and insulated at the tips with foam rubber, was used for tail pinch. Testing consisted of five 20-second, predrug screening trials, each separated by 5 to 8 minutes, and, after an appropriate interval, five trials after the drug (or vehicle) treatment. All animals demonstrated eating, gnawing, or licking (hereafter referred to as tail-pinch behavior) within 20 seconds on 98 to 100 percent of both predrug and vehicle trials. All statistical comparisons are between drugs and vehicles.

Our initial experiment sought to determine the effects on tail-pinch behavior of pharmacological blockade of both brain NE and brain DA receptors. Haloperidol was chosen for this condition as it is known to antagonize both NE and DA receptors at moderate doses (*3, 4*). Doses of 0.2 and 0.4 mg/kg significantly blocked tail-pinch behavior on 44 and 52 percent of the total trials, respectively (Table 1); a dose of 0.1 mg/kg was ineffective in preventing this behavior. The blocking effect could not be attributed to nonspecific debilitation since the animals vocalized and moved about the testing chambers in an alert manner. During those trials in which tail-pinch behavior was initiated, it was typically maintained until the hemostat was removed. Nevertheless, animals treated with drugs at all doses showed significantly longer latencies to begin tail-pinch behavior than those receiving the vehicle (vehicle median, 2 seconds; 0.1 mg/kg median, 4 seconds; 0.2 mg/kg median, 10 seconds; 0.4 mg/kg median, 7.5 seconds; $P < .0005$ in all cases, U-test).

Since the results with haloperidol suggested catecholamine involvement in tail-pinch behavior, we attempted to parcel out effects that may have been due primarily to the action of NE or DA. We proceeded first by measuring the effect of the specific DA-receptor blocking agents, spiroperidol and pimozide (*3*), on tail-pinch behavior. Each of these agents significantly blocked the display of this behavior (spiroperidol: $F = 14.35$; d.f. = 3,19; $P < .01$; pimozide: $F = 4.09$; d.f. = 3,17; $P < .05$). As shown in Table 1, spiroperidol significantly blocked tail pinch–induced consummatory behavior on 50 percent of all trials at a dose of 0.125 mg/kg and virtually eliminated tail-pinch behavior at a dose of 0.25 mg/kg. The 0.062 mg/kg dose was without effect.

Pimozide, at 1 and 2 mg/kg, significantly reduced the display of tail-pinch behavior to 60 percent of the trials, whereas a dose of 0.5 mg/kg had no significant blocking action. During those trials in which tail-pinch behavior did occur, latencies were significantly extended after 0.125 mg of spiroperidol per kilogram (vehicle median, 3 seconds; 0.125 mg/kg median, 17 seconds; $P < .0005$, U-test) and all doses of pimozide (vehicle median, 2 seconds; 0.5 mg/kg median, 5 seconds; 1 mg/kg median, 12 seconds; 2 mg/kg median, 8 seconds; $P < .0005$ in all cases, U-test).

Spiroperidol and pimozide produced a moderate degree of ptosis in most, but not all, animals tested. There was no correlation, however, between the appearance of ptosis and blockade of tail-pinch behavior. Discontinuous lurching movements, observed with each drug, were more pronounced after spiroperidol and may be related to the extrapyramidal side effects ob-