AMP from adenosine triphosphate (*12*). Exposure of enzyme to the fresh gas phase of cigarette smoke resulted in responses similar to these. That is, when smoke was present, either Mn^{2+} or Mg^{2+} was effective as the sole cation cofactor. Also guanylate cyclase from the supernatant fraction of lung homogenates catalyzed the formation of cyclic AMP as well as cyclic GMP in the presence of the appropriate nucleotide substrate and 4 m*M* Mn^{2+} (56 and 818 pmole per milligram of protein per minute, respectively).

Our results thus suggest that the fresh gas phase of cigarette smoke activates guanylate cyclase and increases tissue levels of cyclic GMP by a mechanism involving the NO in cigarette smoke. However, we cannot rule out the contribution of other nonfilterable components in the gas phase of cigarette smoke to the stimulation of guanylate cyclase.

In view of the emerging role of cyclic GMP in tissue proliferation, and the recent evidence suggesting that the tumorigenic agents 1-methyl-1-nitrosourea and nitrosamines activate guanylate cyclase (*3*), it is possible that NO in cigarette smoke may be in some way related to the suspected carcinogenicity of cigarette smoking. Furthermore, some of the effects of cigarette smoke on ciliary function and mucosal secretion in lung may in part be attributable to cyclic nucleotide metabolism (*13*).

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Free Radical Increases in Cancer: Evidence That There Is Not a Real Increase

Abstract. *The controversial finding of an increase in free radicals with the development of cancer was restudied with Walker 256 carcinosarcoma cells. It was confirmed that such an increase appears to occur, but it was also demonstrated that it is not a real increase. With growth of the tumor, the electron spin resonance lines for lyophilized samples became narrower, resulting in an increase in peak-to-peak height measurements, but there was no change in the total number of spins. The signals for lyophilized tumor samples were different from those for the same samples before lyophilization. Changes in line shape indicated that lyophilized tumor samples contain a different mixture of radicals than lyophilized samples of normal tissue.*

There have been conflicting reports on the free radical content of tissues during the development or growth of malignant tumors (*1*). In a number of studies, usually with lyophilized samples (*2*, *3*), an increase in free radical content was found during the early phases of tumor growth, with a subsequent fall in concentration as the tumors become large. Studies in which tumor tissues were frozen but not lyophilized indicated that free radical concentrations decrease during tumor growth (*4*). It is important to resolve this conflict because several hypotheses, including some suggested new treatment schemes, associate free radicals with cancer, partly on the basis of increased free radical contents during tumor growth.

To our knowledge, no studies have been reported in which both methods of sample preparation were used for the

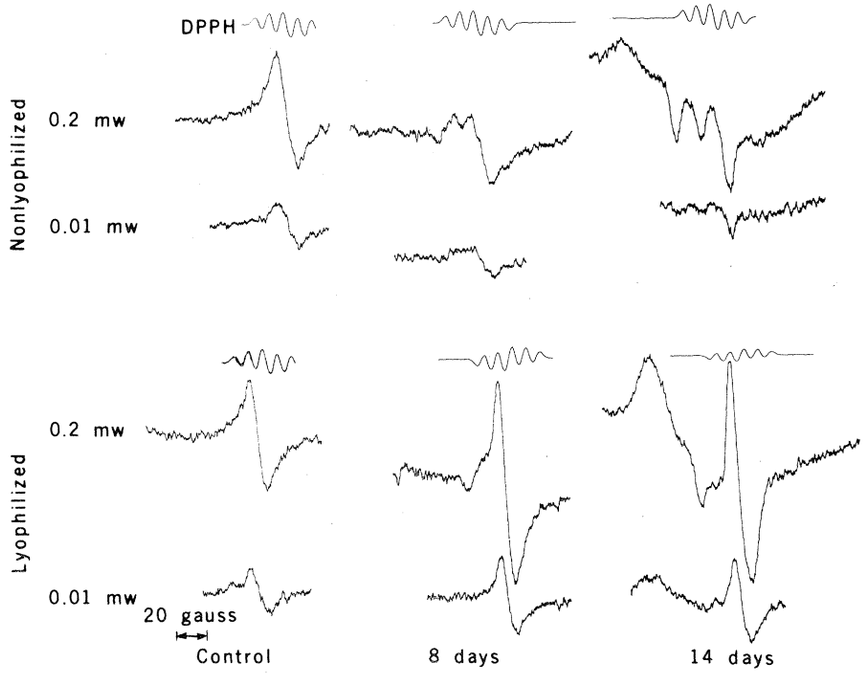

Fig. 1. Representative ESR spectra of nonlyophilized and lyophilized samples. Conditions: temperature, 77°K; incident microwave power levels to a TE_{104} cavity in a Varian E-9 spectrometer, as indicated; frequency, 9.1 Ghz; modulation, 8 gauss at 100 khz; relative signal amplitudes, all the same; the spectra of the DPPH (α,α-diphenyl-β-picrylhydrazyl) standard (g = 2.0036) indicate the g-factor. Spectra are shown for samples taken at 0, 8, and 14 days after inoculation with 10^4 Walker tumor cells, before and after lyophilization. After lyophilization the samples retained the same configuration as in the nonlyophilized condition and were transferred to liquid nitrogen with minimal exposure to air. Care was taken in the lyophilization procedure to avoid artifacts that could appear in this type of preparation (*11*).

same samples. Such an experiment could help resolve the apparent discrepancy between previous results and permit an evaluation of the significance and usefulness of these findings. We report here the results of such a study.

We used the Walker 256 carcinoma because it was one of the tumors for which a dramatic increase in free radical content was observed during the early phases of tumor development (*2*). The method described in the legend of Fig. 1 was developed in order to study the sample in the same configuration, first as a frozen specimen and then after lyophilization (*5*). The tumor line was carried intraperitoneally in female Sprague-Dawley rats (*6*). Experimental tumors were obtained by injecting 10^4 cells (the minimum number that would give 100 percent takes of tumor) into the lateral thigh muscles of the animals. This was the site used in the original electron spin resonance (ESR) experiments with this tumor (*2*). Control samples were taken from the same area on the other rear leg of the tumor-bearing animals and from similar areas of uninjected animals, animals injected with Earle's balanced salt solution, and animals injected with heat-inactivated tumor cells. All controls gave similar results.

Figure 1 shows typical first-derivative, X-band spectra of tumors at 77°K at various intervals after the intramuscular injection of 10^4 cells. At low power levels (0.01 mw) the *g*-values did not change as the tumors developed. At high power, higher apparent *g*-values were observed and the line shapes often differed from those observed at low power. The line shapes at high powers varied from sample to sample because of the superimposition of variable amounts of additional, less saturable signals (*7*). When there were large amounts of nonsaturating components, the line shapes often resembled those obtained with an NO-heme complex (*8*). A nonheme complex of iron, sulfur, and nitrogen oxide may also have been present; such a signal has been observed for a number of tumors (*1, 9*). Although nonsaturating components were sometimes observed at low power levels, in most samples a signal undistorted by these components could be delineated at 0.01 mw. This is the signal and power level we used for the quantitative measurements. The microwave power required to reduce the signal to half what it would have been in the absence of saturation was 0.4 mw. The shape and power saturation characteristics of this signal are typical for free radicals in biological systems at 77°K (*7*).

The signal intensities (as measured by peak-to-peak heights) of the spectra of tumor and control tissues for 30 days after injection of tumor cells are shown in Fig. 2. While the signal intensities for frozen samples gradually decreased as the tumors grew, when the same samples were lyophilized the intensities apparently increased, as previously reported in the literature. [We did not observe a later decrease in the signals from the lyophilized samples, in contrast to the previous reports (*2*).] The line widths observed with tumor samples, however, were significantly narrower than those from control samples. This makes the use of simple peak-to-peak height measurements invalid for these studies. Double integration or an equivalent procedure is needed to quantitatively compare samples with different line shapes. Using the method of Andrew (*10*), we manually integrated representative undistorted spectra at 0.01 mw and obtained the results summarized in Table 1 (lyophilized samples). These results indicate that the apparent increase in signal heights observed with lyophilized tumor samples is actually only a reflection of narrower line widths. Within the limits of our experimental method, the total number of spins in lyophilized samples was constant during the entire experiment and was the same in both tumor and control samples.

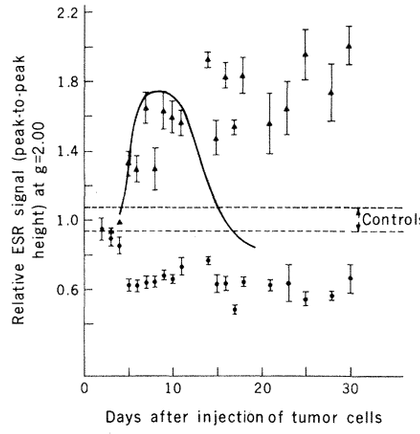

Fig. 2. Peak-to-peak height measurements for nonlyophilized and lyophilized Walker tumors at 77°K. The ESR conditions were as described for Fig. 1 except that the power level was 0.01 mw. Each point is the mean value of data from five or six animals; error bars show the standard errors of the means. Similar results were obtained in two other experiments. (•) Nonlyophilized samples; (▲) lyophilized samples. The solid line is the pattern reported for a similar study of lyophilized samples by Saprin *et al.* (*2*).

We reexamined our data for the frozen samples by performing similar integrations (Table 1, nonlyophilized samples). We did this because, although the line widths did not change, line shape changes could have occurred, which would make peak-to-peak height analyses invalid for these samples also. The results, however, were consistent with the overall trends calculated from peak-to-peak height measurements, although there was no simple correlation between the two types of measurements.

These results help to explain some of the conflicting results reported by different groups, although they also raise new questions. For lyophilized tissues, the increase in free radicals as tumors develop has been shown to be only ap-

Table 1. Comparison of peak-to-peak heights of ESR signals with integrated signal intensities for Walker 256 tumors. All integrations were performed with spectra taken at 0.01 mw and 77°K for which there was no obvious distortion of the line shapes by additional components. Numbers in parentheses are standard errors of the means; *N* is the number of samples.

Day after injection	*N*	Linewidth (gauss)	Peak-to-peak height (arbitrary units)	Integrated intensity ($\times 10^2$)
		Lyophilized samples		
0*	12	13.1 (0.2)	14.4 (1.0)	48 (1.6)
6	2	10.5 (0.4)	20.2 (0.8)	52 (10)
7	3	9.6 (0.4)	24.4 (0.2)	42 (3)
9	3	10.3 (0.9)	28.4 (3.8)	48 (9)
10	3	10.0 (0.3)	24.9 (1.9)	42 (0.6)
15	4	10.3 (0.3)	24.9 (1.1)	41 (3)
17	3	9.8 (0.2)	24.7 (1.0)	41 (4)
21	3	9.7 (0.3)	21.8 (1.5)	40 (1)
28	2	9.5 (0.4)	26.9 (0.8)	58 (3)
30	2	9.0 (0.0)	33.7 (1.4)	48 (0.5)
		Nonlyophilized samples		
0*	9	13.6 (0.2)	13.5 (0.2)	34 (4)
2	3	13.0 (0.6)	13.9 (1.1)	42 (4)
6	3	13.3 (0.3)	8.6 (0.6)	25 (7)
15	3	13.3 (0.3)	8.4 (0.6)	18 (6)
17	3	13.3 (0.3)	6.6 (0.5)	12 (1)
28	3	14.0 (0.6)	8.3 (0.4)	35 (4)

*Controls.

parent. The magnitudes of the signals from frozen and lyophilized samples are initially about the same, but as the tumors develop the number of spins decreases in the frozen samples and remains the same in the lyophilized ones.

Key unanswered questions include (i) the nature of the signal from lyophilized samples; (ii) the nature of the signal from frozen samples; (iii) the relationship between these signals; (iv) whether the signals observed when a tumor is present are qualitatively different from those observed in normal tissues; and most important (v) whether these signals have any practical or theoretical significance in cancer.

It should be possible to obtain definitive answers to at least some of these questions. For example, the changes in intensity, line shape, and line width which we observed indicate that the signal from lyophilized samples is different from that from frozen nonlyophilized samples. There are similar indications that the signals from tumor samples are not identical to those from controls. The signal-to-noise ratios for these samples are probably sufficient to permit more detailed ESR studies.

Admission of oxygen to lyophilized samples provides an additional means of enhancing the signal-to-noise ratios (*11*) and of determining differences between different samples. With the introduction of oxygen, qualitative effects on the line shape, effects on signal intensity, and the time pattern of these changes could be studied.

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Intergeneric Transfer of Genes Involved in the *Rhizobium*-Legume Symbiosis

Abstract. *Genes that seem to be involved in the initial steps of infection of a legume by* Rhizobium *have been transferred, by transformation, to mutant strains of* Azotobacter vinelandii *that are unable to fix nitrogen. These genes code for a surface antigen that binds specifically to a protein from the host plant.*

Bacteria of the genus *Rhizobium* are capable of forming a complex nitrogen-fixing symbiotic association with leguminous plants. Bacteria of the genus *Azotobacter*, on the other hand, fix nitrogen without a requirement for a symbiotic host. Intergeneric transformation between *Rhizobium* and *Azotobacter* was first described by Sen *et al.* (*1*). *Azotobacter* transformants were selected for resistance to crystal violet or streptomycin. Some of these transformants resembled the *Rhizobium* donor strains in several nonselected biochemical properties. More recently, Venkataraman *et al.* (*2*) transformed *R. trifolii* with DNA from *A. chroococcum* and obtained transformants that grew on a nitrogen-free medium and, in the free-living state, reduced acetylene to ethylene. By utilizing DNA from several strains of *Rhizobium*, Page (*3*) was able to transform mutant strains of *A. vinelandii* which are unable to fix nitrogen (Nif^- strains) to Nif^+ strains. We thought that it might be possible to transfer, to *A. vinelandii*, genes of *R. trifolii* that are specifically required for root hair infection of *Trifolium repens* (white clover).

Trifoliin is a protein from clover that seems to be necessary for the initial stages of the infection process by *R. trifolii* (*4*, *5*). There is increasing evidence that lectins, such as trifoliin, are involved in the specificity of a *Rhizobium* species for its natural leguminous host plant (*5*–*7*). Immunochemical studies indicated that infective *R. trifolii* cells and clover roots have unique cross-reactive surface antigens (*7*). Preferential adsorption of infective *R. trifolii* to root hairs of clover may result from cross bridging of the cross-reactive surface antigens by trifoliin (*7*).

We transformed (*8*) three Nif^- strains of *A. vinelandii* to Nif^+ strains with crude DNA preparations from *R. trifolii* strain 0403 (obtained from P. S. Nutman). The transformation frequencies ranged from 1.5×10^{-7} to 2.4×10^{-6} (Table 1). Forty-six Nif^+ transformants from a cross with strain UW10 as the recipient were studied further. These transformants produced a green diffusible pigment characteristic of the recipient strain. The recipient and transformant cells appeared identical to each other and distinctly different from donor cells in both size and morphology when examined by phase-contrast microscopy. Three azotophages that were specific for the recipient strain formed

Table 1. Intergeneric transformation with *R. trifolii* as the donor and *A. vinelandii* as recipient. Transformation was conducted as described by Page and Sadoff (*8*), except that 2 percent sucrose replaced glucose in the transformation medium and the donor strain was lysed in 0.10 percent sodium dodecyl sulfate. The donor strain, *R. trifolii*, was cultured in a defined medium (*12*) with 1 percent D-mannitol as the carbon source. Growth and phenotypes of the *A. vinelandii* Nif^- recipient strains have been described (*13*).

Recipient strains	Nif phenotype*	Reversion to Nif^+†	Nif^+ transformation frequency‡
UW1	I^-II^-	$<1.1 \times 10^{-8}$	1.5×10^{-7}
UW6	I^-II^+	$<6.3 \times 10^{-8}$	2.6×10^{-7}
UW10	I^-II^+	$<2.8 \times 10^{-8}$	2.4×10^{-6}

*I and II represent the Mo-Fe and Fe components of nitrogenase, respectively. The superscripts + or − refer to the presence or absence of activity. †The frequency of reversion was based on the number of cells plated. ‡The Nif^+ transformation frequencies were calculated as the number of Nif^+ transformants per milliliter divided by the total number of cells per milliliter.