ON THE ELECTRON SPIN RESONANCES IN DNA

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ABSTRACT Iron impurities are shown to account for characteristic electron spin resonances observed in samples of DNA. Comparative e.s.r. measurements on lyophilized samples were done in conjunction with static susceptibility measurements, trace analyses, and molecular degradation experiments to establish this correlation. It has not been possible to extract this iron by treatment with a chelating agent. Such resonances were in part accounted for by ferromagnetic iron contamination during extraction and handling. By modifying the method of Kay, Simmons, and Dounce to eliminate or minimize metal contamination—ionic and ferromagnetic—from sources both internal and external to the tissues used, it was possible to prepare iron-free ($<$ 0.0004 per cent Fe), e.s.r.–free ($<$ 0.001 per cent Fe) DNA. The samples showed 20 per cent protein (determined by the indole method), had a molecular weight 4×10^6 and were undenatured according to the density gradient experiment.

INTRODUCTION

The appearance of e.s.r. in nucleic acid samples after high energy irradiation was first described by Shields and Gordy (1959) and Sheng Pei Ken *et al.* (1959). Subsequent to these studies the surprising observation was made by Bliumenfeld and co-workers (1959) that e.s.r. signals were present also in unirradiated nucleic acid and in nucleic acid–protein preparations.

The nucleic acid preparations which they examined were found to give broad (120 to 500 gauss) e.s.r., some of which were asymmetric and had considerable intensity at zero magnetic field. From intensity measurements they estimated an unpaired spin concentration of 10^{21}/gm nucleic acid, and inferred an unpaired spin concentration as high as 6×10^{21}/gm in the case of RNA in complex with protein. They did not find signals in denatured DNA, in protein alone or in the purine and pyrimidine bases. The signals exhibited unusual temperature dependence: at 77°K the signals reversibly disappeared, but boiling DNA in solution and heating in the dry state to 200°C caused an irreversible disappearance of the signal. Paramagnetic transition elements were found to be present by chemical analysis but they were found in quantities insufficient to account for the observed e.s.r. For certain samples the measured static magnetic susceptibility was comparable

to the spin magnetic susceptibility determined from e.s.r. intensity; for others a static magnetic susceptibility much greater than the spin magnetic susceptibility was observed; and in still others there was no spin resonance and the samples were diamagnetic. In addition it was found that the magnetic susceptibility fell off at higher fields indicating a saturation of the magnetization. This suggested a ferromagnetic phenomenon. They were however, unable to detect a zero field moment in their samples (Bliumenfeld and Benderskii, 1960).

Following Bliumenfeld's reports, Redhardt (1961) reported observing similar broad resonances in unirradiated DNA, and Müller, Hotz, and Zimmer (1961) found such resonances in bacteriophage. The latter group reported broad and varied resonances in highly purified and lyophilized samples of T1 and T2 phage, in purified phage nucleic acid and in phage protein as well. The resonances had intensities corresponding to 10^{18} to 10^{19} unpaired electrons/gm phage and were described as combinations of four different resonances; one near zero field, and one near $g = 4$, 100 to 200 gauss broad, a third at $g = 2.2$ about 1200 gauss broad, and a fourth, a narrow line, at $g = 2.0$. The signals were unaffected by vacuum, storage, and irradiation. Cooling to 77°K had no effect. However, heating to 200°C, in air or vacuum caused its irreversible disappearance. It also reversibly disappeared on storage in a saturated atmosphere.

Blois and Maling (1961) reported on studies of signals found in several DNA and RNA samples. Unpaired spin concentrations of 10^{19} to 10^{20}/gm nucleic acid were measured and paramagnetic iron was implicated as the source of the e.s.r. because the iron was present in every sample examined by trace analysis. However the iron content in several of the samples was insufficient by factors of from 4 to 10 to account for the signal intensities observed. Degradation experiments showed that the signal did not depend upon the nucleic acid structure, however. In addition static susceptibility measurements (unreported) indicated a high static magnetic susceptibility present in some samples, too high to be accounted for by either e.s.r. intensities or iron content measured by trace analysis assuming paramagnetic iron. Furthermore, a saturable component of the magnetization was observed in some cases.

Shulman, Walsh, Williams, and Wright (1961) at Bell Telephone Laboratories reported observing such signals in samples of nucleic acids. They observed a temperature-independent intensity down to liquid helium temperature, a saturable static paramagnetism, and a measurable coercive force in one sample. As with the other workers their trace analysis showed insufficient iron to account for the spin resonance intensities observed if one assumed the iron to be paramagnetic. They hypothesised that the signals were due to iron in the ferromagnetic state to account for this discrepancy between signal intensity, static susceptibility of a sample, and the iron content as determined by trace analysis.

Walsh, Shulman, and Heidenreich (1961) further supported this hypothesis by

demonstrating by electron microscopy the presence of 100 A to 1000 A diameter inorganic crystalline particles bound tightly to the nucleic acid molecules, which they proposed to be an oxide or hydroxide of iron, precipitated possibly during the extraction and purification of the material but probably not present in that form in the living cell.

Work in our laboratory, reported in detail below, supports the impurity e.s.r. hypothesis. In addition to results from heating and degradation experiments which indicate an inorganic source of the e.s.r. in question, we have been able to correlate e.s.r. observed in certain samples of ATP and RNA with the presence of copper, manganese, and iron by metal ion chelation experiments where the e.s.r. signals were removed when metal ions were extracted from the samples. (Maling, Taskovich, and Blois, 1963). While in general, being unable to "extract" e.s.r. signals from DNA samples in the same way, a correlation has been established in some cases between certain characteristic signals and ferromagnetic impurities picked up during preparation and handling, and it has been possible to prepare a signal-free, iron-free DNA from calf thymus by trying to minimize metal contamination from sources both "external" and "internal" with respect to the tissues from which the extraction is made.

METHODS AND MATERIALS

A. *Sources of Nucleic Acid*

Salmon sperm and herring sperm DNA were obtained from California Corporation for Biochemical Research (CCBR), Los Angeles, California; a sample of DNA of unknown type was obtained from General Biochemicals, Inc. (GBI), Chagrin Falls, Ohio; a sample of calf thymus NaDNA was kindly provided by Professor H. S. Loring, and several samples of calf thymus DNA were prepared in our laboratory.

B. *Preparation of DNA*

Several samples of calf thymus DNA were prepared by the method of Kay *et al.*, three as the sodium salt, and one as the lithium salt, without attention to metal contamination.

A method of preparation was later designed, to exclude as far as possible, the introduction of either metallic or ionic iron. From the time of acquisition of the fresh thymus at the slaughter house, no metal was allowed to contact the material; we have called this the "stone age" method. Double, glass-distilled water was used; all inorganic salt solutions used were extracted with 8-quinolinol in CCl_4 and then boiled to remove the solvent. All preparations were carried out at 4°C. The procedure was as follows:

(1) 500 gm of fresh calf thymus, was cleaned of extraneous material (hair, etc.), trimmed by hand of most connective tissue, and washed in 0.14 M NaCl–0.05 M Na citrate solution.

(2) 20 gm portions were cut off with a glass knife and ground in a porcelain mortar with 20 ml of 0.14 M NaCl–0.05 M Na citrate solution, using quartz chips to break up the tissue. (The chips, prepared by breaking up quartz tubing, showed no signals when examined for e.s.r.)

(3) The homogenate was centrifuged at 3500 RPM for 30 minutes at 4°C. The sedi-

ment was washed by breaking up the pellet in the mortar with the chloride-citrate solution and centrifuging again. This was repeated four times.

(4) The sediment was then resuspended in 500 ml of distilled water, adjusted to pH 7 with $NaHCO_3$ and centrifuged at 3500 RPM for 30 minutes. This washing was then repeated twice.

(5) The gelatinous sediment was added to 6 liters of distilled water (pH 7) and slowly stirred overnight.

(6) The viscous solution was centrifuged at 3500 RPM for 30 minutes. The supernatant was then divided into four aliquots for the preparation of the deoxyribonucleoprotein, and samples of DNA by each of three methods.

Preparation of the Deoxyribonucleoprotein. (1) To 960 ml of the supernatant (step 6 above) was added 2000 ml of 0.22 M NaCl.

(2) The precipitate was collected after 24 hours by centrifugation and washed, first with 0.15 M NaCl, then with 0.07 M NaCl, and finally with distilled water. The sample was then lyophilized.

Preparation of DNA Using 2.6 M NaCl. (1) To 1500 ml of the supernatant (step 6 above) 1550 ml of 5.3 M NaCl was added, and the solution was slowly stirred for 48 hours.

(2) The solution was centrifuged for 30 minutes at 3500 RPM, and the precipitate removed.

(3) Two volumes of ethanol were added to the supernatant and the fibers were collected with a stirring rod, pressed free of liquid, and washed with 60 per cent ethanol for 2 hours. The fibers were left overnight in 80 per cent ethanol, then placed for 2 hours in 95 per cent ethanol, and finally rinsed for another 2 hours in 100 per cent ethanol. The DNA fibers were then dried *in vacuo* at room temperature.

(4) The supernatant (containing the histone) was dialyzed against water, and then brought to pH 9.6 with ammonia gas. This was left overnight and then centrifuged at 350 RPM for 30 minutes.

(5) The precipitate was washed with 0.07 M NaCl and then with water. The histone was then washed several times with H_2O and lyophilized.

Preparation of DNA using Sodium Lauryl Sulfate. (1) To 1500 ml of the supernatant (step 6 above) 41 ml of 5.3 M NaCl was added to bring solution to 0.14 M with respect to NaCl. 240 ml of 5 per cent sodium lauryl sulfate in 45 per cent ethanol was added and slowly stirred for 48 hours.

(2) 99 ml of NaCl was added to bring the concentration of NaCl to 1 molar, and the solution was stirred for 3 hours. It was then centrifuged at 3500 RPM for 30 minutes, and the sediment set aside.

(3) Two volumes of ethanol were added to the supernant and the DNA fibers were collected on a stirring rod, washed, and dried as above.

Preparation of DNA Using Chloroform. (1) To 1500 ml of the supernatant (step 6 above) 41 ml of 5.3 NaCl was added to obtain a final concentration of 1.4 M with respect to NaCl.

(2) 150 ml of a 50 per cent chloroform solution in ethanol, was added and stirred overnight.

(3) 99 gm of NaCl was added to bring the final concentration of NaCl to 1 molar and the solution was stirred for 2 hours. It was then centrifuged at 3500 RPM for 30 minutes.

(4) Two volumes of ethanol were added to the supernatant and the DNA was centrifuged down—the fibers being too small to be collected by a stirring rod. The DNA was

then washed with 66, 80, and 100 per cent ethanol, being centrifuged after each washing, and finally dried *in vacuo* at room temperature.

C. *Chemical Determination of Iron in DNA*

A colorimetric method for the determination of iron in DNA was adapted from Sandell (1959), and carried out as follows:

1000 mg samples of DNA were weighed and transferred to 250 ml Ehrlenmeyer flasks. 3.00 ml of concentrated HNO_3 and 2.00 ml of concentrated H_2SO_4 were added to each sample. The flasks were covered with funnels (with sealed tips) and left overnight at room temperature. The samples were then heated and the ashing carried on as indicated by Sandell.

Total HNO_3 used in each sample: 5.00 ml. When the ashing was completed, 3 ml of H_2O were added, heated to boiling and cooled. The samples were then filtered, using glass wool filter paper, and transferred quantitatively into 25 ml volumetric flasks for the colorimetric determination of Fe by the o-phenanthroline method. The OD of the samples were measured in a Cary spectrophotometer model 14. The standard curve was carried on through the ashing in the same manner as the DNA samples. (Average OD of blank *versus* H_2O = 0.038)

$$\text{OD of } 1\ \mu\text{g Fe/ml}(1.79 \times 10^{-5}\ \text{moles Fe/liter}) = 0.20$$

$$\epsilon = \frac{0.20}{1.79 \times 10^{-5}} = 1.12 \times 10^{4}$$

$$\text{sensitivity } \frac{\text{M.W.}}{\epsilon} = 0.005\ \mu\text{g/cm}^2$$

Minimum detectable amount by this method for 1 gm sample of DNA brought after ashing to a final volume of 25 ml if read in a cell of 1 cm path length is $0.005 \times 25 = 0.125$ ppm (or 0.125×10^{-4} per cent Fe by weight in DNA).

To determine the variability of these analyses (due to handling, etc.) the OD of a typical DNA sample was taken and its iron concentration determined using the lowest and the highest standard curve obtained during several analyses.

Basis of analysis	Sample	Weight	Volume	OD	Fe/ml	Fe/gm	Per cent	Diff. from actual analysis
		gm	*ml*		*μg*	*μg*		
Actual Analysis	95b	1.00	25	0.145	0.73	18.2	0.0018	0
Lowest standard curve	95b	1.00	25	0.145	0.64	16.0	0.0016	(−0.0002)
Highest standard curve	95b	1.00	25	0.145	0.85	21.3	0.0021	(+0.0003)

The e.s.r. measurements were made on a Varian V-4500 x-band spectrometer using 100 kc modulation. All samples were examined for e.s.r. in the dry state, either as a loose lyophilyzed powder or as a pressed pellet. Sample heating was done in tubes open to the air, or in evacuated tubes that were being continuously pumped. They were held at the desired temperature for 8 to 16 hours, then cooled and examined for e.s.r. at room

temperature. Low temperature measurements were made with a cold-gas flow system using a liquid nitrogen immersed heat exchanger.

THE e.s.r. OF DNA

A. *Characteristics of the Nucleic Acid Resonances*

We have studied the e.s.r. in a number of DNA samples, both from commercial sources and from our own and other laboratories. Electron spin resonances invariably have been observed and there have been generally four characteristic components to the resonances (see also Müller *et al.*, 1961 and Walsh, Rupp, and Wyluda, 1963).

(*a*) A component centering near or at zero field with a tail which sometimes stretches several thousand gauss.

(*b*) A relatively narrow (100 to 200 gauss) asymmetric resonance near $g = 4$.

(*c*) Broad asymmetric resonances between $g = 2.0$ and 2.5. Intensity relatively independent of temperature.

(*d*) Weak complex structure 400 to 500 gauss in extent, centering at $g = 2.0$ in width.

The resonances can vary from sample to sample in position in field, and all components need not be present in any given sample. Often position and/or shape of resonance will change markedly when sample is rotated in cavity about the axis parallel to H_{rf}.

Fig. 1 illustrates a resonance which is a combination of types *c* and *d*. The

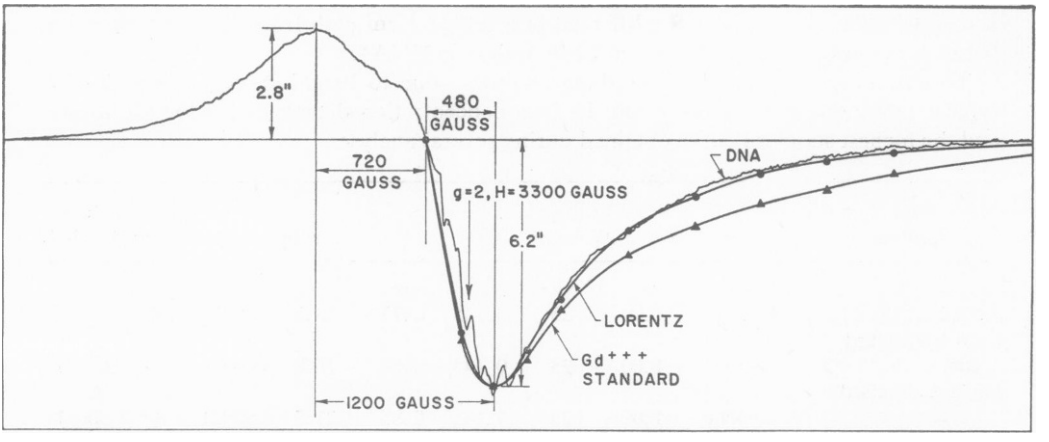


FIGURE 1 An example of the "anomalous" electron spin resonance of nucleic acid, seen in a commercial preparation of salmon sperm DNA. The high field tail (to the right) fits a Lorentzian function. The high field portion of the resonance of an S-state rare earth, ion, Gd^{+++}, in glycerine solution is included for a line shape comparison. The Gd^{+++}-glycerine system was used as a standard to determine the spin concentration in the DNA sample. The Gd^{+++} line is 200 gauss wide and has been scaled up to fit the high field portion of the line in width and amplitude.

sample of salmon sperm DNA (CCBR) $E_{260}^{1\%} = 204$, N/P = 1.67 and as can be seen the signal is a broad complex absorption of approximately 1200 gauss in width, centered near $g = 2$ with some structure centering about $g = 2$. This is due to a manganese ion impurity (see Maling, Blois, and Taskovich, 1963, and section E below). Note that the high field tail has a Lorentzian shape; compared with it is the line from an S-state ion, Gd+++, in glycerine solution. The Gd+++ line is actually 200 gauss broad and has been scaled up to fit the DNA resonance in width and amplitude in order to compare line shapes. Unpaired electron spin concentration was estimated to be 1.0×10^{20}/gm nucleic acid (Blois and Maling, 1961). This spectrum, and those subsequently shown are the first derivative of the resonance absorption.

B. *Effect of High Temperature on e.s.r.*

We have observed both irreversible disappearance and non-disappearance of the e.s.r. at higher temperatures. For example, a sample of the salmon sperm DNA was pressed into a flat, cylindrical pellet, broken up into small fragments and placed in a sample tube. Spectra were then taken as this sample was heated to progressively higher temperatures, and these are shown in Fig. 2. The curve

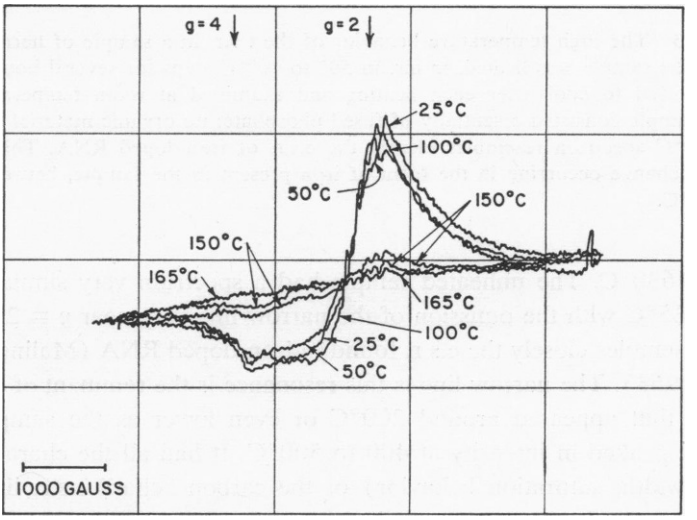


FIGURE 2 The effect of heating on the e.s.r. of a sample of salmon sperm DNA. The apparent loss of the signal at the relatively low temperature of 165°C is to be contrasted with the results illustrated in Fig. 3 (also see text).

labeled 25°C was taken before heating and corresponds to that of Fig. 1. The heating was accomplished by placing the open sample tube, for short periods, in an oven which had been raised to the indicated temperature. It was noted that the solid DNA fragments had liberated moisture which had condensed on the cooler

portions of the sample tube. At first glance, Fig. 2 seems to be in agreement with the original report of Bliumenfeld; at something over 100°C, the e.s.r. signal appears to vanish. Upon repeating the heating experiments (of the dry DNA preparations) under conditions involving the maintenance of the sample at the indicated temperature for extended periods (several hours) it was found that while signals often changed in shape they did not disappear. A crude herring sperm DNA sample (CCBR) was heated to temperatures of over 600°C, while exposed to the air, with the results shown in Fig. 3. Note the change in the e.s.r. occurring between

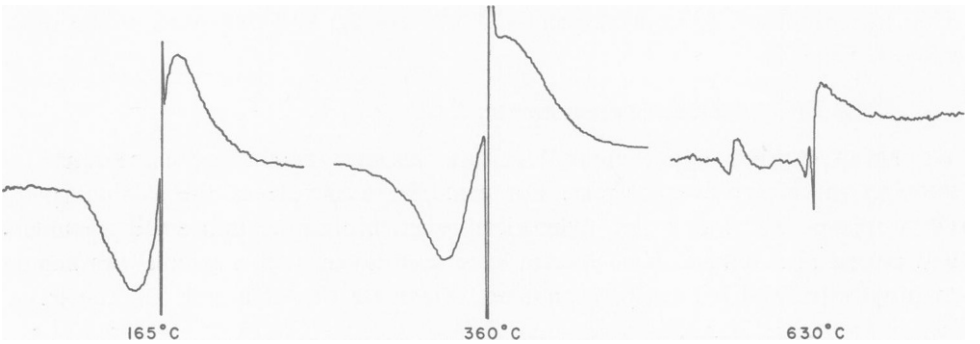


FIGURE 3 The high temperature behavior of the e.s.r. in a sample of herring sperm DNA. The sample was heated, in air, in 50° to 100°C steps for several hours at each step, allowed to cool after each heating and examined at room temperature. The 630°C sample consisted essentially of fused phosphate; no organic material remained. The 630°C spectrum resembles closely the e.s.r. of iron-doped RNA. This may indicate a change occurring in the form of iron present in the sample, between 500°C and 600°C.

360°C and 630°C. The unheated sample had a spectrum very similar to the one heated to 165°C with the omission of the narrow line very near $g = 2$. The 630°C spectrum resembles closely the e.s.r. found in iron-doped RNA (Maling, Taskovich, and Blois, 1963). The narrow line is this resonance is the remmant of a very strong "char" line that appeared around 200°C or even lower as the sample began to oxidize, and peaked in intensity at 400 to 500°C. It had all the characteristics (g-value, line width, saturation behavior) of the carbon "char" e.s.r. line produced by charring sugar. It always appeared when the nucleic acid samples were heated, and disappeared only when the carbon was gone and the samples were white glasses of fused phosphate. These heating experiments were done both in tubes open to the air and at about 10^{-4} mm pressure and no significant difference was found between the results from the two types of experiments. In one case a pyrimidine base which gave a strong signal was heated. Eventually only a tiny residue remained, a fraction of a milligram in weight, but the signal was undiminished in intensity. Its shape altered considerably during the heating, however, as did the signals of the

heated nucleic acids. The persistence of an e.s.r., despite heating while open to air, has been observed by Isenberg (1961) who noted that the e.s.r. in protamine nucleate when the sample was heated *in vacuo* to 200°C disappeared irreversibly, while the signal in the same material charred by heating over a hot air bath to 220°C underwent changes in shape but not in intensity. On the other hand, using T2 phage which had been found to give an e.s.r. signal quite similar to DNA, Müller *et al.* (1961) reported the irreversible disappearance of the signals of vacuum-dried phage particles upon heating above 200°C.

The conclusion is that the dry heating of DNA to temperatures of the order of 200°C or above, in the majority of instances, did not eliminate the broad e.s.r. resonance.

C. *Effect of Low Temperature on e.s.r.*

The effect of sample cooling on the e.s.r. was varied. Often a marked change in line shape would take place, particularly in the low field region. The $g = 4$ resonance increased in intensity with decreasing temperature and its intensity usually had within experimental error a Curie Law dependence. The $g = 2.2$ resonance (whose position in field sometimes showed a dependence on sample orientation) usually was independent of temperature. It had relatively constant intensity over a large temperature range. As an example, salmon sperm DNA was observed at liquid nitrogen temperature; the spectra are shown in Fig. 4. It is evident that the signal does not disappear although there are alterations to line shape. These data were not included at the time this low temperature behavior was reported. (Blois and Maling, 1961). The area under the absorption curve is constant (to within a ±30 per cent experimental error) down to 77°K. A similar persistence of the e.s.r. signal upon cooling to −185°C, was noted for the T2 phage by Müller *et al.* (1961), and Shulman *et al.* (1961) reported a constant amplitude for their signals within experimental error down to liquid helium temperature.

D. *Metal Content*

The most logical source of such a resonance would be a paramagnetic ion in some crystalline environment, in particular an S-state ion because of the position of the resonance in field, the temperature at which it is observed (295°K), and the fact the resonance persists in samples heated to very high temperatures. The ion could have a ground state of high multiplicity and possibly nuclear spin because of the resonance breadth, asymmetry, extension to zero field and an occasional weak complex structure that is observed (Fig. 1).

It has been shown by Wacker and Vallee (1959) that the trace metals, particularly elements of the first transition series, several of whose ions are paramagnetic, are found in both DNA and RNA prepared from a number of sources. They and Loring (1957) have demonstrated that these elements can be reduced in amount by

treatment of the nucleic acids with EDTA, or by other metal extraction techniques, but only to certain minimum values.

We attempted to correlate the e.s.r. observed with metal ion content by measuring e.s.r. intensities in a number of DNA samples in the dry state, measuring their static susceptibility and analyzing for the presence of paramagnetic elements. The nucleic acid samples examined were the following:

(1) Salmon sperm DNA (CCBR) (described further above).

(2) Calf thymus DNA (prepared by Deranleau (1958) by a modification of the Kay, Simmons, and Dounce method (1952) and previously analyzed for trace metals).

(3) Crude herring sperm DNA (CCBR).

(4) DNA, source unspecified (GBI).

A number of transition elements were analyzed for in samples (1) and (2) by conventional chemical methods and by x-ray fluorescence spectroscopy. Table I shows the results of these analyses, and it was clear that a comparable iron con-

TABLE I

PARAMAGNETIC ELEMENT COMPOSITION OF SAMPLES (PER CENT BY WEIGHT)

	Cr	Mn	Fe	Co	Ni	Cu	Mo	Eu	Gd	Re
(1) Salmon sperm DNA (CCBR)	<0.066	<0.004	0.005 to 0.012	<0.004	<0.004	<0.003	<0.07	<0.03	<0.03	<0.015
(2) Calf thymus DNA (Deranleau)	<0.0005	<0.0005	0.012 to 0.039	<0.004	<0.004	<0.003	<0.07	<0.03	<0.03	<0.015

< = not detected at the indicated sensitivity.

tent is common to all samples tested, establishing a tentative correlation with the e.s.r. The comparison between the observed e.s.r. signal intensities, the static magnetic susceptibility, and the iron content for these from DNA samples is shown in Table II.

The theoretical magnetic susceptibility was calculated from Pascal's constants using average values for the relative base composition. The predicted susceptibility is of course diamagnetic. All the DNA samples but one gave a positive susceptibility and this exception was a positive shift from the expected value.

If the assumption is made that the resonance signals of DNA are *paramagnetic* resonances, then the amount of Fe necessary to account for a spin density of 10^{20} spins/gm is 0.08 per cent by weight. From Table II it will be noted that no samples contained this much iron. These analytical results and the conclusion that transi-

TABLE II

	Fe by analsyis (per cent by weight)	χ_m theoretical (iron free) (T = 300°K)	χ_m theoretical (with max. amount of iron, assuming it to be paramagnetic and in 3+ oxidation state)
(1) Salmon sperm	0.005 to 0.012	-0.45×10^{-6}	-0.42×10^{-6}
(2) Calf thymus	0.012 to 0.039	-0.45×10^{-6}	-0.35×10^{-6}
(3) Herring sperm	0.032	-0.45×10^{-6}	-0.36×10^{-6}
(4) DNA (unspecified)	0.042	-0.45×10^{-6}	-0.34×10^{-6}

	χ experimental* (T = 300°K)	E.s.r. signal (equivalent per cent by weight, Fe^{+++})
(1) Salmon sperm	$+(0.09 \pm 0.05) \times 10^{-6}$	0.06
(2) Calf thymus	$+(0.57 \pm 0.05) \times 10^{-6}$	0.10
(3) Herring sperm	$+(0.30 \pm 0.05) \times 10^{-6}$	0.10
(4) DNA (unspecified)	$-(0.08 \pm 0.05) \times 10^{-6}$	0.01

* Susceptibility measured at a field of 7000 gauss.

tion elements could not account for the observed spin density were in accord with Bliumenfeld's original report and the report of Shulman *et al.* (1961).

A similar result in protamine nucleate was reported by Isenberg (1961). He pointed out that if instead of acting independently, the transition element ions present in these substances were coupled so as to produce a "superparamagnetism", the resulting increase in the susceptibility did indeed afford a mechanism whereby the Fe known to be present could in fact account for the intensity of the observed resonances.

Shulman *et al.* (1961) reported the e.s.r. signals seen in commercial DNA preparation to be *ferromagnetic*. This conclusion was based primarily upon the *g*-value, magnetic susceptibility measurements, and the temperature-independent behavior of the observed spin resonance as the sample was cooled to 4.2°K, and it was supported by their finding of ferromagnetic particulate material in one of their less pure samples. This temperature independence of the e.s.r. resonance is that shown in Fig. 4 and it is found to persist to liquid helium temperatures. These workers also carried out an x-ray fluorescence analysis of their DNA samples, showing again that iron is the principal paramagnetic metal present. They found 1×10^{18} atoms Fe/gm; our own results are 1×10^{18} to 5×10^{18} atoms Fe/gm.

E. *Molecular Degradation Experiments*

A variety of molecular degradation experiments were carried out and no signifi-

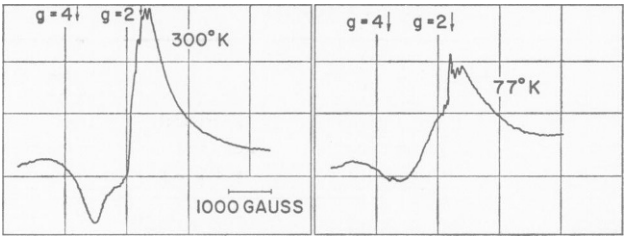


FIGURE 4 The low temperature behavior of the e.s.r. in a sample of salmon sperm DNA. The spin concentration is effectively unchanged between 300°K and 77°K. The line broadens slightly and decreases in amplitude while the structure at $g = 2$ (due to manganese ion) sharpens and becomes better resolved.

cant change in signal intensity was observed, corroborating the results of the heating experiments.

A sample of calf thymus DNA (sample 2 previously described) showed the control, e.s.r. absorption of Fig. 5, upper left. Comparison with Fig. 1 shows this calf thymus DNA preparation to have a more asymmetric absorption extending to zero field. A sample of this same preparation was then sonicated for 2 minutes (at a frequency of 10 kc and nominal power input of 250 watts) in 0.15 M NaCl. The salt was dialyzed out and the lyophilized DNA then gave the spectrum of Fig. 5, upper right. Despite the qualitative changes in line shape, there has been no significant change in spin concentration.

A second sample of the same preparation was then boiled in water for 15 minutes, and after lyophilization gave the signal of Fig. 5, lower left. Upon comparing this with the control it will be seen that again there is no significant change.

A third sample (160 mg) was made up in 500 ml of 0.15 M NaCl, buffered to pH 7.0 with phosphate buffer, and 10 mg of deoxyribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) was added. This was incubated at 37°C for 72 hours, and then dialyzed against deionized H_2O. The lyophilized material was then observed in the e.s.r. spectrometer and the spectrum shown in Fig. 5, lower right, was obtained. This treatment has not essentially altered the spectrum. In general, the differences which exist between these individual spectra are not beyond those existing between different aliquots of the same DNA preparation.

A further sample was made up in aqueous solution and taken to dryness at 86°C in an oven. This treatment did not significantly alter the signal.

In summary, a variety of degradative treatments had essentially no effect upon the broad e.s.r. of DNA.

F. *Metal Extraction and Addition Experiments*

It was hoped that a further, more definite correlation between metal content

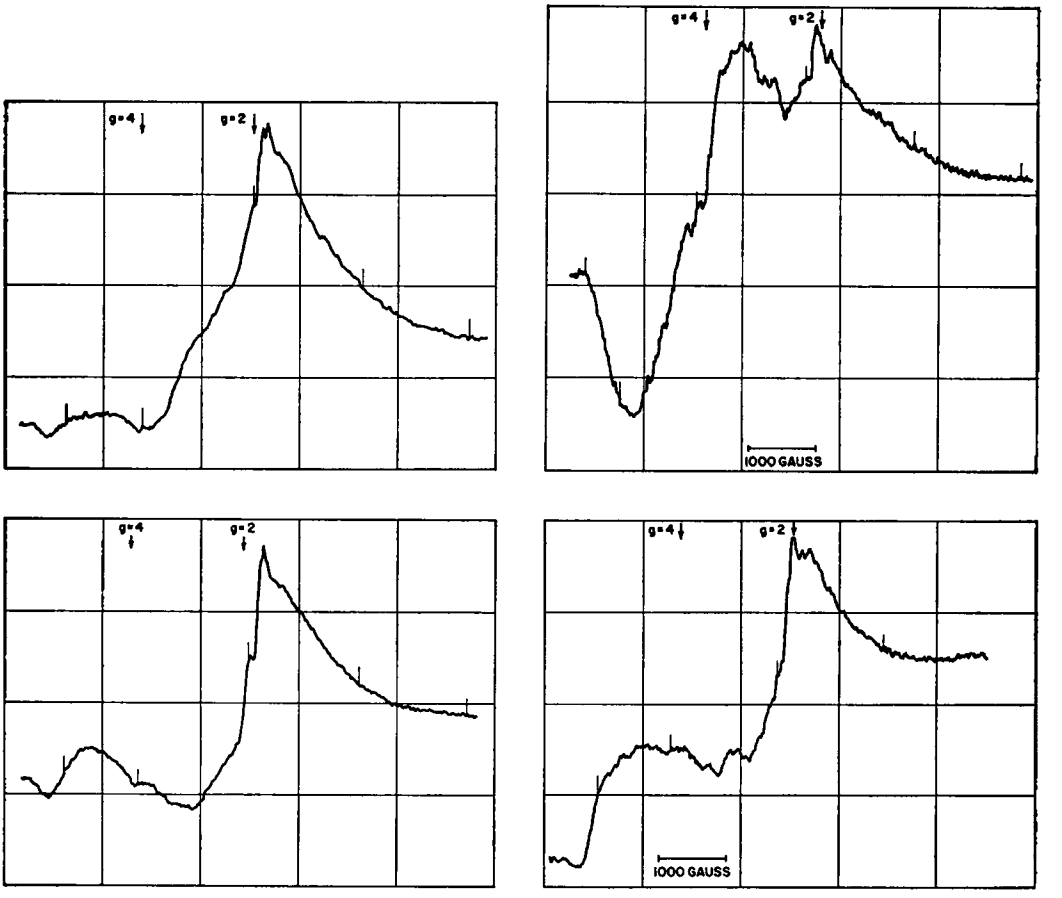


FIGURE 5

Upper left The e.s.r. of a calf thymus DNA preparation. The control for subsequent spectra.) Modulation amplitude is 7.5 gauss.

Upper right The e.s.r. of calf thymus DNA which was sonicated for 2 minutes at 25°C in 0.15 M NaCl. Modulation amplitude is 4.8 gauss. Effective mass of sample is about 50 mg.

Lower left The e.s.r. of calf thymus DNA which has been boiled in deionized water for 15 minutes, then lyophilized. The effective mass of the sample is about 50 mg.

Lower right The e.s.r. of calf thymus DNA after treatment with deoxyribonuclease. The effective mass of sample is about 50 mg.

and e.s.r. spin density might be obtained through the extraction of metals from the commercial DNA samples. Such an approach had been fruitful in the case of RNA and ATP.

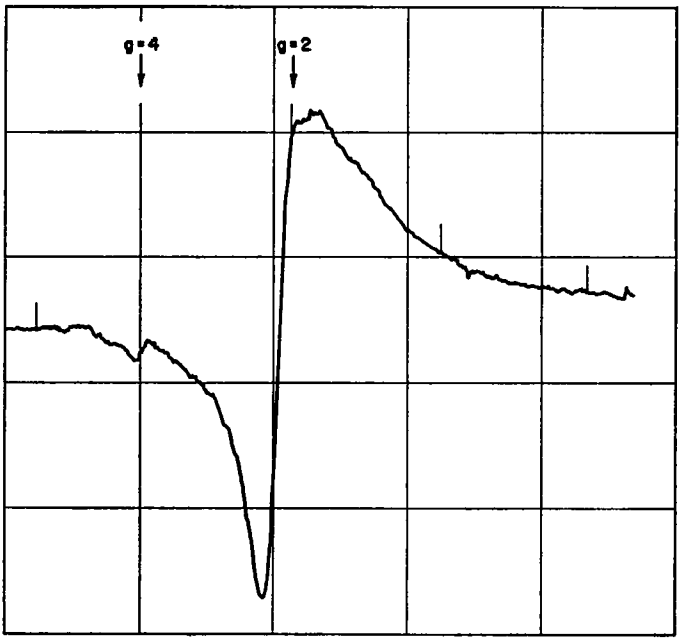
These were generally unsucessful although a variety of techniques (EDTA, 8-hydroxyquinoline, over a range of pH's) were tried. Shulman *et al.*, 1961)

report similar results. One technique, however, did produce a DNA (somewhat degraded, to be sure) which had a reduced e.s.r. signal and iron content in three out of four experiments. This consisted in sonication at alkaline pH, followed by a prolonged centrifugation. The sediment, which upon analysis consisted largely of histone, displayed a greatly enhanced spin concentration over the starting material, and enhanced iron content, and the DNA in the supernatant, a correspondingly reduced spin concentration.

This result fits well with the findings of Walsh *et al.*, (1961) who report finding crystalline particles bound tightly into the nucleic acid samples. One might expect that the sonication breaks the nucleic acid molecules and frees the particles to some extent. At pH 10 a precipitation of residual protein occurs and any free iron oxide-hydroxide colloidal particles are adsorbed onto the surfaces of the protein precipitate particles and then these are centrifuged out and concentrated, leading to a residue high in protein, iron, and e.s.r. intensity.

The addition of known paramagnetic ions to DNA was carried out (Fe^{++}, Fe^{+++}, Cu^{++}, Co^{++}, Ni^{++}, etc.). The addition of an iron salt to calf thymus DNA failed to affect the original spectrum appreciably; the addition of a copper salt to the DNA altered the original spectra only to the extent of introducing a narrow, asymmetric, paramagnetic absorption typical of copper and quite unlike the broad resonances observed (Fig. 6). A very recent report by Walsh and colleagues (1963) however, has described elegant experiments where such additions have produced signals with similar properties to the unknown ones. They have identified at least three different forms of iron from the spectra: one, a micelle of $Fe_3O_4 \cdot n\,H_2O$, and two different binding sites for ionic iron on the nucleic acid molecule itself. Iron present in an aggregated form can explain a variety of resonances we have observed. The e.s.r. of powders of tiny ($< 1\ \mu$ in diameter) ferromagnetic particles of Fe_2O_3 by Valstyn, Hanton, and Morrish (1962) have line shapes very similar to the type *d* resonance (Fig. 1). They show experimental data on powders of three types of particles: one, of nearly circular shape, and two others acicular in shape, with different average axial ratios. A powder of nearly spherical particles gives, at 9.4 kmc, a nearly symmetric, very broad (1200 gauss) resonance absorption peaking at $g = 2.2$. The powders of acicular particles varying in length from 0.1 to 1.5 μ and having axial ratios of 3:1 and 6:1, give even broader, more asymmetric resonances, peaking at about $g = 2.5$. The low field tail of the line has finite amplitude at zero field. The dispersion mode for these systems peaks at zero field and has a tail that extends for several thousand gauss, very similar to our type *a* resonance.

A distribution of particle sizes and shapes in our nucleic acid samples could therefore lead to the complex resonance line shapes that have been observed and also explain the marked sample-orientation effect on the resonances that has often been observed (Figs. 8 and 9). A non-uniform distribution of particles in size and



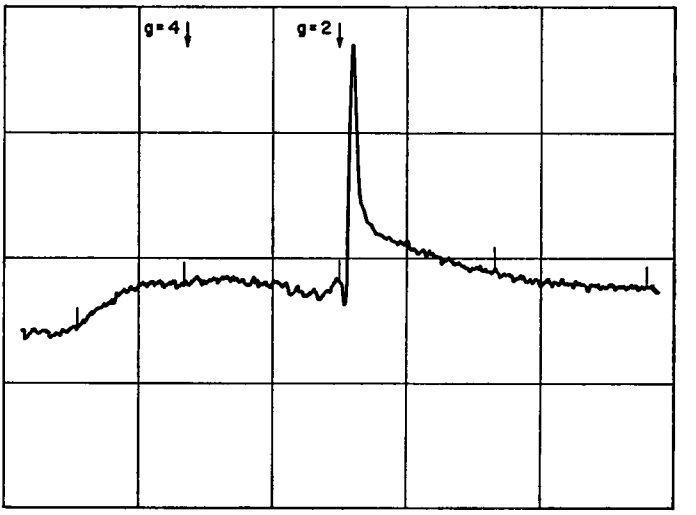


FIGURE 6

Upper The e.s.r. of calf thymus DNA to which has been added 50 mg $Fe(NO_3)_3 \cdot H_2O$/gm DNA. The salt was added in solution and the sample dialyzed and lyophilyzed. Modulation amplitude 3.8 gauss. The effective mass of sample is about 50 mg.

Lower The e.s.r. of calf thymus DNA with 3 mg $CuCl_2 \cdot 2\ H_2O$/gm DNA. The salt was added in solution and the sample lyophilyzed. Modulation amplitude is 0.48 gauss. The effective mass of sample is about 50 mg.

orientation would give rise to this effect. An additional complication arises if particles exist in which there is an rf phase shift so that the resonance then will be a mixture of dispersion and absorption. Such may be the case with type *a* resonances, which have in certain cases been definitely associated with ferromagnetic particles due to external contamination.

G. *Possible Sources of Paramagnetic and Ferromagnetic Contamination.*

The general features of the signal (similar to Figs. 1 and 5) have been amply confirmed in several laboratories, and the possibility of the signal arising from some unique feature of the DNA molecule such as a charge transfer process or a collective electron property has been eliminated; a variety of experiments, in which the DNA molecule was uncoiled, broken, hydrolyzed, and even ashed, had not markedly affected the e.s.r. signal. An iron impurity has been shown to be the source of the signal, and it has been assumed that the iron is in the ferromagnetic state in a hydroxide or oxide form. What then is the source of the iron?

The ease with which ferromagnetic contaminants can be introduced with samples was forcibly impressed upon us by a series of experiments in which purines, pyrimidines, nucleosides, and nucleotides were pressed into pellets for e.s.r. observation. These substances, all diamagnetic when obtained, were found to give e.s.r. resonances remarkably similar to those of DNA after compression. Since a variety of other compounds unfortunately failed during the first series of experiments to show this effect when compressed, it was assumed to be a real phenomenon and one possibly related to the DNA resonances (Blois and Maling, 1961). With the accumulation of further data, it became clear, however, that at least some of the signals seen were due to ferromagnetic contamination: that is, by microscopic flakes of steel alloy from the die used for the compression and from the stainless steel spatula used in handling the samples. Previous attempts to evaluate this possibility by examining small iron filings had failed, and in retrospect, this was probably a failure to use a thin enough or tiny enough sample so as to maximize the absorption while minimizing the detuning of the cavity. The failure to observe in iron particles, signals similar to the broad DNA resonance, was also reported by Müller *et al.* (1961).

The hypothesis that the broad DNA resonances arose from ferromagnetic particles raised new questions: were these particles simple external contaminants, did they form from individual Fe ions either intracellularly, as proposed by Shulman, or perhaps by formation of crystals of iron oxides of hydroxides from ionic iron impurities present in reagents used during the extraction of the DNA? Or does intracellular metallic iron exist *in vivo*? The introduction of ionic iron and/or steel particles from an external source, or the reactions of intracellular ionic Fe incident to sample preparation seemed the most likely. To test these two possibilities, several DNA preparations were made in which the inadvertent introduc-

tion of metallic and ionic iron from sources external to the tissues from which the nucleic acid was extracted was greatly minimized. Also, all important fractions (supernatants, washings, etc.) were retained and examined for the presence of iron and e.s.r. The DNA samples and the other fractions prepared by the above methods were then examined in the e.s.r. spectrometer, by static susceptometry, and were chemically analyzed for iron. The e.s.r. spectra of these samples are shown in Fig. 7. They are nucleoprotein (Fig. 7*b*), protein (histone) extracted in two ways (Figs. 7*c*, 7*d*) and DNA extracted in three ways from the nucleoprotein (Figs. 7*e*, 7*f*, 7*g*). It is apparent that e.s.r. absorptions are very weak (if present at all) when

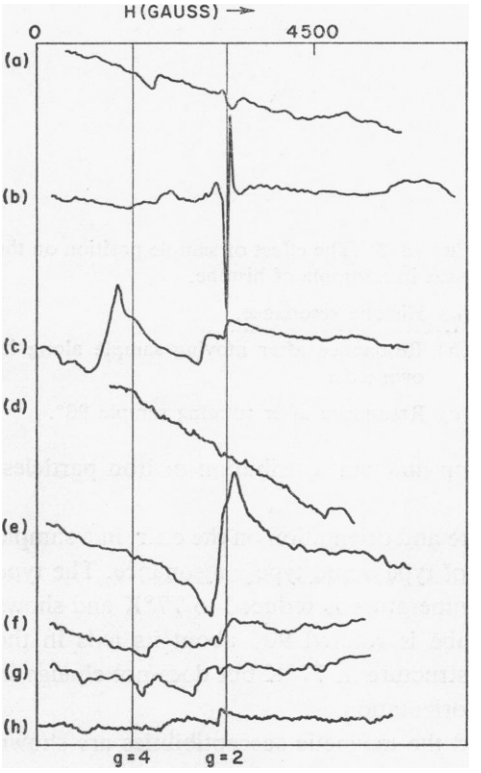


FIGURE 7 E.s.r. of the important fractions of a calf thymus DNA extraction using purified reagents and non-metal tools.

(*a*) Empty cavity.

(*b*) Nucleoprotein.

(*c*) Protein (histone, undenatured).

(*d*) Protein (histone, denatured).

(*e*) DNA, sodium chloride–extracted.

(*f*) DNA, sodium lauryl sulfate–extracted.

(*g*) DNA, chloroform-extracted.

(*h*) DNA extracted from calf thymus using the additional precaution of a chelating agent at the homogenizing stage of the preparation.

compared with any of the conventionally prepared DNA samples previously examined.

The effect of sample position in cavity on the e.s.r. of histone (Fig. 7*c*) is shown in Fig. 8. The component near $g = 4$ increases markedly in intensity as the sample is moved along its axis (parallel to H_{rf}) (Fig. 8*a* to Fig. 8*b*) and it shows a marked anisotropy as the sample is rotated 90° (Fig. 8*b* to Fig. 8*c*). At the same time, the $g = 2.5$ component disappears, while the $g = 2.0$ component remains relatively

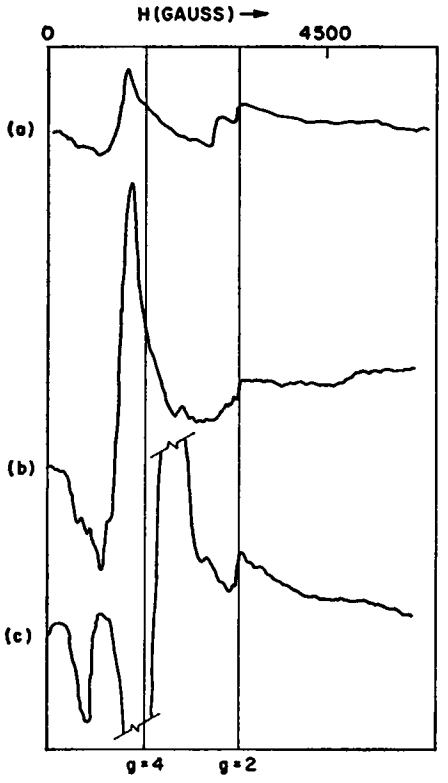


FIGURE 8 The effect of sample position on the e.s.r. in a sample of histone.

(*a*) Histone resonance.

(*b*) Resonance after moving sample along its own axis.

(*c*) Resonance after rotating sample 90°.

unchanged. This can be explained by a non-uniform distribution of iron particles, both in space and orientation.

Fig. 9 shows the effect of low temperature and orientation on the e.s.r. in a sample of DNA. The resonance is a combination of type *a* and type *c* resonance. The type *a* resonance doubles in amplitude when temperature is reduced to 77°K and shows a marked anisotropy when the sample tube is rotated 90° about its axis in the cavity. The type *c* resonance shows some structure at 77°K but does not change in intensity nor does it show a dependence on orientation.

The results of the chemical analysis and the magnetic susceptibilities are shown in Table III. The magnetic susceptibilities measured by the Gouy method were negative and corresponded, within experimental uncertainty, to the diamagnetic susceptibility calculated for an "average" DNA using Pascal's constants, and assuming equal amounts of A, T, G, and C. The iron content was about the same for nucleoprotein, protein, and DNA, and it was quite low, a factor of 20 or less than the DNA samples we had previously produced by standard methods or in the commercial samples. They were lower by about the same value than the levels reported previously (Wacker and Vallee, 1959) for beef liver RNA and DNA.

Two controls were run on these preparations to investigate the effects of the

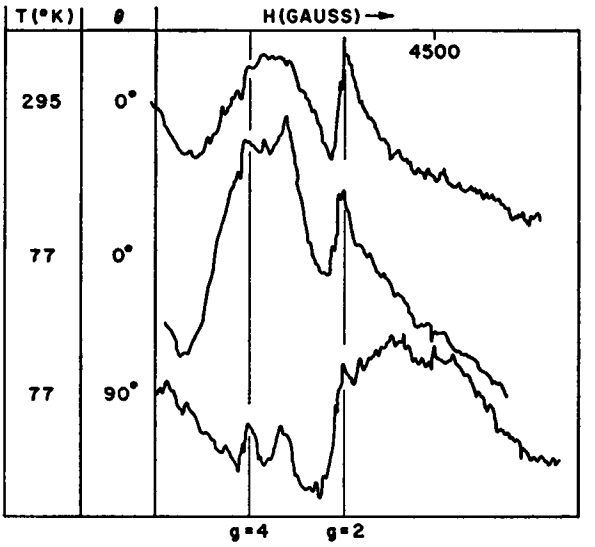


FIGURE 9 The e.s.r. of a calf thymus DNA as a function of temperature and orientation in the cavity. The low field component–type a resonance, increases in amplitude by about a factor two and it shows a marked anisotropy with rotation of the sample in the cavity about the rf field direction.

TABLE III

	Sample	Measured* Magnetic Susceptibilities × 10^6	Theoretical Magnetic Susceptibilities × 10^6	Fe (per cent by weight)
98d	DNP (thymus)	−0.56 ± 0.05	...	0.0014 ± 0.0004
98g	DNA (by NaCl method)	−0.37 ± 0.05	−0.45	0.0014 ± 0.0004
98k	DNA (by sodium lauryl sulfate method)	−0.47 ± 0.05	−0.45	0.0012 ± 0.0004
98n	DNA (by $CHCl_3$ method)	−0.37 ± 0.05	−0.45	0.0013 ± 0.0004
98a	Soluble material from first homogenate	−0.51 ± 0.05	...	0.0049 ± 0.0004
98c	Soluble material in supernatant of DNP washing	−0.37 ± 0.05	...	...
98h	The histone fraction of the DNP	−0.49 ± 0.05	...	0.0016 ± 0.0004
100	DNA (by sodium lauryl sulfate, EDTA method)			<0.0004

* The susceptibilities were measured at an applied field of 7000 gauss.

Waring blendor: one, calf thymus DNA was prepared with purified reagents, but the tissues were ground with the Waring blendor; and two short pieces of cheese cloth, simulating calf thymus tissue, were ground in the Waring blendor. In both cases characteristic e.s.r. signals were observed. Fig. 10 shows the e.s.r. found in

cheese cloth before and after (Figs. 10*a* and 10*b*) grinding in the Waring blendor, demonstrating clearly that it is a source of external contamination.

One interesting exception occurred in this series of signal-free preparations; one lyophilized preparation (a centrifuged sediment discarded during the preparation) showed a typical broad e.s.r. as with the earlier DNA samples. It happened that the lyophilizing flask which contained this sample had been accidentally broken by allowing a pair of pliers to fall upon it!

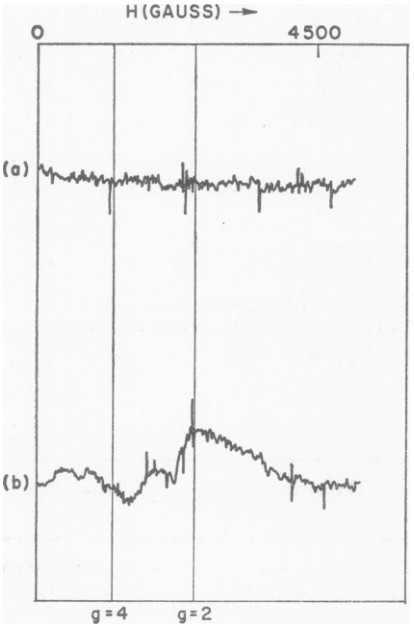


FIGURE 10 The e.s.r. found in a fibrous, inert, material (cheese cloth) after processing in the Waring blendor. The cheese cloth was chosen because it would offer a resistance to the blades of the blendor similar to that of the calf thymus tissue.

(*a*) E.s.r. of the untreated cheese cloth (control).

(*b*) After processing in the blendor.

The conclusions reached from the observations on this series of samples is that, if one rigorously attempts to keep iron out of the DNA, the e.s.r. absorption is greatly diminished, the magnetic susceptibility approaches the predicted value, and the iron content is seen to fall. It was clear, however that since DNA binds ionic iron readily, the intracellular iron released by enzymatic breakdown or by something more violent when homogenizing the tissue could be a source of internal contamination.

To test this hypothesis, a further modification was made in the preparation procedure, and another preparation of thymus DNA was made. All previous precautions regarding metal instruments, freedom from ferro- or paramagnetic impurities in the reagents, etc., were taken. The thymus gland was washed in 0.12 M NaCl–0.02 M ethylenediaminetetraacetic acid (EDTA), and then homogenized in this NaCl—EDTA solution. Washing and resuspending of the homogenate in the same solution was carried out five times. Finally the nucleoprotein was salted out of a 1

M NaCl–0.02 EDTA solution, and then washed several times with 0.14 M NaCl. The DNA was then prepared by the detergent method as before.

No e.s.r. was observed in this sample (shown in Figure 7*h*) and chemical analysis showed the iron content to be less than 0.0004 per cent. By taking steps to eliminate the possibility of both "external" and "internal" contamination during extraction, both iron content and e.s.r. were no longer detectable in a sample of DNA.

DISCUSSION

It seems possible to reconcile at least some of the disparate reports that have been made. First, as to the origin of the broad signal—it does not seem possible that the broad signal arises from some unique structural feature of the DNA molecule. With a sample which initially shows the broad resonance (and in the absence of special precautions, all DNA preparations appear to) a series of progressively severe degradations—"melting," molecular scission, hydrolysis, or ashing, fail in general to eliminate this resonance. Yet comparison of metal content and spin density, in several laboratories, shows that paramagnetic ions alone cannot account for signals of these intensities.

The temperature dependence studies of the Bell Telephone Laboratories group have shown that this resonance is not paramagnetic. This leaves a variety of exchange systems, ferromagnetic, antiferromagnetic, ferrimagnetic, etc., as possible candidates. For such a system to be antiferromagnetic or antiferrimagnetic it would have to have a Neel temperature below that of liquid helium as Shulman *et al.*, (1961) have shown the persistence of the signals to these temperatures. This seems improbable. There has so far been only one systematic way of getting rid of this resonance—that is by the preparation of the DNA in a manner which avoids the introduction of ionic iron (as well as other paramagnetic metals) or ferromagnetic particles into the sample. It seems to us most probable that the origins of these resonances are

(1) External contamination by ferromagnetic particles.
(2) External contamination by ionic iron which during the preparation may form ferromagnetic micelles (as of the hydroxide).
(3) Internal contamination by intracellular iron, followed by subsequent micelle formation.
(4) A residual contamination by intracellular ionic iron and sometimes manganese, which gives a superimposed weak paramagnetic resonance.

The elimination of external contamination is by no means a simple task as the history of this problem attests. For example, reagent grade NaCl that we used at an early point, upon careful analysis showed trace amounts of Fe sufficient to account for the amount found on analysis. It is possible that iron or other metal ions may exist in association with DNA *in vivo*, but the e.s.r. evidence sheds no light upon

this. Walsh, Rupp, and Wyluda (1963) have studied the e.s.r. signals produced in DNA by the addition of metal ions and have been able to distinguish two types of binding sites. These conclusions relate to the chemical properties of DNA, but there is as yet no evidence that binding of such ions plays a biological role.

Because of the increasing role of e.s.r. in the study of transition element ions in biochemistry, and of preparations obtained from tissue homogenates, it is probably not belaboring the point to proscribe absolutely the Waring blendor, the razor blade or knife and the forceps in handling these materials.

Finally, something can be said about the contradictory data which has now found its way into the literature. The failure of an iron filing to give an e.s.r. resembling those associated with DNA is not evidence against the probable ferromagnetism of these signals, as was earlier supposed. The reasons for this are associated with the microwave and magnetic properties of such relatively massive particles, and if tiny iron particles are produced, they may be shown to give the 'DNA type" broad resonances.

The occasional disappearance of the e.s.r. signal of a DNA sample (as in Fig. 2) may be rationalized in the following way. The supposedly dry DNA sample has a fair amount of water of hydration which is bound to the molecule in such a way that its dielectric properties are not those of bulk water. As this is heated, droplets of water form by coalescence and the sample now becomes dielectrically lossy, the Q of the cavity is reduced, and the sensitivity of the e.s.r. decreases so that the signal amplitude falls. Close inspection of Fig. 2 will convince one that the resonance is still present.

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