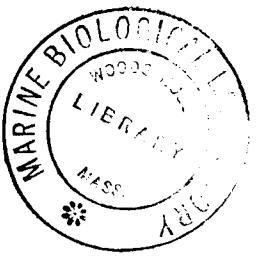

ELECTRON SPIN RESONANCE IN ATP AND RNA

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ABSTRACT Cu^{++}, Mn^{++}, and Fe^{+++} account for the electron spin resonances observed in certain samples of ATP and RNA. The copper ion seems more loosely bound to these substances than either iron or manganese. A striking similarity is observed between the manganese spectra in manganese RNA, ATP, and ADP suggesting that the binding sites are similar in the three compounds. The similarity of the e.s.r. spectra of iron ATP and of iron and manganese RNA, except for hyperfine spectrum (hfs) in the latter, suggests that the two ions bind similarly in the two compounds. A detailed interpretation of the spectra is lacking however and these conclusions can only be tentative. When manganese TPP and ATP are heated or pH changed the e.s.r. alters indicating a change in the environment of the ion. The sharp 6 line manganese spectrum in both TPP and ATP at pH 1 suggests an almost "free" ion at this pH in the sense of an almost isotropic average environment.

INTRODUCTION

In the course of work on electron spin resonances in lyophilized samples of nucleic acids and related compounds, a variety of resonance intensities and line shapes has been observed. The discussion here will be restricted to two resonances, quite complex and very similar to one another, one observed in a sample of ATP, and one observed in two different samples of yeast RNA. This resonance is referred to as the "ATP-RNA" resonance in the discussion. For a more general discussion of nucleic acid resonances see Bliumenfeld, Kalmanson, and Sheng Pei-Ken (1959), Blois and Maling (1960), Shulman *et al.* (1961), Walsh *et al.* (1962).

Weak resonances have been reported in ATP and ADP by Isenberg and Szent-Györgyi (1959) and those authors suggested that the resonances were due to either a paramagnetic impurity or to an unpaired electron associated with a charge-transfer-complex formed between the terminal phosphate and the adenine of the same ATP molecule. Since a strong resemblance was noted between the "ATP-RNA" resonance and the ATP resonance of Isenberg and Szent-Györgyi, and because it has been suggested that a charge-transfer-complex might also be the

source of the spin resonances observed in nucleic acids (Bliumenfeld, 1959), an analysis of this particular "ATP-RNA" spectrum was undertaken.

MATERIALS AND METHODS

The e.s.r. studies were done with a V-4500 Varian X-band spectrometer with 100 kc modulation, using a Varian 6-inch magnet.

Chemicals were obtained from the Nutritional Biochemical Corp. (NBC), Cleveland; the Sigma Chemical Co. (SCC), St. Louis; Delta Chemical Works, Inc. (DCW), New York; the Fisher Scientific Co. (FSC), Pittsburgh; The California Corporation for Biochemical Research (CCBR), Los Angeles; and C. F. Boehringer and Soehne (BS), Mannheim, Germany.

All samples were made up in solution and the pH was adjusted with HCl or NH_4OH and measured with a Beckmann model G pH-meter. All samples were lyophilized before examination in the spectrometer.

The abbreviations used in this paper are adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), sodium tripolyphosphate (TPP), and ribonucleic acid(RNA).

Preparations of TPP and ATP at pH 3 or lower were very hygroscopic. A lyophilization of 48 hours (at room temperature) was not enough to bring them to dryness; therefore they were examined in the e.s.r. spectrometer in a viscous state. The acidity of the most acid preparations (below pH 2) was determined by back titration.

Copper was extracted from the water solutions of ATP and RNA with 0.01 per cent (DCW) zinc dibenzyldithiocarbamate in carbon tetrachloride at pH 5 — 7. Iron and manganese were extracted from basic (pH 10) water solutions of ATP and RNA with several aliquots of a 0.1 per cent solution of 8-hydroxyquinoline (8-quinolinol).

Spin density estimates were made by comparing the integrated area of the resonance curves under study with integrated areas of the resonance curves for known concentrations of both Gd^{+++} in glycerol, and Mn^{++} in water.

Sample heating was done in tubes open to the air, in a small electric furnace. They were held at the desired temperature for 8 to 16 hours; the samples were then removed from the furnace and after cooling their e.s.r. were examined at room temperature. Samples were studied at low temperature in the e.s.r. cavity with a cold gas flow system using a liquid nitrogen immersed heat exchanger.

EVIDENCE FOR PARAMAGNETIC ION IMPURITIES[1]

Definite evidence for a copper ion impurity giving an e.s.r. in ATP has been found by Russel and Wyard (1961). These authors concluded that a resonance they observed in a sample of ATP was due to copper from the shape of the e.s.r. and its g-value, and by a quantitative correlation between trace amounts of copper present (75.2 ± 4 μgm copper/gm ATP) and the corresponding amount of copper in

[1] The word "impurity" is used advisedly since the reason for the presence of the metal ions is uncertain. A functional role played by the ion in the cell in complex with the molecule in question cannot be ruled out (See Wacker and Vallee 1959) although the most reasonable possibility at this point is that the ions have been picked up in the purification process, either externally or from the organic material from which the extractions have been made.

copper phthalocyanine which gave a resonance comparable in magnitude to the original resonance. In addition, Isenberg (1961) has recently discussed the possibility that an impurity is the source of the resonance in ATP previously reported by Isenberg and Szent-Györgyi (1959).

The characteristic appearance of the spectra in the case of the NBC ATP (Figure 1*c*) which we undertook to analyze argues strongly that an S-state paramagnetic ion is the source of the spectrum. The low intensity of the spectrum, its

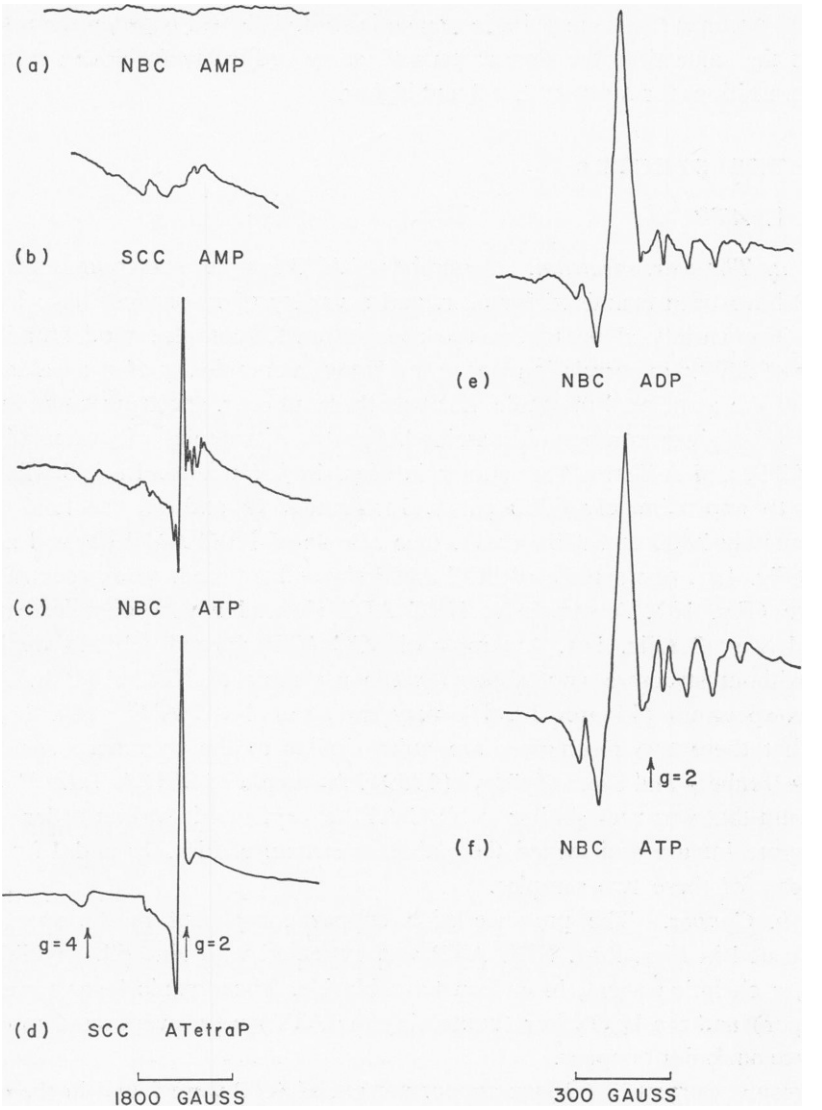

FIGURE 1 Electron spin resonance in samples of AMP, ADP, ATP, and A-Tetra-P.

general breadth and asymmetry, its position near $g=2$, and the fact that the resonance is obtainable at room temperature are consistent with this. Because of the structure, the source could be an ion of multiplicity higher than two or with nuclear spin; because of the breadth of the line as well as the structure, it is probably both. At the same time, the general breadth of the resonance, its asymmetry, and the presence of the $g=4$ component, suggests that the ion is in a strong electric field. This can cause a splitting between spin energy levels of different $|m_j|$ even in zero magnetic field. As a result the strongly allowed ($\Delta m = \pm 1$) transitions will no longer all occur at the same point in magnetic field. This will broaden the resonance line. At the same time the interaction will allow ordinarily forbidden transitions, that is, transitions for $\Delta m = \pm 2, \pm 3$ and higher.

THE SPECTRA

1. *ATP*

a. The e.s.r. spectrum. A number of ATP samples and compounds related to ATP have been examined for e.s.r. and a variety of resonances have been observed. The variety alone makes the charge-transfer-complex model unlikely in the case of ATP. No correlation was found between complexity of spectra and complexity of the associated molecule, nor was there an e.s.r. spectrum common to all samples of a given compound examined. Spectra of samples of untreated AMP, ADP, ATP, and A-Tetra-P are shown in Figs. 1*a-f*. (In Figs. 1*a-d*, the field scan is zero to approximately 6000 gauss. In Figures 1*e* and 1*f*, the field scan is approximately 2800 to 3800 gauss). One sample of NBC AMP showed no resonance (Fig. 1*a*), one sample of SCC AMP showed a broad, weak spectrum with structure (Fig. 1*b*). A sample of NBC ADP showed a very complex structure centered at $g=2$ (Fig. 1*e*). A sample of SCC ATP showed a broad weak resonance without structure (not shown) while a sample of NBC ATP has a very complex spectrum (Figures 1*c*, 1*f*) very similar to NBC ADP (Fig. 1*e*). It is noted that these two resonances are quite similar to the resonance reported in ATP by Isenberg and Szent-Györgyi (1959). A sample of SCC A-Tetra-P showed a spectrum that was very similar to NBC ATP except that it was an order of magnitude more intense and lacked the elaborate structure. Figs. 1*c* and 1*d* compare the spectra of these two samples.

b. Copper. The presence of a copper component in the spectrum is demonstrated in Figs. 2*a-f*. NBC ATP was extracted with zinc dibenzyldithiocarbamate, a chelating agent, in carbon tetrachloride. The organic layer (containing the copper) and the H_2O layer (containing the ATP) were evaporated to dryness for a trace analysis of copper.

The results were: (1) 60 μgm copper per gm of ATP were found in the organic layer while the ATP (in the aqueous layer) was copper-free; (2) the sharp, asym-

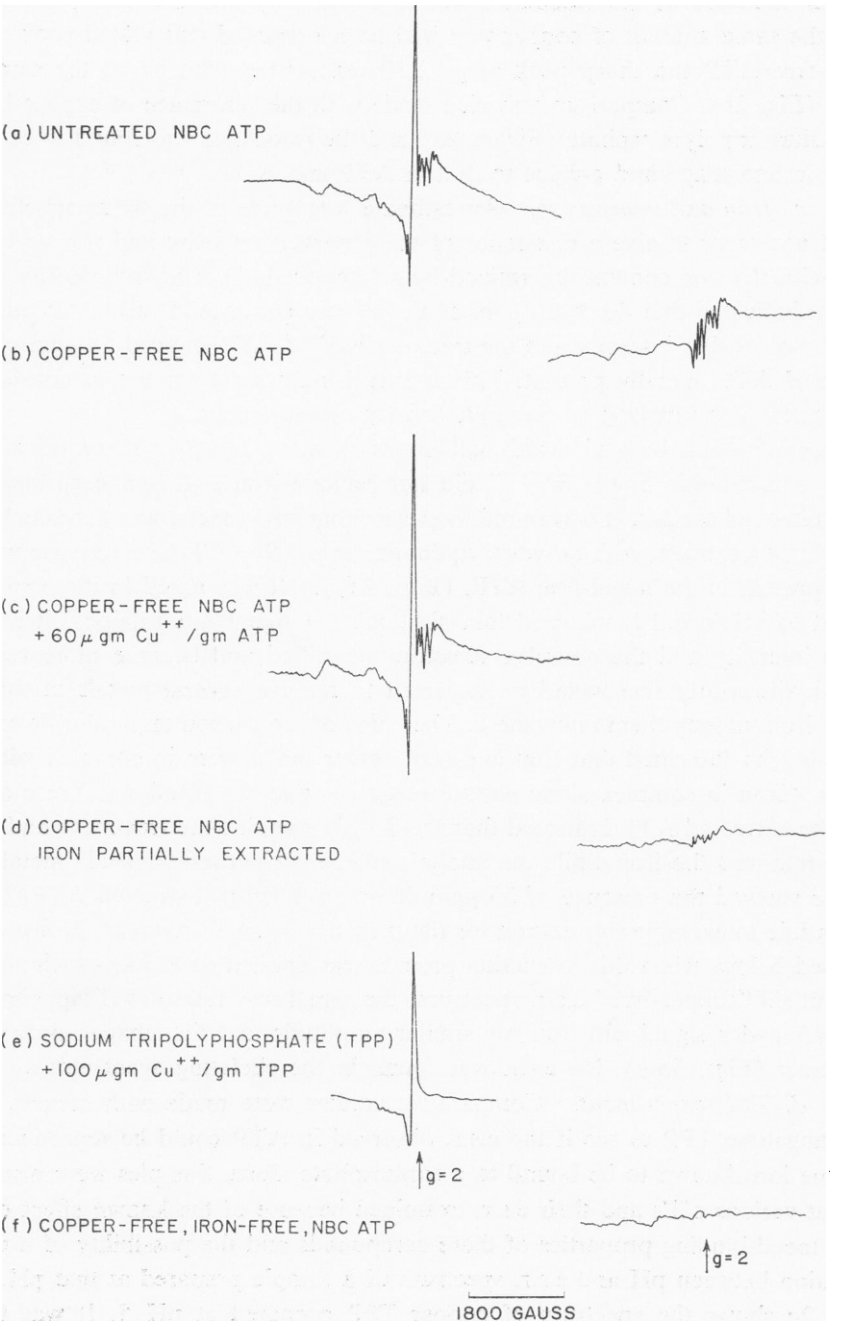

FIGURE 2 Electron spin resonance in untreated and metal-extracted ATP.

metric resonance at $g=2.10$ disappeared from the ATP e.s.r. (Figures 2*a*, *b*). (The complex structure in the resonance was unaffected by this treatment, however.) When the same amount of copper that had been extracted was added back to the copper-free ATP the sharp peak at $g=2.10$ reappeared with about the same intensity (Fig. 2*c*). Comparison was also made with the resonance of copper bound to disodium tripolyphosphate (Figure 2*e*) and the resonance was found to be quite similar in line shape and *g*-value to that in ATP and ADP.

c. Iron and manganese. An estimate was made of the paramagnetic iron present necessary to give a resonance of the observed intensity and this was compared with the iron content determined by a trace analysis. The spin density measurement indicated that 40 ± 20 μgm of Fe^{+++} per gm of ATP would account for a resonance of that intensity and the trace analysis of ATP showed 53 μgm of iron per gm of ATP actually present. This is surprisingly good agreement considering the uncertainties involved in the spin density measurement.

Extractions with both zinc dithiodibenzylcarbamate and 8-hydroxyquinoline in carbon tetrachloride at pH 4 – 7 did not remove iron although both chelating agents removed copper. It was found, however, that when metal was extracted from the ATP by treatment with 8-hydroxyquinoline at pH 9 – 10 the resonance was no longer present in the metal-free ATP. (Figs. 2*d*, *f*.) It was noted in those samples brought to pH 9 and lyophilized that although the signal was retained, it seemed to lose intensity and the complex structure simplified and became more regular. The 8-hydroxyquinoline would be expected to remove several metals at this pH besides iron, among them manganese. The color of the carbon tetarchloride extract (green-black) indicated that iron and some other metal were in complex with the reagent. (Iron in complex alone gives a black color to the solution). Trace analysis of the extracted ATP indicated that the 8-hydroxyquinoline treatment had completely removed the iron while the analysis of the carbon tetrachloride metal concentrate showed the presence of 55 μgm of iron per gm of extracted ATP. It was not possible to examine this extract for the presence of an e.s.r. signal. However, as described below, when this extraction process was applied to RNA, which showed the identical "copper-free" e.s.r. spectrum, the signal was found to disappear from the RNA and a signal with roughly similar amplitude and line shape appeared in the extract (Figs. 5*a-e*). No e.s.r. was found in the chelating agent alone.

d. TPP experiment. Comparative studies were made with copper, iron, and manganese TPP to see if the e.s.r. observed in ATP could be reproduced by the same ions known to be bound to a triphosphate alone. Samples were also prepared at various pH's and their e.s.r. examined because of the known effect of pH on the metal-binding properties of these compounds and the possibility of a useful correlation between pH and e.s.r. spectrum of a sample prepared at that pH.

Fig. 2*e* shows the spectrum of copper TPP prepared at pH 5. It was found to be unchanged in samples made at pH 1. In Figs. 4*a-c* the resonance spectrum

of Fe+++ TPP (pH 1 and pH 4) is shown. As the pH is lowered the ratio of the amplitude of the $g=4$ component to that of the $g=2$ component becomes less and this effect is reversible. Because of the complex line shape and poor signal-to-noise ratio it is difficult to determine in detail how the line shape varies. It would seem that the $g=2$ peak loses area possibly to the tails, at least to the "tail" extending to zero field, while the $g=4$ component remains relatively constant in both shape and amplitude. One should note the structure which appears at pH 1 and disappears again at pH 4. This structure has identical splittings (85 ± 5 gauss) and

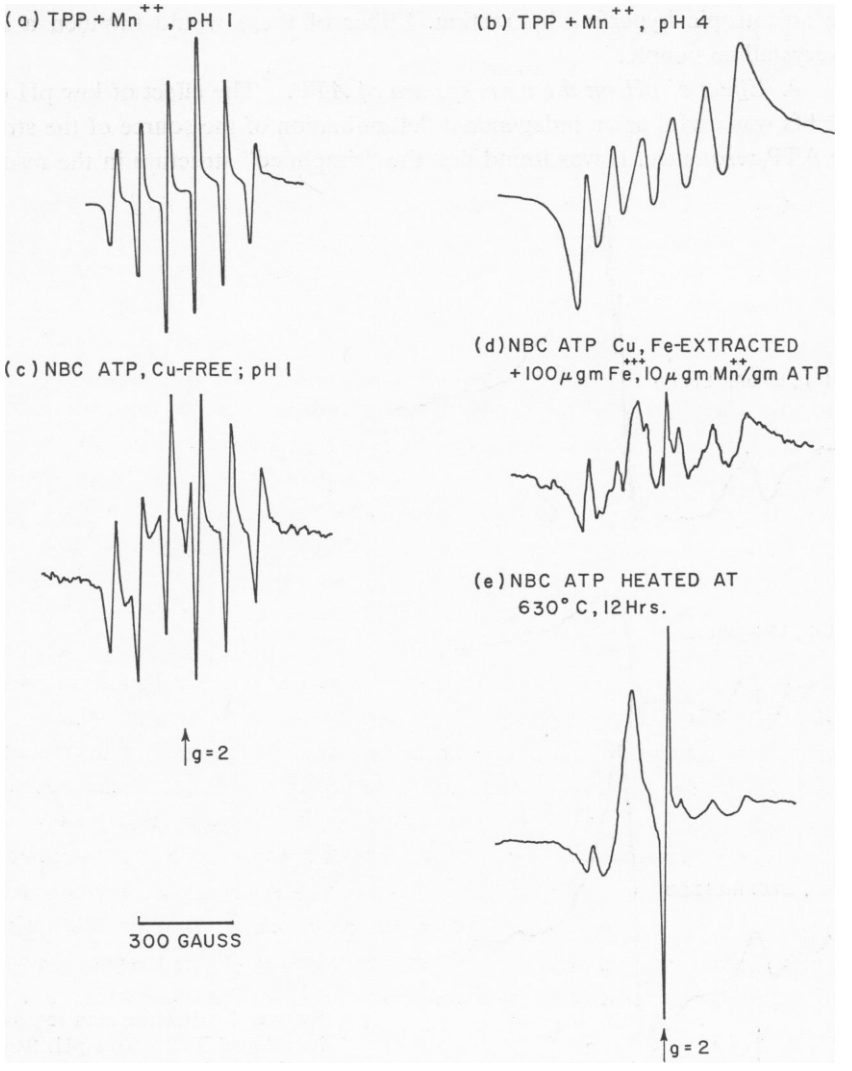

FIGURE 3 Electron spin resonance in treated TPP, ATP.

g-value ($g=2.006 \pm 0.002$) and the same relative amplitudes of hyperfine lines as that of the manganese TPP to be discussed below. It therefore must be due to a manganese impurity. In the e.s.r. of manganese TPP the six hyperfine components are seen to narrow markedly and increase in amplitude as the pH is lowered from 4 to 1. (Figs. 3*a*, *b*). This explains the appearance and disappearance with change in pH of the weak Mn++ hyperfine spectrum (hfs) in the spectrum in Fig. 4. The reason for the observed line width variation with varying pH is not certain. It is possible that change in pH causes a variation in strength of binding of the ion to the TPP, leading in turn to a variation in fine structure splitting or a modification of the anisotropic hyperfine interaction. Either of these would broaden a line in a polycrystalline sample.

e. Effect of pH on the e.s.r. spectra of ATP. The effect of low pH on the Mn++ hfs was useful as an independent determination of the source of the structure of the ATP resonance. It was found that the "simplified" structure in the resonance

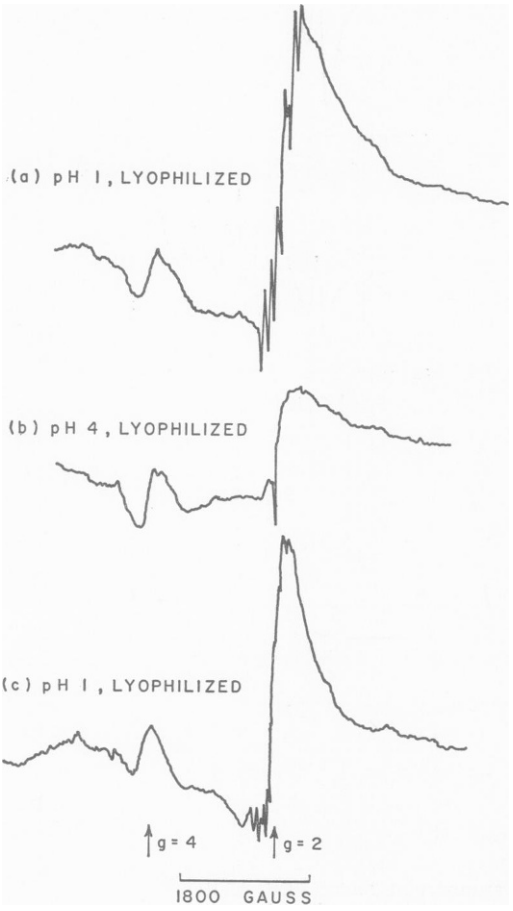

FIGURE 4 Electron spin resonance in iron-doped TPP *versus* pH. Sample is put in solution, pH adjusted, and sample is lyophilized.

of iron-ATP with 10 μgm of Mn per gm of ATP (Fig. 3*d*) disappeared when the pH of the sample was lowered to 1, and a six line spectrum appeared in its place which correlated exactly with the Mn hyperfine structure in manganese TPP, pH 1 (Fig. 3*a*). The simplified structure is certainly due, therefore, to the 0.001 per cent Mn impurity present in the ATP.

The original NBC ATP sample was then tested in this way for manganese without resorting to further trace analysis. When a sample which contained the complex structure (Figs. 1*c*, *f*) was taken to pH 1 and lyophilized, the e.s.r. showed the characteristic sharp 6 line hfs of manganese, with about one-fourth the intensity of the 0.001 per cent manganese ATP spectrum (Fig. 3*c*). It was concluded that the NBC ATP had 2 to 3 μgm of manganese per gm of ATP, in addition to 60 μgm of copper and 53 μgm of iron per gm of ATP.

2. *RNA*

a. The e.s.r. spectrum. A broad, asymmetric spin resonance matching the resonance found in the sample of copper-free NBC ATP was found in two different samples of RNA: one, yeast nucleic acid (grade c, CCBR); and two, a very pure yeast RNA (BS).

b. Copper. It was concluded that little or no copper was present in these samples and this conclusion was borne out by a trace analysis.

c. Iron and manganese. Following the analysis applied to the copper-free ATP it was proven that this e.s.r. spectrum was characteristic of a combination of e.s.r. spectra from traces of Fe+++ and Mn++ ions.

Trace analysis indicated roughly 400 to 500 μgm of iron per gm of RNA in both samples of RNA which agreed within experimental error with the amount of paramagnetic Fe+++ (predicted from a spin density measurement) to account for a resonance of that intensity. As with NBC ATP, the e.s.r. could be eliminated from both RNA's by extended treatment with 8-hydroxyquinoline at pH 11, and a signal appeared in the extract with approximately the expected amplitude; *i.e.*, the signal strength was conserved in transferring from sample to extract. Figs. 5*a-e* illustrate this for the pure yeast RNA. The line shape altered to a certain extent in transfer and the elaborate structure is not present in the extract e.s.r. Note that the resonance in RNA at pH 8.85 is weakened and the structure is simpler and less prominent.

The color of the extract was green-black indicating that iron and one or more other metals were in complex with the chelating agent and therefore at least one of these metal ions, probably iron, was responsible for the resonance. Samples of metal-free RNA (RNA that had been extracted with the reagent) were then doped with iron to see if the original resonance would reappear. The results are shown in Fig. 6*a*. The iron RNA spectrum resembles very closely the spectra of the original samples, including a weaker replica of the complex structure at $g=2$. From the

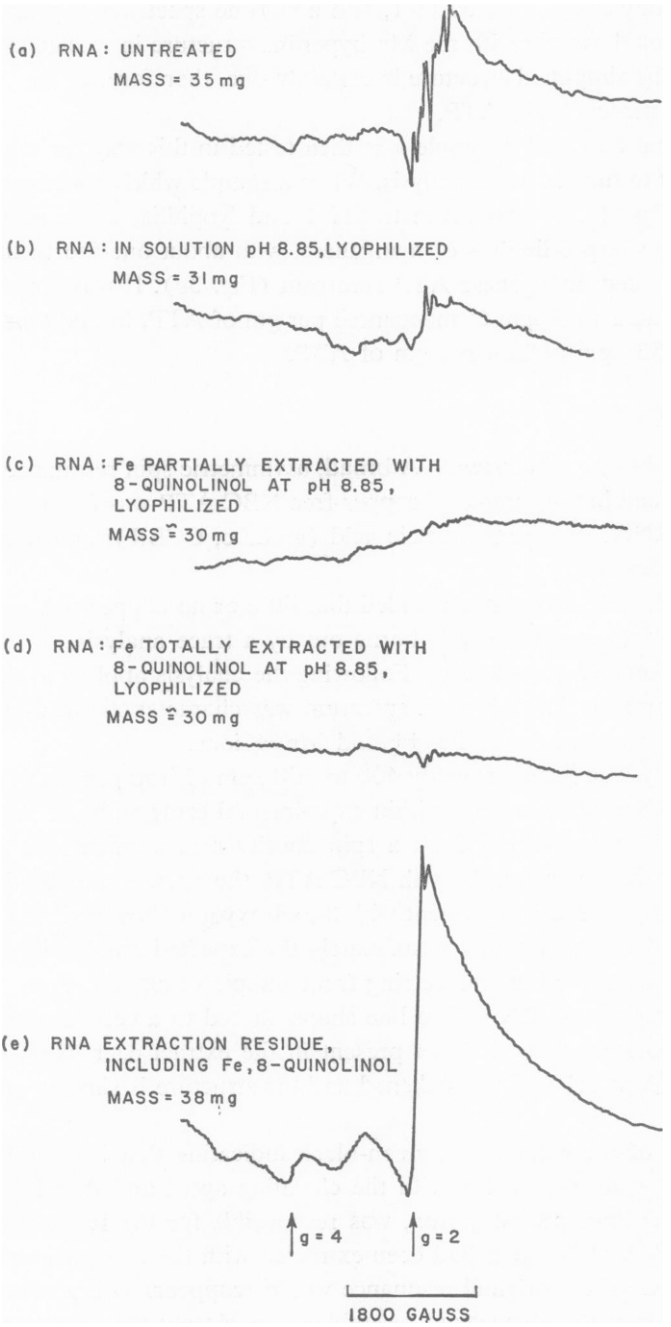

FIGURE 5 Electron spin resonance in metal-extracted pure yeast RNA (Boehringer).

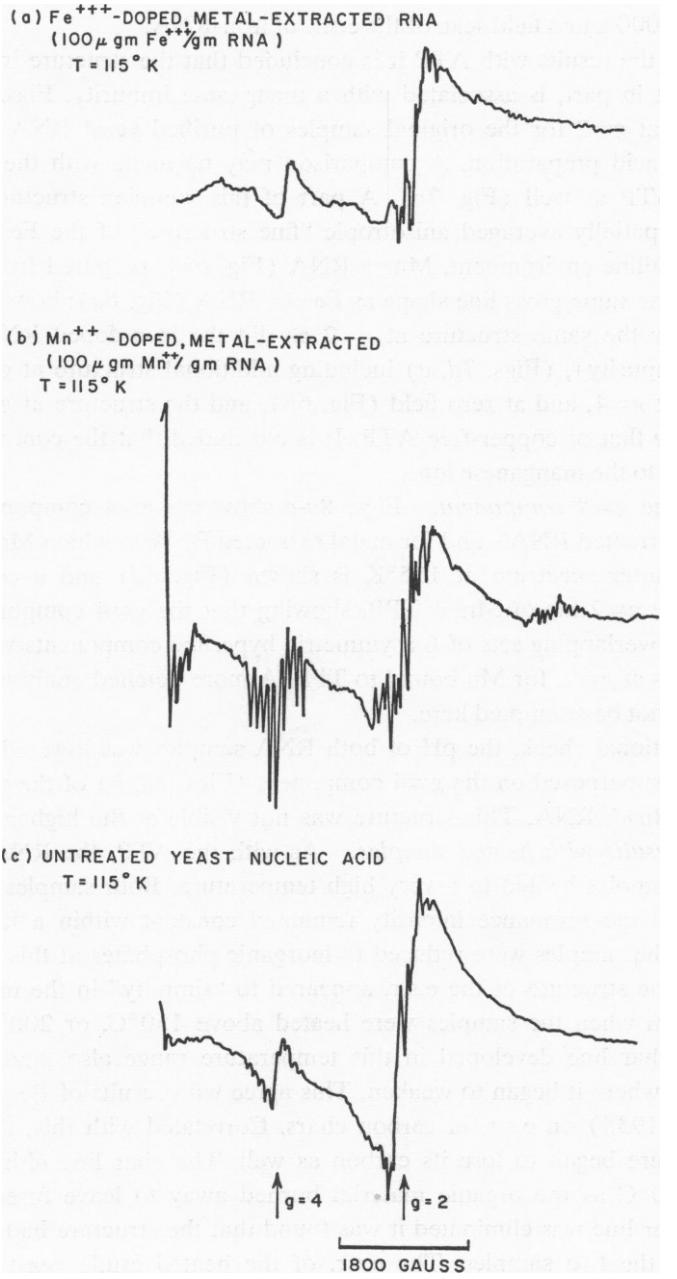

FIGURE 6 Electron spin resonance in treated and untreated RNA. (Samples treated in solution at pH 1, and then lyophilized.)

ATP results we believe that this structure is due to a Mn impurity in the iron salt used to dope the sample or due to a slight residue of Mn in the sample itself. Fig. 6*c* shows a 0 to 6000 gauss field scan of the e.s.r. of iron-RNA.

Because of the results with ATP it is concluded that the structure in the original RNA, at least in part, is associated with a manganese impurity. Figs. 7*a*, *b* show the structure at $g=2$ for the original samples of purified yeast RNA and a crude yeast nucleic acid preparation. A comparison may be made with the structure of copper-free ATP as well (Fig. 7*c*). A part of this complex structure might still be due to a spatially averaged anisotropic "fine structure" of the Fe+++ ion in a suitable crystalline environment. Mn++ RNA (Fig. 6*b*), prepared from metal-free RNA shows the same gross line shape as Fe+++ RNA (Fig. 6*a*); however, it shows almost *exactly* the same structure at $g=2$ as did the iron-doped RNA (with the manganese impurity), (Figs. 7*d*, *e*) including additional structure at $g=1.3$, possibly at $g=3$, at $g=4$, and at zero field (Fig. 6*b*), and the structure at $g=2$ matched almost exactly that of copper-free ATP. It is concluded that the complex structure is due entirely to the manganese ion.

d. The g=4 component. Figs. 8*a-c* show the $g=4$ component in detail for the two untreated RNA's and for metal extracted RNA to which Mn++ has been added. The latter spectrum at 115°K is shown (Fig. 8*d*) and a comparison is made with the $g=2$ hfs of Mn++ TPP, showing that the $g=4$ component is really two partially overlapping sets of 6 asymmetric hyperfine components with the same splitting as hfs at $g=2$, for Mn bound to TPP. A more detailed analysis of the e.s.r. structure will not be attempted here.

As an additional check, the pH of both RNA samples was lowered and a weak hfs appeared superposed on the $g=4$ component (Figs. 8*a*, *b*) of the spectrum just as with the Mn++ RNA. This structure was not visible at the higher pH's.

e. Results with heated samples. As with the ATP, the RNA resonances persisted in samples heated to a very high temperature. Both samples were heated to 630°C and the resonance intensity remained constant within a factor of two, even though the samples were reduced to inorganic phosphates at this temperature. In addition, the structure of the e.s.r. appeared to "simplify" in the manner of the ATP spectrum when the samples were heated above 150°C, or 200°C. A strong free radical char line developed in this temperature range also, and persisted to about 550°C where it began to weaken. This agree with results of Bennett, Ingram, and Tapley (1955) on e.s.r. in carbon chars. Correlated with this, the sample at this temperature began to lose its carbon as well. The char line ultimately disappeared at 600°C as the organic material burned away to leave fused phosphate. When this char line was eliminated it was found that the structure had taken different forms in the two samples. The e.s.r. of the heated crude yeast nucleic acid resembled the e.s.r. of heated ATP except for the presence of the copper component in the latter's spectrum, and it also resembled the spectrum of Mn++ TPP

at pH 4 (Fig. 3*b*). The 630°C purified yeast RNA spectrum was altered from that of the crude yeast NA although initially the two spectra were identical.

DISCUSSION

1. *Source of the ATP-RNA e.s.r. Spectra*

Trace amounts of 3 paramagnetic ions, copper, iron, and manganese, have been shown to be responsible for the broad asymmetric e.s.r. observed in the samples of ATP and RNA analysed. The ion system is assumed to be a paramagnetic one because there was agreement within experimental error between amount of paramagnetic iron in the ferric state, estimated to be present from e.s.r. intensity measurements, and the amount of iron actually present as determined by trace analysis. However, the intensity of the resonances as a function of temperature in general does not appear to obey the Curie law; line intensity is enhanced less than a factor $1/T$ with decreasing temperature. In addition the $g=4$ component seems to be disproportionately enhanced with respect to the broad $g=2$ resonance at lower temperatures. With the relatively poor signal-to-noise ratio available in these broad signals, it is difficult to determine exact changes in line shape (almost impossible in the tails), so that intensity measurement could easily be in error because of an inapparent change in line shape.

2. *Metal Ion Binding*

The chelation experiments suggest that manganese and iron are bound tightly to the ATP, while copper is less tightly bound. Both zinc dithiodibenzylcarbamate and 8-hydroxyquinoline, in excess, at pH 7, removed copper but failed to remove iron and manganese. Since these reagents do not preferentially remove copper at this pH (Sandell, 1959), the iron and manganese must bind much more tightly to the molecule than the copper. In addition Mn and Fe were removed together, suggesting that these ions are bound in a similar way. These results are compatible with NMR measurements by Cohn and Hughes (1962) on copper and manganese ATP. Their chemical shift measurements on phosphorus in manganese ATP in solution indicate that copper binds to the β and γ phosphates while the manganese either forms a single species of complex with ATP where it binds to all three phosphates or it forms a mixture of coexisting complexes, giving the appearance of a single three-phosphate complex.

Conclusions based on similarities and differences in e.s.r. spectra are at best tentative because no definite model has been established which explains in detail these very complex resonances. Such a detailed explanation involves a discussion of the microscopic environment of the ion, the symmetry of the binding site, the strength of interaction and the multiplicity of the ion. A knowledge of the spin-Hamiltonian is needed (see Bleaney and Stevens, 1953, for a description of the

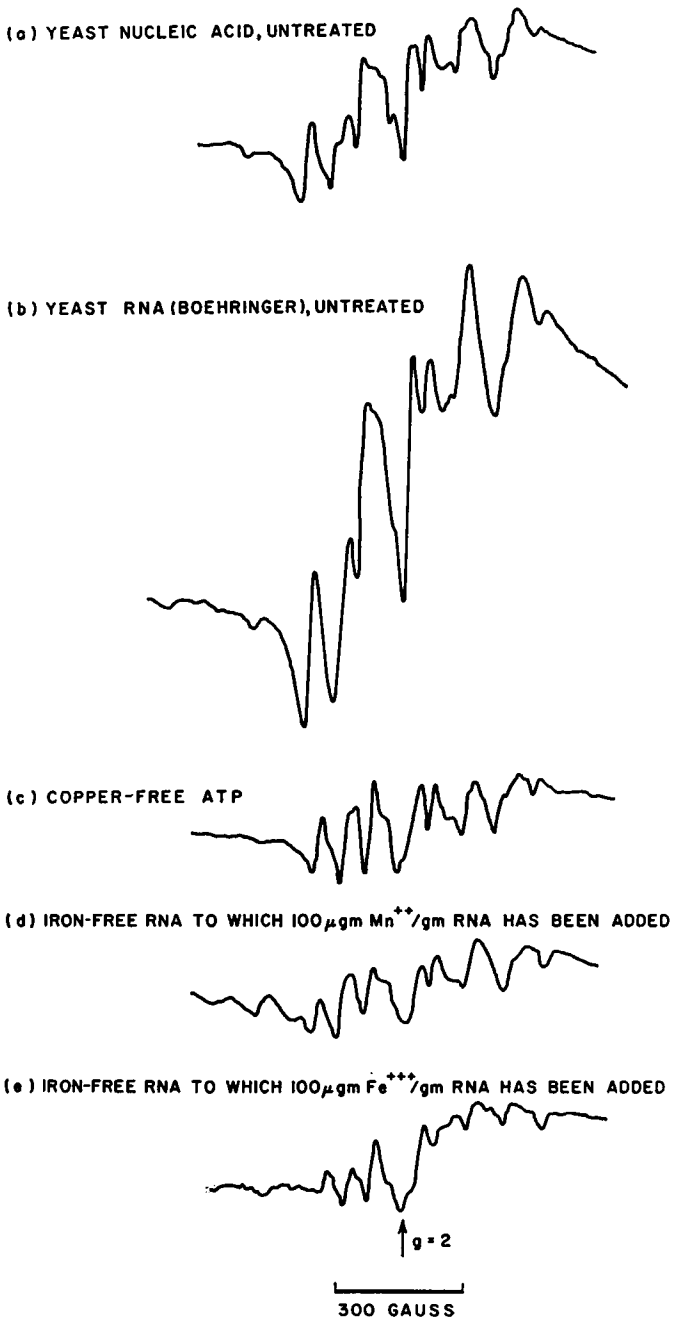

FIGURE 7 Structure at $g = 2$ in the e.s.r. of treated and untreated ATP and RNA.

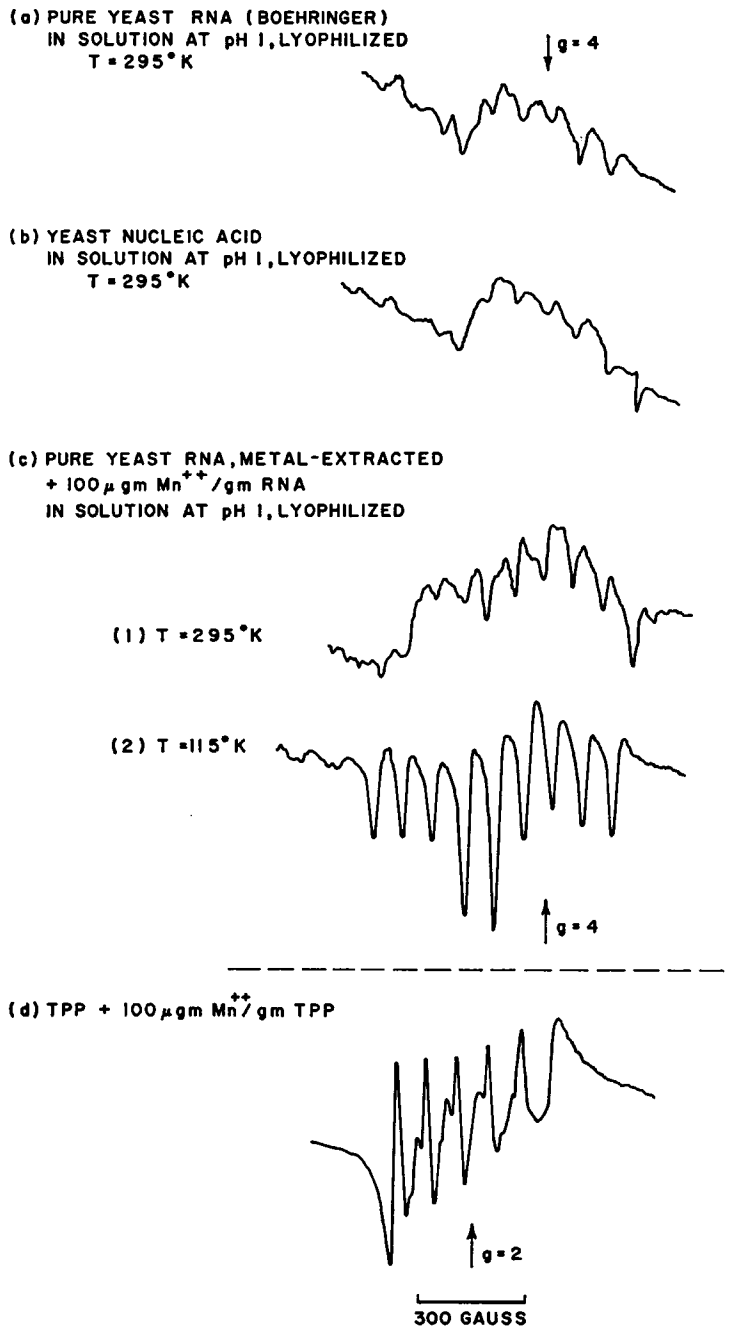

FIGURE 8 Structure at $g=4$ in the e.s.r. of RNA.

spin-Hamiltonian formalism). The origin of the complex structure of Mn ATP and RNA, other than the fact that it is due to an anisotropic hyperfine coupling with the nuclear spin of Mn, is not certain. The spectrum in such a case depends upon the orientation of the microscopic environment of the ion with respect to the external field. These are polycrystalline samples and can easily produce very complex e.s.r. line shapes because of contributions to the e.s.r., from all possible orientations of the crystal axes. Additional complication is also possible due to a g-value anisotropy. This type of problem is discussed in detail for example in H. M. McConnell's theory of hyperfine line widths in the e.s.r. of Mn^{++} in solution, based on a microcrystalline model (McConnell, 1956); by O'Reilly (1958, 1959) for vanadyl etioporphyrin I; by Roberts, Koski, and Caughey (1961) for vanadyl etiophorphyrin II; and for Vo^{++} by Rogers and Pake (1960).

Iron RNA and manganese RNA gave surprisingly similar e.s.r. spectra except that structure due to hyperfine coupling with the manganese nucleus was superposed on the latter at $g=1.3$, 2, 3, 4, and at zero field. In addition the complex structure at $g=2$ due to manganese is the same in a sample of ATP, two samples of RNA, and is strikingly similar to a spectrum in a sample of ADP. This suggests that the binding site for manganese in the three substances is the same. Suppose the site involves three phosphates, it is possible for the ion to bind to a single molecule in the case of ATP and, of course with RNA, although it is not clear why the binding site should have the same symmetry. In the case of ADP two molecules at least would be necessary; unless the ion were associated with contaminating amounts of ATP in the ADP sample. Spatial configuration might again be expected to differ yet the spectra are similar. Bonding by manganese to two phosphates instead of three would therefore be more compatible with our data.

3. *The "Simplified" Spectrum*

This spectrum was seen in manganese TPP at pH 4 and above, in heated manganese RNA and ATP, and in manganese RNA and ATP at pH 9. Its appearance in ATP and RNA must reflect a change in environment of the Mn^{++} ion due to these treatments. Since the complex structure was never reproduced in the Mn TPP the binding site of the ion in ATP and RNA may involve a ribose and/or the base. Cohn and Hughes (1962) show with NMR measurements, that paramagnetic ions bound to ATP interact with H-8 on the adenine ring as well as with the triphosphate moiety.

The spectra of Mn ATP and Mn TPP prepared at pH 1 indicate a drastic change in the environment of the metal ion. Variation in line widths and symmetry of the sharp 6 line spectrum indicate both a slight anistropic hyperfine coupling and a slight g-value anisotropy. The hyperfine coupling is essentially isotropic however. Sharper manganese hyperfine structure is seen in e.s.r. of manganese in single crys-

tals but these acid-polyphosphate samples are viscous liquids. This suggests that the ion is almost free or in an environment of high symmetry. The ion could be in a complex where tumbling or exchange of surrounding molecules bonded to it is rapid enough to average any effect of an anisotropy in environment (almost) to zero. This possibility seems unlikely, however, because the spectrum changes very little from 300°K to 77°K, and the resonance at room temperature shows some saturation at 100 milliwatts rf power, indicating spin lattice relaxation time increased over that at the higher pH. No saturation was detected at this power level for the higher pH e.s.r. of manganese-ATP.

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BIBLIOGRAPHY

BENNETT, J. E., INGRAM, D. J. E., and TAPLEY, J. G. 1955, *J. Chem. Physics*, **23**, 215.

BLEANEY, B., and STEVENS, K. W. H., 1953, *Rep. Progr. Physics*, **16**, 108.

BLIUMENFELD, L. A., KALMANSON, A. E., and SHENG PEI-KEN, 1959, *Dokl. Akad. Nauk SSSR*, **124**, 1144 (A59); *Biofizika*, **4**, 263.

BLIUMENFELD, L. A., 1959, *Biofizika*, **4**, 515.

BLOIS, M. S., JR., and MALING, J. E., 1960, *Biochem. and Biophysic. Research Communications*, **4**, 252.

COHN, M., and HUGHES, T. R., JR., 1962, *J. Biol. Chem.*, **237**, 176.

ISENBERG, I., 1961, *Biochem. and Biophysic. Research Communications*, **5**, 139.

ISENBERG, I., and SZENT-GYÖRGYI, A., 1959, *Proc. Nat. Acad. Sc.*, **45**, 1232.

MCCONNELL, H. M., 1956, *J. Chem. Physics*, **25**, 709.

O'REILLY, D. E, 1958, *J. Chem. Physics*, **29**, 1188.

O'REILLY, D. E, 1959, *J. Chem. Physics*, **30**, 591.

ROBERTS, E. M., KOSKI, W. S., and CAUGHEY, W. S., 1961, *J. Chem. Physics*, **34**, 591.

ROGER, R. N., and PAKE, G. E., 1960, *J. Chem. Physics*, **33**, 1107.

RUSSEL, D. B., and WYARD, S. J., 1961, *Nature*, **191**, 65.

SANDELL, E. B., 1959, Colorimetric Determination of Traces of Metals, 3rd edition, New York, Interscience Publishers, Inc., 180.

SHULMAN, R. G., WALSH, W. M., WILLIAMS, H. J., and WRIGHT, J. P., 1961, *Biochem. and Biophysic. Research Communications*, **5**, 52.

WACKER, W. E. C., and VALLEE, B. L., 1959, *J. Biol. Chem.*, **234**, 3257.

WALSH, W. M., JR., SHULMAN, R. G., and HEIDENREICH, R. D., 1962, data to be published.