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*NUCLEIC ACIDS AND METALS, II: TRANSITION METALS AS DETERMINANTS OF THE CONFORMATION OF RIBONUCLEIC ACIDS**†

BY KEIICHIRO FUWA,‡ WARREN E. C. WACKER, ROBERT DRUYAN§, ANTHONY F. BARTHOLOMAY, AND BERT L. VALLEE

BIOPHYSICS RESEARCH LABORATORY, DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL, AND THE PETER BENT BRIGHAM HOSPITAL, BOSTON

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RNA isolated from a wide variety of biological sources, extending from simple microorganisms to mammals, contains significant amounts of metals. Metals of the first transition series, especially, are bound so firmly to RNA that they are not readily removed by chelating agents which are known to bind these ions quite avidly.[2] The functional significance of this observation has been uncertain.

The present studies demonstrate that metals in RNA maintain the secondary helical structure of the molecule through the formation of intramolecular bonds. The stabilization of the conformation of RNA by metals has been established through an examination of their effect on the characteristics of the phase-transition

which RNA undergoes on heating. As the temperature is raised, a phase-transition is observed which has been interpreted to reflect the melting-out of the ordered helical structure of RNA due to the disruption of intramolecular hydrogen bonds between the component nitrogenous bases. This melting curve may be measured either by an increase in absorbancy at 258 mμ or by a decrease of specific optical rotation.[3-6] We now find that metals of the first transition series of the periodic system affect a different parameter of the phase transition curve, i.e., A_{max}, the maximum increase in absorbancy attained as a result of heating. Variations in A_{max}, as a function of metal ion concentration, indicate that metal bonds, in addition to hydrogen bonds, play a decisive role in the stabilization of the molecular conformation of RNA.

Materials and Methods.—RNA was prepared by phenol extraction from beef liver,[7] rat liver,[8] and tobacco mosaic virus.[9] Beef liver RNA was successively precipitated with 2 volumes of ethanol 5 times from 0.1 versene and 3 times from 1×10^{-3} *M*, 1,10-phenanthroline to prepare "metal-free" RNA. Ammonium acetate buffer was prepared by titration with acetic acid. Both this buffer and NaCl were extracted 3 times with 0.01 per cent dithizone at acid, neutral, and alkaline pH to remove contaminating metals. Urea was recrystallized twice. Metals were added as the chloride salts; all, except magnesium, were prepared from reagent grade chemicals of known purity; magnesium chloride was prepared by dissolving magnesium metal (Johnson-Matthey Ltd., London, "Specpure") in distilled HCl and heating to dryness. Dialyses were performed in Visking tubing, prepared before use.[10] Metal-free water and acid-cleaned glassware were used throughout.[11]

RNA was dissolved in NaCl less than 48 hr before use and stored at 4°. One half to 2 hr prior to heating, this stock solution was diluted to 25 μg of RNA per ml of buffer (0.025 *M* NH_4 ($C_2H_3O_2$) in 0.025 *M* NaCl, pH 6.8); metals were added at 4°.

A Beckman DU Spectrophotometer equipped with a thermospacer and connected to an external circulator was employed for spectrophotometric measurements. Temperature was measured by a thermometer in the cell compartment. The melting curves were observed while the temperature changed at a rate of 1° per min.

Specific optical rotation was measured with a model 200S-80Q photoelectric spectropolarimeter with an oscillating prism (O. C. Rudolph and Sons) using a 10 cm water-jacketed cell; the RNA concentration was 1.25 mg/ml in 0.025 *M* NH_4 ($C_2H_3O_2$), pH 6.8, and 0.025 *M* NaCl. After the circulating water bath reached the stated temperature, ten minutes were allowed for equilibration.

Results.—The absorption of radiation by beef liver RNA, measured at 258 mμ, shows marked hypochromicity compared to that of its component bases, measured on the hydrolysate: an increase in absorbancy of 1.31 is obtained reproducibly on alkaline hydrolysis, expressed as the ratio of the absorbancy at 258 mμ after hydrolysis to the absorbancy at 10°. While ribonucleic acids from different sources have been shown to behave qualitatively similarly, quantitative differences in this hypochromic effect are characteristic of each RNA.[12]

On heating, RNA also becomes hyperchromic by asymptotically approaching a limiting value of absorbancy which is significantly less than that observed on hy-

drolysis.[12a] For native beef liver RNA there is a reproducible maximal increase of absorbancy of 1.22, expressed as the ratio of the maximal absorbancy to the absorbancy at 10°. We shall henceforth refer to this maximal increase as A_{max}.

A_{max} is markedly affected by metals of the first transition series. It can be augmented to 1.26 on removal of some of the metals intrinsic to native RNA, as described under *Methods*.[12b] The transition metal content intrinsic to native beef liver RNA provides a concentration of these metals equal to 5×10^{-6} *M* under the conditions here employed. The striking increase in A_{max} of the "metal-free" RNA is brought about by lowering the content of the intrinsic transition metals to only 1×10^{-6} *M*. Conversely, the addition of Ni^{++} ions, e.g., results in a marked *de-*

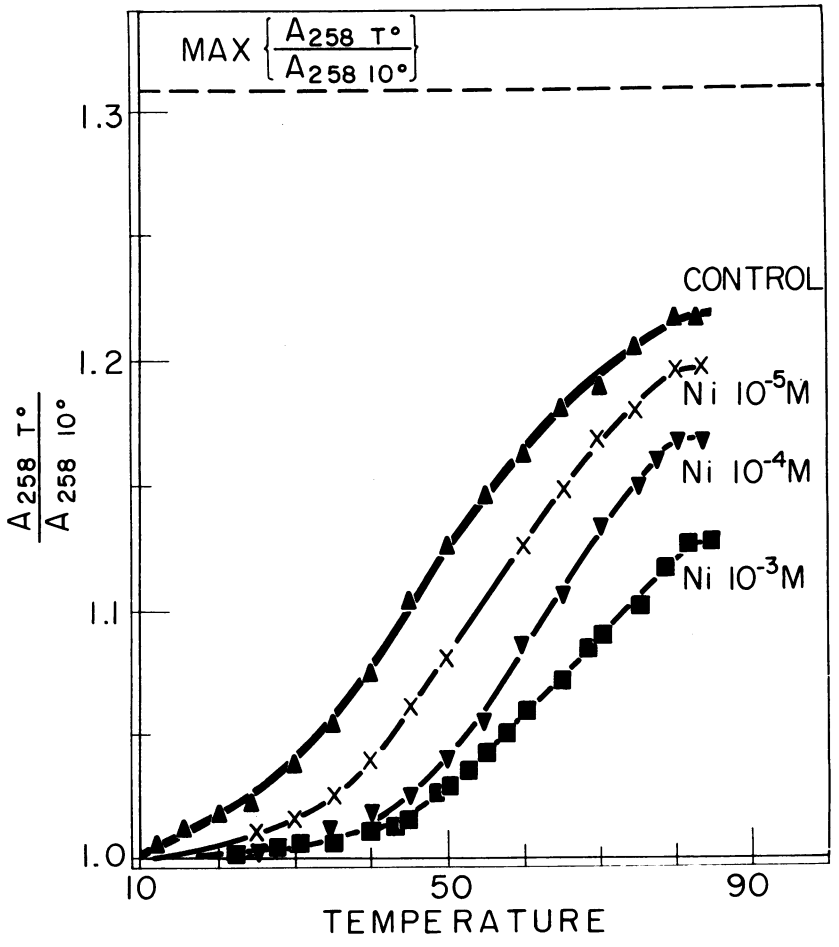

FIG. 1.—The effect of nickel on the phase transition curve of beef liver RNA. In this and subsequent figures, the increase in A_{258}, as a function of temperature is expressed as the ratio of the absorbancy at any given temperature to the absorbancy at 10°, $A_{258\,T°}/A_{258\,10°}$. The dashed line at the top indicates the maximum hyperchromicity obtained by alkaline hydrolysis. All experiments were performed under identical conditions: RNA content = 25 μgm/ml; Buffer = 0.025 *M* $NH_4(C_2H_3O_2)$ + 0.025 *M* NaCl, pH 6.8. Control of temperature as in the text. A_{max} of control (▲) = 1.22; plus 10^{-5} *M* Ni^{++} (X) = 1.20; plus 10^{-4} *M* Ni^{++} (▼) = 1.16; plus 10^{-3} *M* Ni^{++} (■) = 1.13.

pression of A_{max}, the extent of the depression being dependent on the concentration of the ion (Fig. 1). This effect, however, is not limited to nickel; in fact, it is observed with other ions of the first transition group of the periodic system shown in Table 1 which lists the hypochromic effect, induced by Cr^{+++}, Mn^{++} and Zn^{++}.

TABLE 1

EFFECT OF ADDED METALS ON A_{MAX} OF THE MELTING CURVE OF BEEF LIVER RNA

pH 6.8	A_{MAX}	pH 6.8	A_{MAX}
Control*	1.22	Li^{+} 10^{-3} M	1.22
Cr^{+++} 3×10^{-4} M	1.14	Na^{+} 10^{-1} M	1.20
Mn^{++} 3×10^{-4} M	1.17	K^{+} 10^{-3} M	1.22
Ni^{++} 10^{-3} M	1.12	Mg^{++} 10^{-3} M	1.19
Zn^{++} 10^{-3} M	1.13	Ca^{++} 10^{-3} M	1.21

* 0.025 M $NH_4(C_2H_3O_2)$ + 0.025 M NaCl, pH 68.

In the presence of 10^{-5} Cu^{++} and Fe^{+++}, precipitation occurred on heating, precluding similar observations with these metals.

In contrast neither Na^{+}, K^{+}, or Li^{+}, exemplifying the alkali metals, nor Ca^{++} and Mg^{++}, representing the alkalin eearths, in concentrations varying from 10^{-1}

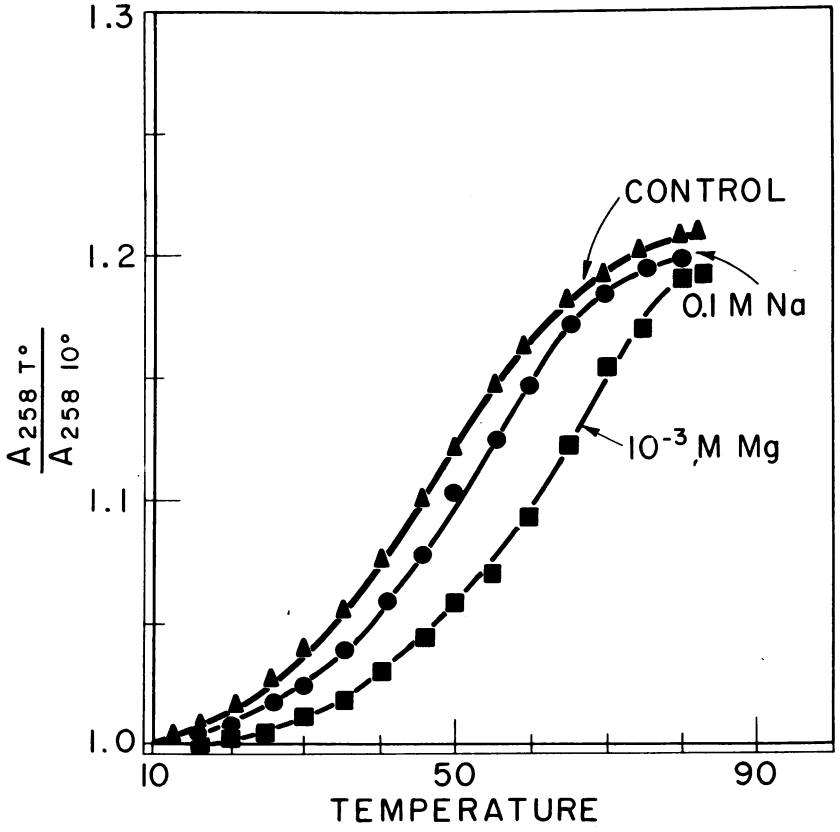

FIG. 2.—The effect of sodium and magnesium on the phase transition curve of beef liver RNA. Experimental conditions as in Figure 1. A_{max} is not altered significantly by the addition of either 10^{-1} M Na^{+} (●) or 10^{-3} M Mg^{++} (■) compared to the control (▲) even though T_m is increased.

to 10^{-3} M bring about a similar depression of A_{max} (Table 1). These elements, however, do affect T_m, the temperature at the midpoint of the phase transition curve. Results similar to those previously described have been obtained in this study as shown in Figure 2. Such changes in T_m in the presence of these elements have been interpreted in terms of hydrogen bonding which is enhanced in their presence and is diminished by urea.[3-6]

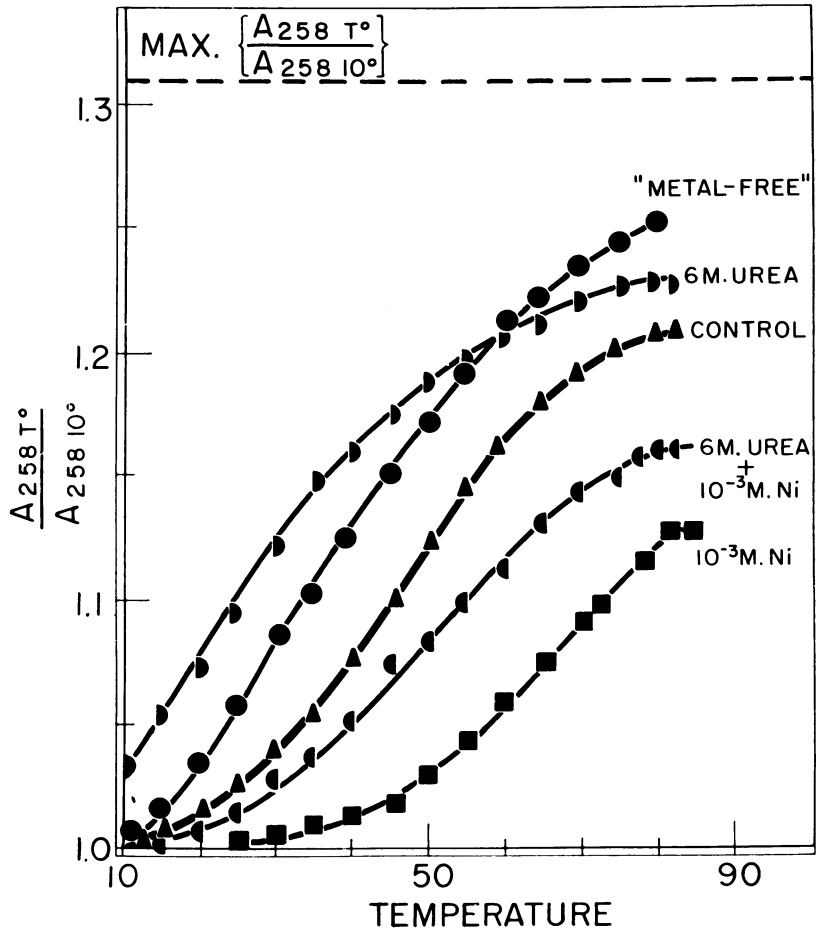

FIG. 3.—Conservation of hydrogen bonding through the establishment by Ni^{++} of tertiary folding in RNA. The experiments represented by the individual phase transition curves are indicated in the graph. The separate and joint contributions of the metal and hydrogen bonding are evident.

Parameters affecting hydrogen bonding, as expressed by T_m, appear to be independent of those which affect metal bonding, as expressed by A_{max}. This is apparent from the data shown in Figure 3. The increase in A_{max}, brought about by the removal of metals intrinsic to beef liver RNA, is here contrasted with the effect of 6 M urea which causes a marked decrease in T_m without A_{max} being affected significantly. A_{max} is, however, reduced by 10^{-3} M Ni^{++}, as previously shown in

Figure 1. In fact, an analogous depression of A_{max} by 10^{-3} M Ni^{++} is still seen, even in the presence of 6 M urea.

The specificity of metals in stabilizing the structure of RNA, and their action in this regard, as apart from hydrogen bonding, can be discerned from an experimental extension of this reasoning shown in Figure 4. When RNA is heated to the temperature at the midpoint of the phase transition curve, 49° in this instance, 50 per cent of the hydrogen bonds would be expected to be disrupted. The addition of 10^{-3} M Ni^{++}, at this juncture, instantaneously depresses the ab-

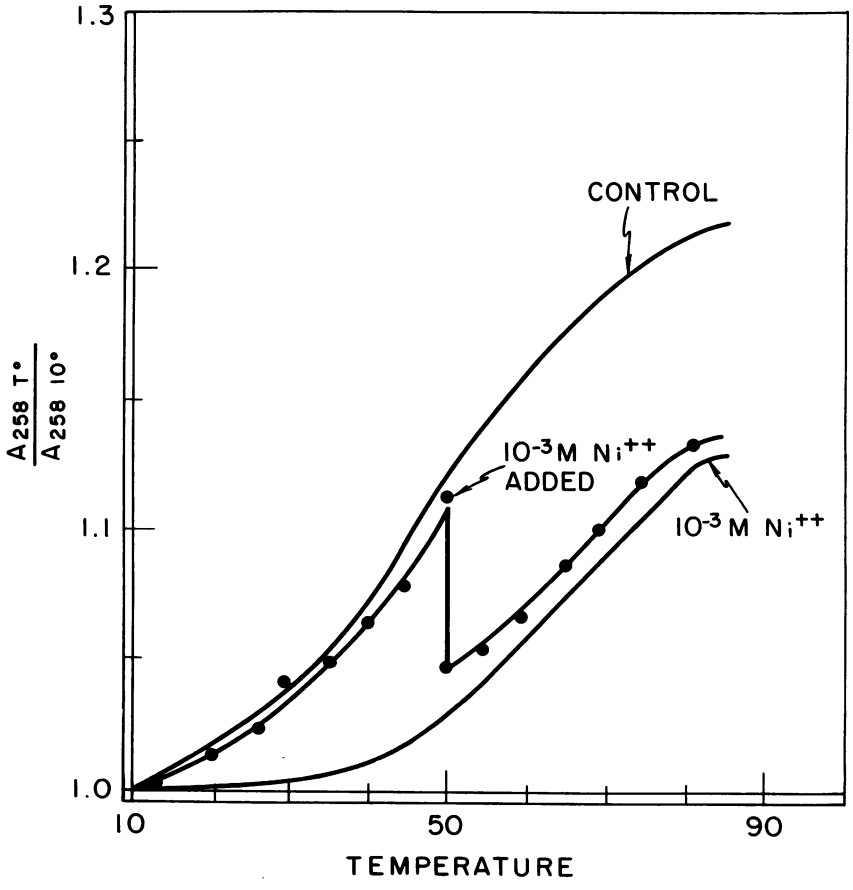

FIG. 4.—Reformation of ordered structure of native beef liver RNA at the T_m of its phase transition on addition of 10^{-3} M Ni^{++} ions. Addition of 10^{-3} M Na^{+} ions (not shown) had no effect.

sorption of radiation at 258 mμ to equal that observed in the presence of this concentration of Ni^{++} ions added prior to heating. Moreover, the phase transition eventually reaches the same value of A_{max} predicted for this concentration of Ni^{++} ions. Thus the bonds attributed to the metal can be formed even as hydrogen bonds are being destroyed.

Such findings as here presented are not limited to beef liver RNA. Analogous observations have been made with RNA from rat liver and tobacco mosaic virus.

A_{max} of TMV·RNA under the standard conditions of pH and ionic strength employed in these experiments is 1.26 while in the presence of 10^{-4} M Cr^{+++} it is markedly reduced to 1.07. The details of these experiments will be the subject of a separate communication.[13]

Discussion.—The existence of secondary structure in RNA has been deduced largely from the behavior of this molecule under conditions analogous to those known to affect hydrogen bonding in proteins, polymers, and copolymers.[14] On increasing the temperature, a phase transition is observed the midpoint of which is affected drastically by denaturing agents, ionic strength, solvents, and the presence of certain metals. The extinction coefficient at 258 mμ, the specific optical rotation, and the specific viscosity have served as indices of the helix-coil transition.[3-6] These experimental findings are in good agreement with expectations from theoretical treatments of such systems.[15]

Under the conditions which progressively abolish hydrogen bonding, metal ions of the first transition period are here shown to preserve and enhance the stability of the molecule as judged by the characteristics of the phase transition curves.

Metal ions of the first transition period, previously found to be constituents of native RNA prepared from many sources of widely diverging stages of evolutionary development,[2] seem to endow this molecule with the capacity to achieve intramolecular bonding by means additional to hydrogen bonding. The increase in ordered structure is apparent from the comparison of maximal chromicity of the free bases with that of all the possible constellations affecting hydrogen or metal bonding in RNA. Our results, demonstrating that metal linkages stabilize the molecule even while hydrogen bonds are being disrupted, may be viewed in the light of the numbers of conformational states available to a denatured molecule capable of resuming helical structure, as predicted by Gibbs and di Marzio.[15] The slopes of the phase transitions and the asymptotic maximum toward which each tends, observed experimentally on addition of transition metal ions to RNA, may be compared to the curves calculated, assuming the cleavage of discrete numbers of bonds of known energy content. On this basis, both a decreasing slope and maximum of absorption, as here observed with increasing concentration of transition metals, denotes increased rigidity, due—presumably—to greater preservation of helical content. Such stabilization implies the formation of intramolecular bonds in RNA which are of sufficient strength to resist the disrupting effects of increased temperature or concentrated urea.

A question now arises as to the characteristics and the number of this new species of bonds. The addition of metals to RNA in concentrations sufficient to cause a marked lowering of A_{max} neither affects the absorbancy at 258 mμ nor the specific optical rotation when measured at 10°. Thus neither the total number of internucleotide bonds nor the helical content of the molecule, dependent upon them, is increased solely by the addition of metals at low temperatures. The possibility that heating induces the formation of metal bonds is obviated by the reversibility of the melting curves on cooling. This stabilizing effect only becomes apparent on heating, when hydrogen bonds are being broken, but while persisting metal bonds attenuate the degree of the helix—coil transition. Therefore, it may be concluded that metals act to *preserve* the helical conformation of the molecule. Several interpretations may be offered to account for these observations: metal bonds may

simply be *exchanged* for existent hydrogen bonds such that the total number of bonds at low temperature remains constant, but at high temperature the metal bonds are preserved while hydrogen bonds are being broken. This view is challenged, however, by the marked effect of the metals intrinsic to RNA on A_{max}. The transition metal content of native beef liver RNA corresponds to about 1 metal atom for every 100 pairs of nucleotides.[2] After removal of some of these intrinsic metals by exposure to chelating agents, the ratio of transition metals to pairs of nucleotides is only decreased to 1:500. Despite the relatively small percentage of the total nucleotides affected directly by the intrinsic metals in native RNA and by their removal, a significant decrease in the stability of the molecule results, as evidenced by the observed increase in A_{max}. The intrinsic metals in RNA therefore exert a greater effect upon the helical structure than is accounted for on the basis of a simple, quantitative substitution of metal for hydrogen bonds.

In fact, the basis of the phenomenon appears to be different in *kind* from that implied by the above remarks. Thus, an alternative explanation for this enhanced effect of metals is required.

Characteristically, in proteins the formation of cross-linkages between peptide chains results in tertiary folding of the molecule.[16] This is exemplified most prominently by disulfide bridges[16] but also by hydrogen bonding between certain electro positive and negative groups along the chain,[17-19] hydrophobic bonds,[20] amide bonds,[21] phosphate diesters,[22] van der Waals interactions,[23] and finally by metal bridges as in trypsin[24-25] or amylase.[26] The data here reported suggest to us the existence of tertiary folding in RNA, the transition metal atoms serving as crosslinking devices, through chelation or sandwich complexes.[2] A metal bond, serving as a cross-link between nucleotide residues on adjacent turns of the helix, may be expected to fix or stabilize the hydrogen-bonded secondary structure enclosed within the tertiary loop, a well documented effect of tertiary folding in proteins.[16-27] In RNA this is recognized by the failure to achieve complete randomization of secondary structure by the rupture of hydrogen bonds on heating, i.e., a reduction of A_{max} of the melting curve, as seen in these experiments. The concept that metals stabilize RNA in this manner is strengthened by the experiments (Fig. 4) which demonstrate the ordering of the molecular structure by the addition of nickel at a temperature sufficient to have caused the disruption of half of the existent hydrogen bonds. Thus the establishment of tertiary structure allows the reformation of the helical conformation in the regions susceptible to stabilization through the creation of tertiary folds, even under conditions where the naked secondary structure is being destroyed.

That macromolecules are endowed with structural rigidity seems to be a general principle since this property is apparently required for effective biological function. Rigidity is accomplished, however, by means which seem to be adapted to the functional needs of the individual macromolecular species. Thus DNA whose function is mainly concerned with replication achieves rigidity through the two helically interwoven polynucleotide strands, held in juxtaposition by low energy hydrogen bonds.[28] The linear stability thus attained still permits the necessary separation of the two strands. In proteins, tertiary folding maintained chiefly by disulfide bridges, provides for the rigidity needed to induce the configurational specificity characteristically required in the function of enzymes, immune

bodies, and hormones.[16] It is probable that a mechanism for structural stabilization exists in RNA which promotes the specificity required for its postulated function as a template in protein synthesis.[29] The present data suggest that metals of the first transition series may be the agents responsible for this configurational property.

Since the biological activity of RNA, unlike the catalytic activity of many proteins, has not been defined in chemical terms, the role of metals in such ultimate function of RNA has not been assessed. However, metals of the first transition series stabilize the biological activity of RNA as expressed by the infectivity of TMV-RNA.[30] The constancy of metal content of TMV-RNA observed in successive analyses of different preparations eliminates the possibility that their presence is spurious.[13] The effect of their addition on A_{max} are in conformity with those observed for beef liver RNA. Studies on the implications of these compositional and conformational changes on the function of TMV-RNA are in progress.

Summary.—Certain metals of the first transition period of the periodic table are found to stabilize the ordered structure of RNA. These findings are interpreted to denote the existence of tertiary structure in RNA which is produced and maintained by metal bonds.

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† A preliminary report has been given (see ref. 1).

‡ Fellow of the Kettering Foundation, Dayton, Ohio.

§ Post-Doctoral Fellow of the National Institutes of Health of the Department of Health, Education and Welfare.

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[12a] In the melting curves to be shown, the phase transition has been measured by the increase in A_{258}, it has also been measured using the decrease in $[\alpha]_{546}$ as the indicator of the helix-coil transition. In comparable experiments, a correlation coefficient (r) of -0.9 between the changes in A_{258} and $[\alpha]_{546}$ has been obtained, suggesting that these parameters reflect the same physical-chemical process. Studies of viscosity also indicate a more ordered structure of RNA at increased temperatures in the presence of transition metals.

[12b] The metal content of native beef liver RNA, expressed as μgms of metal per gram RNA is as follows: Mg 500, Ca 930, Sr 26, Ba 99, Al 37, Cr 86, Mn 81, Fe 370, Ni 63, Cu 147, Zn 291.[2] After five successive precipitations from 0.1 *M* versene and three successive precipitations from 0.001 *M* 1,10-phenanthroline, the metal content is reduced: Mg 78, Ca not detected, Sr not detected, Ba 4, Al 37, Cr 31, Mn 26, Fe 155, Ni not detected, Cu not measured, Zn not detected.

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*SPIN RESONANCE STUDY OF SEROTONIN-FMN INTERACTION**

BY I. ISENBERG, A. SZENT-GYÖRGYI, AND S. L. BAIRD, JR.

INSTITUTE FOR MUSCLE RESEARCH, MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS

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A variety of indoles form complexes with riboflavin.[1, 2] These complexes have been interpreted as ones involving electron transfer, the indoles acting as an electron donor and the riboflavin as an acceptor. Theoretical studies[3] have shown that the positions of the energy levels of these molecules are consistent with this interpretation. The indoles have moderately high occupied energy levels, thus making them fairly good donors, while riboflavin has a low unoccupied energy level, thus making it a good electron acceptor.

This paper will report a study of serotonin and flavin mononucleotide (FMN) by the method of electron spin resonance (ESR). Tryptophan and FMN were also studied, but, for reasons stated below, not as extensively. To help identify the spectra obtained, the semiquinone of FMN at acid pH was prepared by reduction with zinc, by reduction with dithionite and photoreduction.

If an electron donor complexes with an electron acceptor, the donor giving an electron to the acceptor, the complex will not necessarily yield a signal in SR. In such a complex the donated electron will not necessarily be completely uncoupled from its partner and the two electrons will probably form a weak covalent bond