Studies on the Structure of Ribosomal Ribonucleic Acid from Normal and Tumour Tissues

VI. Dissociation Properties

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The dissociation properties of ribosomal RNA from liver and hepatoma were found to be dependent upon the temperature, ionic strength and cationic valency of the solvent. On increasing the temperature the pK values of the $N^1H-C^6=O$ and $N^1=C^6-NH_2$ groups of nucleobases were displaced towards neutrality due to the thermal cleavage of H-bridge linkages. Consequently, as the temperature is increased the nucleic acids bind increasingly more equivalents of acid or alkali. On forward- and back-titration hysteresis is observed in the acid pH range and the degree of hysteresis is found to decrease as the temperature is increased. Increasing the ionic strength of monovalent cations from $\mu=0.005$ to $0.05 Na^+$ leads to a reduced binding of equivalents of acid and alkali brought about by the displacement of pK values towards more extreme pH -values and to an increase in the stability of secondary structure.

Bivalent cations exert a stronger stabilizing effect on the molecular conformation of nucleic acids than monovalent cations of equal ionic strength. The more pronounced displacement of the pK values of ionizable groups on titration from pH 7 to pH 3 may be attributed to the additional action of bivalent cations as chelate formers.

Significant differences in the acid-base properties of ribosomal RNA from liver and hepatoma were observed especially at low temperatures and high ionic strength. At $5^\circ C$ and $\mu=0.1 NaCl$ less equivalents of acid and alkali are bound by hepatoma RNA than by liver RNA due to a lower degree of dissociation particularly of the $N^1=C^6-NH_2$ and $N^1H-C^6=O$ groups.

The acid-base properties of nucleic acids are different from the behaviour of a simple polyelectrolyte. The ionization of bases is largely dependent upon secondary structure i. e. upon the form and stability of the conformation of polynucleotides. Changes in the conformation and interaction of the bases of nucleic acids are reflected in their dissociation properties.

In previous experiments¹⁻³ we described differences in the optical behaviour of ribosomal RNA from liver and hepatoma, particularly increased T_m -values of ribosomal RNA from hepatomas on thermal denaturation under different ionic conditions² and in the presence of urea³. In view of the effect of secondary structure upon the degree of ionization i. e. upon the dissociation constants of the base residues, it is considered that studies on the dissociation properties of nucleic acids under different ionic conditions and at different temperatures are suitable to provide information about their structure and conformational changes.

Hence, studies have been carried out to investigate the acid-base properties of ribosomal RNA from liver and hepatoma and the effect of ionic strength, ionic valency and temperature upon the dissociation properties, in order to gain further information about the conformation of ribosomal RNA and its stability.

Experimental

Material

High-molecular-weight ribosomal RNA obtained from normal rat liver (Wistar strain) and from transplantable hepatocellular carcinomas of rats of the same strain primarily induced by feeding diethylnitrosoamine was used in these experiments.

The RNA preparations were polydisperse having sedimentation constants of $S_{20,W}^0 = 30.8 \pm 0.48$ and 17.4 ± 0.37 for liver and 31.4 ± 0.29 and 17.1 ± 0.14 for hepatoma.

In a sucrose gradient (5–20%) ribosomal RNA was found to sediment in two homogenous fractions.

¹ H. BIELKA, I. SCHNEIDERS, and A. HENSKE, *Acta biol. med. german.* 13, 13 [1964].

² H. BIELKA, I. JUNGAHN, and I. SCHNEIDERS, *Z. Naturforschg.* 19b, 1121 [1964].

³ I. JUNGAHN and H. BIELKA, *Naturwissenschaften* 52, 62 [1965].

Preparation of Ribosomal RNA

The tissues were homogenized in 0.1 M NaCl/0.01 M Tris-buffer (pH 7.2) at 0 °C in a Potter homogenizer fitted with a Teflon pestle. Microsomes and ribosomes were sedimented from the 10 000 *g*-postmitochondrial supernatant by ultracentrifugation (Spinco L 2, 105 000 *g*, 60 minutes at 0 °C). The RNA was isolated from the resuspended pellet by stirring with dodecyl sulfate (0.1%) and water-saturated redistilled phenol (pH 6.7) for 30 minutes at 20 °C. Denatured proteins were separated by centrifugation at 12 000 *g* for 10 minutes. The RNA was precipitated from the aqueous phase after addition of potassium acetate at a final concentration of 2% with 2.5 volumes of 96% ethanol at 0 °C. At a concentration of 1 mg/ml the RNA precipitate was transferred to the solutions used for titration and dialyzed against the corresponding solvent for 24 hours at 0 °C. A stream of purified nitrogen was passed through the dialysis bath.

Spectrophotometric Measurements

The RNA-concentrations were determined by UV absorbance measurements at 260 nm with an extinction coefficient of $E_{260}^{1\%} = 210$ and $\epsilon_{(p)} = 6.800$ in 0.1 M NaCl/0.01 M Trisbuffer pH 7.2 at 20 °C.

Analytical Ultracentrifugation

A Spinco Model E Ultracentrifuge with U.V. absorption optics was used for sedimentation analyses. Records were made at 2 minutes' intervals after reaching maximum speed (= 42 040 rev./min.). The temperature was 4 °C. Densitometric analyses were carried out in a Beckman Analytrol densitometer RB. Sedimentation coefficients were corrected to 20 °C for viscosity and density.

Nucleic acids were dissolved in 0.1 M NaCl/0.01 M Tris-buffer (pH 7.2) at concentrations of 20 to 80 $\mu g/ml$.

Electrometric Titrations

Titrations were carried out with a titration device supplied by the firm "Radiometer". The device consists of the titrator TTT 1 (pH meter), the titrator SBR 2 (recorder), a micrometer syringe SBU 1 and a microtitration device TTA 31. A calomel electrode K 4012 and a glass electrode G 2222 C covering the pH -range 0–12 were used as measuring devices. Errors in the electrodes were corrected for $pH > 11$ at $\mu = 0.01 Na^{\oplus}$ and for $pH > 9.5$ at $\mu = 0.1 Na^{\oplus}$. The accuracy of the titrator was to within 0.02–0.05 pH units and ± 0.2 °C.

Calibration of the pH meter was carried out with a phthalate buffer pH 4.0, a phosphate buffer pH 6.88, and a borate buffer pH 9.22, at 20 °C. For calibration at temperatures other than 20 °C, the same buffer solutions were employed and the values used for the cor-

responding temperatures were those indicated by the National Bureau of Standards (USA). The titration vessel was constructed of polyethylene and was flushed during the experiment with purified nitrogen.

Titrations were carried out with 0.05 N HCl and 0.05 N NaOH with stirring. In each case a carbonate-free sodium hydroxide solution was freshly prepared by dilution of a stock solution. The concentration of RNA solutions was 1 mg/ml. According to Cox and LITTAUER⁴ the titration properties are independent of RNA concentration over the range 0.5–4 mg RNA/ml. In each case volumes of 2 ml were titrated and one blank solution was titrated on each titration cycle. The titration values of RNA solutions were corrected against the blank values. Additionally, the "water correction" by the method of JORDAN and TAYLOR⁵ was carried out below pH 4 and above pH 10 according to the following equation

$$V_s'(\text{corr.}) = \frac{V_s' \cdot V_g}{V_g'}, \quad \text{where } V_g = V_A + V_s.$$

(V_g = total volume of RNA solution; V_g' = total volume of blank solution; V_A = initial volume; V_s = quantity of acid or alkali added to the RNA solution; V_s' = quantity of acid or alkali added to the blank solution).

The true difference between RNA solution and blank solution can be derived from

$$V_s - V_s'(\text{corr.}).$$

The experimental results described are represented as curves derived from the mean values of a number of separate titrations. Data denoting the ratio — number of preparations tested/number of separate titrations — are given in parentheses in the legends of the figures e. g. (3/6). A complete titration cycle consists of titration with acid from pH 7 to pH 2.5, subsequent titration with alkali to pH 11.5 and backtitration with acid to neutrality.

Determination of Phosphorus

Phosphorus was determined by the method of BEE-RENBUM and CHAIN and FISKE-SUBBAROW.

Results

Dependence of Acid-Base Properties upon Temperature at Constant Ionic Strength

Titrations were carried out in 0.1 M NaCl at 5 °C, 20 °C and 50 °C. The results of the experiments described below show that within definite pH ranges the dissociation behaviour of nucleic acids is dependent upon temperature.

At 5 °C the titration curve for ribosomal RNA from liver is found to be reversible within the ex-

⁴ R. A. COX and U. Z. LITTAUER, *Biochim. biophysica Acta* [Amsterdam] **72**, 188 [1963].

⁵ D. O. JORDAN and H. F. W. TAYLOR, *J. chem. Soc. [London]* **1946**, 994.

Fig. 1. Titration of ribosomal RNA from rat liver in 0.1 M NaCl-solution. (a) at 5°, (b) at 20°, (c) at 50°. ● titration with acid from p_H 8 to p_H 2.75; ▲ titration with alkali from p_H 2.75 to p_H 11.25; □ titration with acid from p_H 11.25 to p_H 2.75; △ a second titration with alkali from p_H 2.75 to p_H 11.25. Mean values of 6 separate titrations with 3 different RNA-preparations = 3/6.

perimental error over the alkaline range (figure 1 a). At p_H 11.35 50% of the titratable $N^+H-C^6=O$ groups are ionized. In the acid p_H range 50% of the titratable groups dissociate within the range to p_H 3.54. After titration to p_H 2.5 hysteresis is observed on back-titration. More equivalents of acid are bound on back-titration than on forward-titration, the difference at p_H 4 being 0.13 equivalents per 4 P-gramatoms (table 1).

Curves for ribosomal RNA from liver and hepatoma found on forward- and back-titration at 5° are represented in figure 2 a. Essentially, both types of RNA reveal the same pattern although certain quantitative differences are observed indicating

that less equivalents of acid or alkali are bound by ribosomal RNA from hepatoma. Consequently, the p_K values of ionizable groups are displaced towards more extreme p_H values both in the alkaline and acid ranges of p_H (table 1). At p_H 2.75 the difference between the two curves is -0.33 gram equivalents per 4 P-gramatoms. The difference is statistically significant ($p < 0.01$).

At 20° the titration pattern was about the same as that at 5° (figure 1 b). Significant differences, however, are found with regard to the larger quantity of acid necessary for titration to p_H 2.5 and the larger amount of alkali required for titration to p_H 11.25. In the alkaline range of p_H the curves found

Fig. 2. Comparison of the titrations of ribosomal RNA from liver (—) and hepatoma (---) in 0.1 M NaCl-solution. (a) at 5°, (b) at 20°, (c) at 50°.

Experimental conditions	pH for 50% dissociation in the acid alkaline range		Hysteresis (Δ equivalents of acid at pH 4 on forward and back titration)	Equivalents of acid bound per 4 P-atoms at pH 2.75
5° Liver-RNA	3.5	11.4	0.13	1.65
Hepatoma-RNA	3.2	11.6	0.15	1.32
20° Liver-RNA	3.9	10.8	0.25	2.00
Hepatoma-RNA	3.8	11.1	0.15	1.85
50° Liver-RNA	4.1	9.9	0.10	2.2
Hepatoma-RNA	4.1	10.6	0	2.2

Table I. Dissociation properties of rRNA from liver and hepatoma in 0.1 M NaCl-solution depending upon different temperatures.

ond forward- and back-titration are again reversible; at p_H 10.84 50% of the $N^1H-C^6=O$ groups are dissociated. Hence, this value is about 0.6 p_H units less than that obtained at 5 °C.

In the acid range 50% of the titratable groups are already ionized at p_H 3.87 (table 1). The degree of hysteresis observed at p_H 4 is 0.25 equivalents per 4 P-gramatoms.

Furthermore, the measuring points of a second titration cycle with alkali from p_H 2.75 to p_H 11.25 are plotted in figure 1 b. The shape of the second titration curve is coincident with that of the first cycle.

On comparing the titration curves of ribosomal RNA from liver and hepatoma represented in figure 2 b it will again be noticed that the curve for ribosomal RNA from hepatoma is displaced towards more extreme p_H values (table 1). At a temperature of 20° less equivalents of acid are bound by ribosomal RNA from hepatoma than by ribosomal RNA from liver, particularly in the acid p_H range, but the differences (at p_H 2.75 – 0.15 gram equivalents per 4 P-gramatoms) are not significant.

At 50° (figure 1 c) more equivalents of acid are bound by RNA on titration to p_H 2.75 than at 20 °C. This is due to a further shift in the p_K values of ionizable groups. 50% of these groups are already ionized at p_H 4.1. This indicates a displacement by 0.23 p_H units compared with the values obtained at 20°. Moreover, a decrease in hysteresis is observed in the acid range of p_H . The difference between forward- and back-titration at p_H 4 is 0.1 gram-equivalent per 4 P-gramatoms. Over the alkaline range of p_H the titration curves are again found to be reversible, within the experimental error although at 50° significantly more equivalents of alkali are bound by ribosomal RNA than at 20 °C.

50% of the $N^1H-C^6=O$ groups are dissociated at p_H 9.9. Hence, this value is 0.94 p_H unit less than that found at 20 °C and 1.45 units less than that at 5 °C. A comparison of the titration curves for ribosomal RNA from liver and hepatoma shown in figure 2 c reveals that no difference in titration properties are observed at 50 °C, particularly in the acid range of p_H . On forward-titration to p_H 2.75 no differences in acid consumption are noted.

Fig. 3. Titration of RNA from liver (a) and hepatoma (b) at different temperatures in 0.1 M NaCl-solution. ···· at 5°, — at 20°, --- at 50°.

Consequently, the acid-base properties of ribosomal RNA are largely dependent on temperature (figure 3). With increasing temperature more equivalents of acid and alkali are bound by RNA within a particular p_H range. In the acid range of p_H the difference is found to be greatest between p_H 4.8 and 2.75 especially when the temperature is raised from 5° to 20°. Conformational changes brought about by changes in temperature are mainly observed below p_H 4.8 and, in the alkaline range, above p_H 8.5. With increasing temperature, particularly with a rise in temperature from 20 °C to 50 °C the degree of hysteresis is diminished within the acid p_H range. Simultaneously, the p_K values

of ionizable groups are displaced towards neutrality (table 1).

Dissociation due to change in temperature occurs at $p_H < 5.5$ and at $p_H > 8$ while dissociation over the range $p_H 5.5-8$ is largely independent of changes in temperature.

The experimental results show that increasing temperatures lead to a higher sensitivity of the helical structures of RNA to extreme p_H values so that at higher temperatures a rupture of H-bonds will already take place at lower H^+ -concentrations. The dependence of dissociation properties upon temperature is more pronounced in ribosomal RNA from hepatoma than in ribosomal RNA from liver (figure 3). On comparing the curves presented in figures 2 a-c and figures 3 a and 3 b it will be noticed that the titration properties of RNA from liver and hepatoma are particularly different from each other in the low temperature range whereas at 50° no differences are demonstrable.

Dependence of Acid-Base Properties upon Ionic Strength at Constant Temperature

Titration curves were performed in solutions of RNA in 0.1 M and 0.01 M NaCl at $20^\circ C$.

On comparing the titration curves for ribosomal liver RNA derived from titration in 0.1 M (figure 4 a) and 0.01 M NaCl solution (figure 4 b) it was found that more equivalents of acid were bound by RNA in 0.01 M NaCl solution than in 0.1 M NaCl solution. Changes in molecular configuration due to a decrease in ionic strength from $\mu = 0.1$ to $\mu = 0.01$ are already observed below a p_H of 5.5. If the ionic strength is increased from $\mu = 0.01$ to 0.1 the acid branch of the titration curve is displaced by about $-0.5 p_H$ units (table 2) i. e. from $p_H 4.4$ to $p_H 3.9$ relative to the 50%-dissociation of groups titratable over this p_H range. Furthermore, it is apparent from titration in 0.01 M NaCl solution that the degree of hysteresis observed on forward- and

Fig. 4. Titration of liver-rRNA at 20° in (a) 0.1 M NaCl-solution and (b) 0.01 M NaCl-solution. Δ 3/6 in every experiment. $\bullet$, $\blacktriangle$, $\triangle$, $\square$ the same as in fig. 1.

Experimental conditions	pH for 50% dissociation in the acid alkaline range		Hysteresis (Δ equivalents of acid at pH 4 on forward and back titration)	Equivalents of acid bound per 4 P-atoms at pH 2.75
3.3 mM $MgCl_2$				
Liver-RNA	3.6	10.6	0.58	1.7
Hepatoma-RNA	3.6	10.7	0.62	1.7
0.1 M NaCl				
Liver-RNA	3.9	10.8	0.25	2.0
Hepatoma-RNA	3.8	11.1	0.15	1.85
0.01 M NaCl				
Liver-RNA	4.4	10.9	0.05	2.3
Hepatoma-RNA	4.3	10.7	0.07	2.4

Table II. Dissociation properties of rRNA from liver and hepatoma at 20° depending upon strength and valency of cations

Fig. 5. Comparison of the titrations of ribosomal RNA from liver (—) and hepatoma (---) at 20° in (a) 0.1 M NaCl-solution, (b) 0.01 M NaCl-solution.

back-titration is considerably less than in 0.1 M NaCl solution. In 0.1 M NaCl solution a difference of 0.25 gram-equivalents per 4 P-gramatoms was found at p_H 4 whereas in 0.01 M NaCl solution the difference found on forward- and back-titration at p_H 4 amounts to only about 0.05 equivalents per 4 phosphorus gramatoms.

Contrary to the behaviour of ribosomal RNA from liver and hepatoma in 0.1 M NaCl solution (figure 5 a) no difference in the acid-base properties over the acid p_H range was found in 0.01 M NaCl solution (figure 5 b).

Acid-Base Properties in the Presence of Mg-Ions

The RNA used in these experiments was dissolved in 3.3 mM $MgCl_2$ solution; titrations were carried out at 20°C. The titration properties of ribosomal

liver RNA in $MgCl_2$ solution are represented in figure 6. The shape of the titration pattern is largely consistent with that obtained in the presence of monovalent cations; a marked shift, however, particularly of the acid branch towards lower p_H values, is quite evident. The value obtained for the 50%-dissociation of the $N^1=C^6-NH_2$ and $N^3-C^2-NH_2$ groups was found to be 3.57 indicating a displacement by about $-0.8 p_H$ units compared with titration values obtained in the presence of Na^+ of the same ionic strength (table 2). Similarly, the degree of hysteresis is much greater than that found on titration in 0.01 M sodium chloride; at p_H 4 the difference between forward- and back-titration in 3.3×10^{-3} M $MgCl_2$ solution is 0.6 equivalents per 4 P-gramatoms. In the presence of Mg ions no difference in the titration properties of ribosomal RNA

Fig. 6. Titration of liver-rRNA in 3.3 mM $MgCl_2$ -solution at 20°. ●, ▲, □ as in fig. 1 (2/4).

Fig. 7. Comparison of the titrations of RNA from liver (—) and hepatoma (---) at 20° in 3.3 mM $MgCl_2$ -solution.

from liver and hepatoma was observed (figure 7). In comparing the titration curves for ribosomal RNA derived in the presence of monovalent and bivalent cations of equal ionic strength ($\mu=0.01$) it will be noticed that less equivalents of acid are bound on forward-titration with acid in the presence of Mg^{2+} (-0.58 gram-equivalents per 4 P-gram-atoms at p_H 4) than in the presence of Na^+ (figure 8). This is also reflected by differences in p_K values. On back-titration with alkali from p_H 2.75 to p_H 7.5 the dissociation behaviour was found to be independent of ionic valency.

Fig. 8. Dependence of acid-base properties of RNA from liver (a) and hepatoma (b) on ion conditions at 20° . — — — 3.3 mM MgCl_2 , — 0.1 M NaCl , — · — 0.01 M NaCl .

In the alkaline range no clear evidence could be provided for the dependence of the dissociation behaviour of nucleic acids upon ionic valency.

Discussion

The molecular conformation and stability of the secondary structure of nucleic acids are largely dependent upon temperature and ionic conditions. This is reflected e. g. in the sedimentation behaviour, the viscosity and the ultra-violet absorption properties of nucleic acids depending upon these factors. Increasing the temperature leads to a labilization and increasing the ionic strength to a stabilization of molecular configuration. Bivalent cations are more effective than monovalent cations.

The present results of electrometric titrations show that the acid-base properties of RNA-nucleotides are essentially dependent upon temperature and the ionic state of the solvent and, hence, upon

the spatial conformation of the polynucleotide. Stabilization of secondary structure brought about by a decrease in temperature or an increase in ionic strength or ionic valency leads to a change in the dissociation of the $\text{N}^1\text{H}-\text{C}^6=\text{O}$, $\text{N}^1-\text{C}^6-\text{NH}_2$ and $\text{N}^3-\text{C}^2-\text{NH}_2$ groups resulting in a displacement of p_K values towards more extreme p_H values and, consequently, in a decrease in equivalents of acid and alkali bound by nucleic acids. Changes of this kind are first and foremost attributed to the formation of H-bonds. The dependence of the dissociation properties of nucleic acids upon temperature and, hence, upon molecular conformation is different in the alkaline and acid ranges of p_H . Dependence upon temperature is found to be more pronounced in the alkaline than in the acid p_H range; this may be due to the great heat of dissociation of $\text{N}^1\text{H}-\text{C}^6=\text{O}$ groups which is higher than that of groups titratable in the acid range of p_H ^{4, 6}. Changes in hysteresis also provide certain evidence for the dependence of molecular configuration and stability upon temperature and ionic conditions. In view of the fact that the curves found on back-titrations are nearly identical under all conditions, differences in hysteresis dependent upon temperature and ionic conditions are to be assigned to differences in the behaviour of nucleic acids on forward-titration. The degree of hysteresis is found to be greatest when nucleic acids are titrated under conditions resulting in a highly ordered state of the polynucleotides so that less equivalents of acid are bound on forward-titration. A diminution in hysteresis with increasing temperature and decreasing ionic strength may thus be regarded as further evidence for the dependence of the acid-base properties of nucleobases upon the conformation of nucleic acids. At low temperatures hysteresis is probably due to rearrangements in molecular conformation in dependence upon p_H . According to Cox and LITTAUER^{4, 7, 8} two different stable conformations of RNA may exist in the neutral and acid ranges of p_H . Hysteresis in the acid p_H range is attributed to a potential energy barrier which, on forward- and back-titration, prevents the thermodynamic equilibrium of the two ordered forms of RNA from being attained spontaneously. In view of the fact that no

⁶ R. A. Cox and A. R. PEACOCKE, J. chem. Soc. [London] **1957**, 4724.

⁷ R. A. Cox, Biochim. biophysica Acta [Amsterdam] **68**, 401 [1963].

⁸ R. A. Cox, Biochim. biophysica Acta [Amsterdam] **72**, 203 [1963].

hysteresis is observed at 50° it may be inferred that at higher temperatures conversion of RNA into another molecular conformation will already take place at about neutral p_H whereas at lower temperatures it will be achieved only at higher concentrations of H^+ . It seems to be evident that above a definite temperature there is no energy barrier which prevents thermodynamic equilibrium between two structural states of RNA from being attained.

Changes in the titration behaviour in dependence upon ionic strength are less pronounced in the alkaline p_H -range compared with those in the acid range. The dependence of the acid-base properties of nucleobases of RNA upon ionic strength is essentially due to an increase in the electrostatic energy of the phosphate group brought about by a decrease in cationic strength. This exerts a direct effect on the p_K values of dissociable groups in the bases shifting them towards higher p_H values. Furthermore intramolecular H-bonds are disrupted by breaking the bond energy and this, in turn, leads to a displacement of the p_K values of dissociable groups involved in H-bonding. For $N^1=C^6-NH_2$ and $N^3-C^2-NH_2$ groups the two effects re-inforce each other in the acid p_H range while in the alkaline range the two effects are superimposed i.e. a decrease in p_K values is observed if H-bridges are split whereas an increase in the p_K value of the $N^1H-C^6=O$ group is noticed if the negative charge of the phosphate group rises. Thus, displacement of the titration curve with ionic strength is found to be less pronounced in the alkaline and more pronounced in the acid range of p_H .

The observed data on the dependence of acid-base properties of nucleic acids upon temperature and strength of monovalent cations are in accord with experiments of COX and LITTAUER on RNA from *E. coli*⁴, studies of COX⁷ and STEINER and BEERS⁹ on poly-(A + U), studies of PEACOCKE and LIFSON¹⁰ and COX and PEACOCKE⁶ on denatured Na-DNA and investigations of FRESCO and KLEMPERER¹¹ on poly-A.

Moreover, particular mention should be made of titration experiments in the presence of Mg ions. Mg^{2+} exerts a much more pronounced stabilizing ef-

fect on molecular conformation than monovalent cations of the same ionic strength. In the acid range of p_H the same degree of dissociation is attained at considerably lower p_H values; a particularly large degree of hysteresis is found on back-titration (the difference between forward- and back-titration amounting to as much as 0.6 gram-equivalente per 4 P-gramatoms). The results of titrations in the presence of Mg ions may be regarded as further indication of the fact that the particularly strong stabilizing effect of bivalent ions upon nucleic acids is brought about by the additional formation of chelate bonds since COTTON and HARRIS¹² have found that chelate bonds account for a marked displacement of the p_K values of amines towards lower p_H values. In the case of nucleobases an interaction of Mg^{2+} with the lone-pair electrons of N and O atoms of dissociable groups must be assumed. In view of the behaviour of RNA on forward- and back-titration in the presence of Mg ions it can be inferred that on titration with acid the chelate bonds are disrupted but form again on back-titration at about p_H 5. Essentially, this is in accord with observations by BANARJEE and PERKINS¹³ who found that the bonding capacity of RNA for bivalent cations increases between p_H 3.5–4.8 and remains constant afterwards. On back-titration below p_H 5 the molecular conformation and stability of RNA and structural rearrangements would then be solely accounted for by ionic strength and not, additionally, by ionic valency which seems to be of importance only at $p_H > 5$.

On comparing the titration curves of RNA from liver and hepatoma found at different temperatures and different ionic strengths it can be seen that in RNA from hepatoma fewer base residues are ionized at a given acidic or alkaline p_H value. With increasing temperature the difference is diminished and is increased with a rise in ionic strength. Consequently, at low temperatures and higher ionic strength the p_K values of the $N^1=C^6-NH_2$ and $N^3-C^2-NH_2$ groups of hepatoma RNA are displaced towards lower p_H values. In view of the dependence of the dissociation properties of bases upon the conformation of nucleic acids described above the lower

⁹ R. F. STEINER and R. F. BEERS, *Biochim. biophysica Acta* [Amsterdam] **33**, 470 [1959].

¹⁰ A. R. PEACOCKE and S. LIFSON, *Biochim. biophysica Acta* [Amsterdam] **22**, 191 [1956].

¹¹ J. R. FRESCO and E. KLEMPERER, *Ann. N. Y. Acad. Sci.* **81**, 730 [1959].

¹² F. A. COTTON and F. E. HARRIS, *J. phys. Chem.* **59**, 1203 [1955].

¹³ K. C. BANARJEE and D. J. PERKINS, *Biochim. biophysica Acta* [Amsterdam] **61**, 1 [1962].

degree of dissociation of ionizable groups in hepatoma RNA may also provide further evidence for an increased stability of the molecular conformation of ribosomal RNA from hepatoma. The present results suggest that the increase in structural stability is particularly due to the NH_2 groups. It may be assumed in this connection that, apart from being involved in H-bonding, the $\text{N}^1 = \text{C}^6 - \text{NH}_2$ groups are also linked up with other bonds in the RNA

molecule. This is in agreement with previous studies on formaldehyde reacting with ribosomal RNA¹; these studies show that the proportion of NH_2 groups of ribosomal hepatoma RNA reacting with formaldehyde is less than that of ribosomal liver RNA.

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Untersuchungen zum endogenen Stoffwechsel von Mykobakterien *

I. Mitt. Zur Trennung phosphorylierter Metabolite der säurelöslichen Fraktion

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1. Bei Untersuchungen des endogenen Stoffwechsels von Mykobakterien wird mit Hilfe eines kombinierten Ogur-Rosen-Schneider-Trennungsganges die säurelösliche Fraktion von *Myc. smegmatis* isoliert und zur Ionenaustausch-Chromatographie vorbereitet.
2. Als Anionenaustauscher wird Wofatit SBW verwendet und die Trennung mittels Gradienten-Elution bei geschlossener Mischkammer durchgeführt. Gute Trennungen werden bei Einsatz eines zu Beginn mit sehr flachem Säure- und Neutralsalzgradienten arbeitenden Elutionssystems erreicht.
3. Durch automatische Messung der ^{32}P -Aktivität und durch eine spektrophotometrische Messung der erhaltenen Fraktionen wird die Auftrennung kontrolliert.
4. Die erhaltenen Banden werden mit Hilfe der Papierchromatographie weiter aufgetrennt und auf die Anwesenheit von Monosacchariden und Aminosäuren geprüft.
5. Folgende Daten dienen zur Identifizierung der aufgetrennten Metabolite: Elutionsfolge, R_f -Werte, ^{32}P -Aktivität, spektrophotometrische Auswertung bei saurer und alkalischer Reaktion vor und nach Hydrolyse. In besonderen Fällen werden spezielle Methoden, z. B. Cyanid-Komplex bei NAD bzw. NADP, zur Erhärtung der Aussage herangezogen.
6. Neben den energiereichen Phosphaten und den Phosphorsäureestern der Embden-Meyerhof-Kette werden in den Zellen von *Myc. smegmatis* alle niedermolekularen Vertreter des Nucleinsäurepools nachgewiesen.
7. Die ^{32}P -Verteilung kann sowohl aus der bei der Ionenaustausch-Chromatographie erhaltenen Kurve abgelesen, als auch durch quantitative Bestimmung des ^{32}P in den einzelnen Peaks ermittelt werden.

In der vorliegenden Arbeit werden folgende Abkürzungen verwendet: A = Adenin, AMP = Adenosinmonophosphat, ADP = Adenosindiphosphat, ATP = Adenosintriphosphat, C = Cytosin, CMP = Cytidinmonophosphat, CDP = Cytidindiphosphat, DNS = Desoxyribonucleinsäure, FAD = Flavinadenindinucleotid, G = Guanin, GMP = Guanosinmonophosphat, GDP = Guanosindiphosphat, GTP = Guanosintriphosphat, H = Hypoxanthin, IMP = Inosinmonophosphat, KF = Kulturfiltrat, LF = Lipid-Fraktion, NAD = Nikotinamidadenindinucleotid, NADP = Nikotinamidadenindinucleotidphosphat, NS = Nucleinsäure-Fraktion, RNS = Ribonucleinsäure, SF = säurelösliche Fraktion, TCE = Trichloressigsäure, U = Uracil, UMP =

Uridinmonophosphat, UDP = Uridindiphosphat, UTP = Uridintriphosphat.

Stoffwechseluntersuchungen an proliferierenden Mykobakterien sind aus verschiedenen Gründen schwierig durchzuführen. Selbst die beim Einsatz radioaktiver Verbindungen zu erwartenden Vorteile lassen sich nur in begrenztem Ausmaß ausnutzen^{1, 2}. Bei Untersuchungen unter Bedingungen des endogenen Stoffwechsels entfallen viele methodisch bedingten Probleme, so daß es aussichtsreich erscheint,

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