Hysteresis and Conformational Changes in Ribosomal Ribonucleic Acid

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Both rat liver and *Escherichia coli* rRNA in 0.1M-sodium chloride were titrated with acid or alkali over the range pH3–7 at approx. 0°C. rRNA did not bind acid reversibly and hysteresis was observed, i.e. the plot of acid bound to rRNA against pH had the form of a loop showing that the amount of acid bound at a particular pH depended on the direction of the titration. Although the boundary curves were reproducibly followed on titration from pH7 to 3 and from pH3 to 7, points within the loop were 'scanned', e.g. by titration from pH7 to a point in the range pH3–4 followed by titration with alkali to pH7. It is inferred that the 'lag' in the release of certain bound protons is at least 1 pH unit, that at least about 9–15% of the titratable groups (adenine and cytosine residues) that are involved in this process and that the free energy dissipated in completing a cycle is approx. 4.2 kJ/mol (1 kcal/mol) of nucleotide involved in hysteresis. The interpretation of the 'scanning' curves was illustrated by means of a cycle of possible changes in the conformation of a hypothetical nucleotide sequence that allows formation of poly(A)·poly(A α H⁺)-like regions in acidic solutions. It is also inferred that the extent of 'hysteresis' might depend on the primary nucleotide sequence of rRNA as well as on secondary structure.

Nucleic acids and polynucleotides have many unusual properties arising from their capacity to form a range of different stable conformations through intramolecular [e.g. as in poly(A-U)] and intermolecular [e.g. as in poly(A)·poly(U)] interactions. The transition from one form to another is generally co-operative. Such transitions take place abruptly because of the simultaneous rupture of a large number of weak bonds. Nevertheless the transition from one form to another is reversible (for reviews see Felsenfeld & Miles, 1967; Michelson *et al.*, 1968).

The first polynucleotide to be isolated in a form that allowed its macromolecular properties to be studied was DNA, and it is ironic that it is atypical because it is often found in a metastable state, the so-called 'denatured' form. In this respect DNA resembles other bio-colloids, e.g. proteins, that also have 'native' and 'denatured' states. Denaturation is often an irreversible process. A particular case is the titration of DNA at 25°C with either acid or alkali, which leads to denaturation at the extremes of pH, and the denatured form persists for a long period when the solution is neutralized. The pH-titration curve followed before denaturation is distinct from that followed after denaturation, and the original curve is never retraced (Gulland *et al.*, 1947; Cox & Peacocke, 1956).

'Hysteresis' is another phenomenon involving metastable states and irreversible transitions. The term was introduced by Ewing (1881*b*) to describe phenomena in which the property being measured 'lags behind' in its efforts to follow changes in conditions. The electric polarization of both DNA (Polonsky *et al.*, 1960) and RNA (Stanford & Lorey, 1968) lags behind the external field strength (ferroelectric hysteresis). The pH-titration curve of rRNA exhibits hysteresis over the range pH3–7. The path followed on bringing the pH from neutrality to pH3 is different from the path followed on adding alkali to neutralize the solution, but a closed loop is formed that is reproduced on successive cycles of titration (Cox *et al.*, 1956; Cox, 1963; Cox & Littauer, 1963; Cox, 1966*a*). Raising the temperature to 38.4°C did not appear to hasten the approach of an equilibrium state (Cox & Littauer, 1963). Further, points inside the hysteresis loop could be reached by means of scanning curves (e.g. Cox, 1963, 1968), a standard procedure for studying hysteresis phenomena. These properties support the view that behaviour found on titration of rRNA is a hysteresis phenomenon that is independent of time under ordinary conditions. This is an example of molecular hysteresis because the conformational changes concerned probably take place within a single rRNA molecule.

The aim of the present work is to relate the hysteresis found on potentiometric titration to the secondary structure of RNA.

Experimental

Materials

rRNA was isolated from *Escherichia coli* by the method of Littauer & Eisenberg (1959) and from rat liver by the method of Laskov *et al.* (1959).

Methods

The procedure for electrometric titration was described by Cox & Littauer (1963). Solutions of rRNA (1.2–3 mg/ml) were titrated with HCl (0.1 M) or NaOH (0.1 M) at the temperature of melting ice. The equivalents of acid bound at a particular pH was measured to within ± 0.01 equiv. of HCl bound/4g-atoms of P. Movements of acid or alkali were sufficient to change the pH by about 0.1 unit. About 30–60 min elapsed as the pH was decreased by 1 unit.

The values of pH did not appear to change with time to any significant extent, i.e. a steady pH was very quickly attained on the addition of acid. On several occasions the pH was observed for periods of up to 12 h and the maximum change noted was 0.05 pH unit compared with the difference between forward- and back-titration curves of 0.26 pH unit.

Results

The significance of the proton-binding curves of RNA is that they may reflect changes of conformation. The shape of the pH-titration curve can be informative about conformational changes, and the reversibility or otherwise of proton binding reveals the extent to which such changes are reversible. Over the range pH 3–7 the titratable groups of RNA are $N_{(1)}=C_{(6)}-NH_2$ groups of adenine and cytosine residues. Guanine residues (for GMP pK 2–3) and diesterified phosphate residues (pK approx. 1.6) are unlikely to contribute appreciably to proton binding. The pK values of adenine and cytosine residues depend on conformation (Table 1), and it is for this

Table 1. Values of pK and $\Delta\epsilon$ used to calculate the pH-titration curves

The values of pK are given for solutions of 0.1 M-sodium phosphate buffer or 0.1 M-NaCl at about 25°C. The base residues appear to titrate as simple monobasic acids according to eqn. (4). Apparently the negatively charged phosphate residues make the major contribution to the electrostatic field, which remains essentially constant above pH 3 (Peacocke & Lifson, 1956). The value of $pK_{A(dh)}$ has not been measured, so $pK_{A(dh)}$ 3.50 and $pK_{A(dh)}$ 4.0 were taken as extreme values. The shape of the potentiometric titration curve was affected, but the area of the hysteresis loop was scarcely altered.

Reaction	pK	Reference	$\Delta\epsilon$	Reference
$AH^+ MP(5') \rightleftharpoons AMP + H^+ + \Delta\epsilon_{AH^+, \lambda}$	3.80	Clauwaert & Stockx (1968)	$\Delta\epsilon_{AH^+, 260} + 500$ $\Delta\epsilon_{AH^+, 280} - 800$ $\Delta\epsilon_{AH^+, 300} 0$	<i>Pabst</i> <i>Circular OR10</i>
$A_{(am)} \rightleftharpoons A_{(am)}H^+ + \Delta\epsilon_{AH^+, \lambda}$	4.35	Clauwaert (1968)	$\Delta\epsilon_{AH^+, \lambda}$ as for AMP	—
$AH^+ \cdot U \rightleftharpoons A \cdot U + H^+ + \Delta\epsilon_{A \cdot U, \lambda}$	3.88*	Clauwaert (1968)	$\Delta\epsilon_{AH^+, \lambda}$ as for AMP	—
$A \cdot AH^+ \rightleftharpoons A \cdot A + H^+ + \Delta\epsilon_{A \cdot U, \lambda}$	6.60	Calc. from data of Steiner & Beers (1959)	$\Delta\epsilon_{AH^+, \lambda}$ as for AMP	—
$AH^+ \cdot AH^+ \rightleftharpoons A \cdot AH^+ + H^+ + \Delta\epsilon_{A \cdot U, \lambda}$	4.80	Based on data of Steiner & Beers (1959)	$\Delta\epsilon_{AH^+, \lambda}$ as for AMP	—
$A \cdot A \rightleftharpoons A_{(am)} + A_{(am)} + \Delta\epsilon_{A \cdot A, \lambda}$	—	—	$\Delta\epsilon_{A \cdot A, 260} + 4700$ $\Delta\epsilon_{A \cdot A, 280} + 500$ $\Delta\epsilon_{A \cdot A, 300} 0$	Holcomb & Tinoco (1965)
$A \cdot U \rightleftharpoons A_{(am)} + U_{(am)} + \Delta\epsilon_{A \cdot U, \lambda}$	—	—	$\Delta\epsilon_{A \cdot U, 260} + 4500$ $\Delta\epsilon_{A \cdot U, 280} + 500$ $\Delta\epsilon_{A \cdot U, 300} 0$	Cox (1970)
$CH^+ MP(2':3') \rightleftharpoons CMP + H^+ + \Delta\epsilon_{CH^+, \lambda}$	4.32	Clauwaert & Stockx (1968); Cox (1966b)	$\Delta\epsilon_{CH^+, 260} + 1100$ $\Delta\epsilon_{CH^+, 280} - 5700$ $\Delta\epsilon_{CH^+, 300} - 2800$	<i>Pabst</i> <i>Circular OR10</i>
$G \cdot CH^+ \rightleftharpoons G \cdot C + H^+ + \Delta\epsilon_{CH^+, \lambda}$	2.5	Cox <i>et al.</i> (1971)	$\Delta\epsilon_{CH^+, \lambda}$ as for CMP	—
$C_{(am)}H^+ \rightleftharpoons C_{(am)} + H^+ + \Delta\epsilon_{CH^+, \lambda}$	4.7	Cox (1966b)	$\Delta\epsilon_{CH^+, \lambda}$ as for CMP	—
$G \cdot C \rightleftharpoons G_{(am)} + C_{(am)} + \Delta\epsilon_{G \cdot C, \lambda}$	—	—	$\Delta\epsilon_{G \cdot C, 260} + 1400$ $\Delta\epsilon_{G \cdot C, 280} + 3350$ $\Delta\epsilon_{G \cdot C, 300}$ Negligible	Cox (1970)

* Calculated value.

reason that conformational changes are apparent from proton-binding curves. Evidently not all the conformational changes effected by titrating rRNA with acid are simply reversed by raising the pH. It is the range in the properties of rRNA secondary structure that accounts for the shape of the hysteresis loop and which the 'scanning' technique is designed to reveal (for review see Everett, 1967).

'Scanning' curves of rat liver rRNA

The potentiometric titration properties of rat liver rRNA were described by Cox (1963), when hysteresis over the range pH 3–7 was reported and it was shown that the loop could be 'scanned'. The family of curves given in Fig. 1 were obtained by starting at pH 8 and titrating with acid to pH x , when the solution was back-titrated with alkali to pH 8. A second cycle was then done, but this time the reversal point was pH

$x - \delta\text{pH}$, and so on for several cycles. The path followed from pH 8 was always curve I (Fig. 1), but the path followed on back-titration depended on the pH at which the direction of titration was reversed. As reported by Cox (1963) curve I was followed reversibly provided that the lowest pH attained was pH 3.8 (see Fig. 2a). When the reversal point was pH 3.6 a different path (curve IIa in Fig. 1), intermediate between curves I and IIe, was traced on back-titration to pH 7. The reversal points for the third and successive cycles were within the range pH 3.0–3.3, and different routes (curves IIb–IIe in Fig. 1) were followed on back-titration. It appeared that curves IIb–IIe merged with curve IIe at approx. pH 4.8 or above. Titration to pH 2.79 led to another curve II (Fig. 2) that enclosed curve IIe (Fig. 1).

The growth of the hysteresis loop is illustrated and typical scanning curves are given in Fig. 2. Curves I and II are included for reference. n_{HCl} is the mol of

Fig. 1. Scanning curves of rat liver rRNA titrated at 0.1°C in 0.1M-NaCl

The family of curves were obtained by starting at pH 8 and titrating with acid to pH x , when the solution was back-titrated with alkali to pH 8. A second cycle then followed, but this time the reversal point was pH $x - \delta\text{pH}$, and so on for several cycles. The titration curves were, curve I: titration with acid from pH 8.0; curves I and IIa: titration with acid from pH 8.0 to 3.6 and back-titration with alkali from pH 3.6 to 8.0; curves I and IIb: titration with acid from pH 8.0 to 3.3 and back-titration with alkali from pH 3.3 to 8.0; curves I and IIc: titration with acid from pH 8.0 to 3.24 and back-titration with alkali to pH 4.86; curves I and IId: titration with acid from pH 8.0 to 3.14 and back-titration from pH 3.14 to 4.86; curves I and IIe: titration from pH 8.0 to 2.99 and back-titration from pH 2.99 to 8.0.

HCl bound/nucleotide, and the difference $n_{\text{HCl}} \times 4$ bound along curves I and II (Fig. 2) is also given because the area enclosed by the plot of Δn_{HCl} against pH is proportional to the free energy degraded or dissipated during a titration cycle (see Appendix 1). At a particular pH the slope $\delta(\Delta n_{\text{HCl}})/\delta\text{pH}$ of the plot Δn_{HCl} versus pH is related to the difference in the buffering capacities along curves I and II at this pH (see Appendix 2). The difference curve II-I (Fig. 2a) was obtained directly and is also the sum of the differences between curves IIa and I, etc., which serve to show the complex make-up of the hysteresis loop.

The difference curves $n_{\text{HCl(I)}} - n_{\text{HCl(IIa)}}$ etc. versus pH serve to show the pH values at which protons

are bound or discharged irreversibly, and also reveal the lag in pH between the uptake and release of protons involved in this process. We infer from the difference curves given in Fig. 2 that this lag is at least one pH unit. The main features revealed by the scanning curves are (a) starting at pH 7-8 titration over the range pH 3.8-7 was reversible; (b) when the reversal point was decreased to pH 3.6 the proton binding properties of RNA were altered irreversibly (Fig. 2b); (c) lowering the reversal point still further, e.g. to pH 3.1 led to an increase in n_{HCl} bound irreversibly; and (d) starting from pH < 3.8, e.g. pH 3.3, a curve intermediate between I and II was attained by titrating first with alkali to e.g. pH 4.7 and then with acid. Similar results were

Fig. 2. Representative scanning curves observed on titration of rat liver rRNA at 0.1°C in 0.1M-NaCl

Curve I was followed on titration from pH 7 to 2.79 and curve II was traced on back-titration. (a) Titration from pH 7 to 3.80 and back to pH 7 (cf. Fig. 7a); (b) titration from pH 7 to 3.59 and back to pH 7 (cf. Fig. 7b); (c) titration from pH 7 to 3.59 to 4.94 and back to pH 3.33; (d) titration from pH 7 to 3.33 to 4.70 to 3.33; (e) titration from pH 7 to 3.24 to 4.86 to 3.24; (f) titration from pH 7 to 3.14 to 4.84 and back to pH 3.14 (cf. Fig. 9b). Curve IIb has the same relation to curve II as in Fig. 1. The continuous line in each figure denotes the observed path in each particular case.

obtained when unfractionated *E. coli* rRNA in 0.1M-NaCl was titrated at 0.1°C (Figs. 3 and 4). Cox (1963, 1966a) noted hysteresis effects on titration from pH 7 to 4 and below and it is over the range pH 3–4 that some of the short double-helical regions of rRNA 'melt'. It appears that the hysteresis effects might have their origin in changes in the double-helical secondary structure of rRNA.

Calculations from the pH-titration curves

The minimum fraction of residues involved directly or indirectly in conformational changes causing hysteresis may be deduced from the difference in $n_{\text{HCl}} \times 4$ bound on the forward- and back-titration curves. For example, adenine and cytosine residues account for about half of the base residues of rat liver RNA and Δn_{HCl} is maximum at about pH 4 and amounts of 0.075 equiv., hence about 15% of the adenine and cytosine residues are affected in one way or another. Values for *E. coli* rRNA and other RNA species are compared in Table 2.

The free energy expended irreversibly in the hysteresis cycle is $T\oint d_i S$ (see Appendix 1) which is given (Katchalsky *et al.*, 1966) by eqn. (1):

$$T\oint d_i S = 2.3 RT \cdot \oint n_{\text{HCl}} \cdot dpH > 0 \quad (1)$$

The cyclic integral $\oint n_{\text{HCl}} \cdot dpH$ is the area under the difference curves given in Figs. 2 and 4 and allows $T\oint d_i S/\text{nucleotide}$ to be calculated. However, not all nucleotides are involved in hysteresis. The estimate of the minimum fraction of residues involved in hysteresis divided into $(T\oint d_i S/\text{nucleotide})$ yields a maximum value for $(T\oint d_i S/\text{nucleotide})$ involved in hysteresis. The estimated values so obtained (see Table 2) are respectively -3.7 and -5.0 kJ/mol (-0.87 and -1.2 kcal/mol) of nucleotide involved in hysteresis for *E. coli* rRNA and rat liver rRNA at 0.1°C compared with the calculated value of -5.9 (s.d. ± 0.4) kJ/mol [-1.4 (s.d. ± 0.1) kcal/mol] of adenine residue for poly(A)·2poly(U) at 20°C (Neumann & Katchalsky, 1970).

Discussion

It is generally accepted that rRNA has a secondary structure in which double-helical segments formed as a result of intramolecular interactions are linked by single-stranded regions (Doty *et al.*, 1959; for reviews see Cox, 1968; Spirin & Gavrilova, 1969). It is probable that the double-helical segments are stabilized by A·U and G·C base pairs. One partner of each base pair, namely an adenine or a cytosine residue, can become protonated in acidic solutions,

Fig. 3. Scanning curves of *E. coli* rRNA in 0.1 M-NaCl at 0.1°C (cf. Fig. 1)

Curve I: titration with acid from pH 8; curves Ia and Ib: titration from pH 8.0 to 3.7 to 5.2 to 3.7; curves Ic, Ib and Ia: titration from pH 8.0 to 3.58 to 4.88 to 3.58; curves Ia and Ic: titration from pH 8.0 to 3.37 to 8.0; curves Ia and Id: titration from pH 8.0 to 2.80 to 8.0.

although double-helical secondary structure tends to suppress ionization (see Table 1). By analogy with DNA and double-helical RNA (Cox *et al.*, 1971), the double-helical part of rRNA secondary structure should decrease in stability as the solution is acidified, as appears to be the case (e.g. Cox & Littauer, 1963; Cox, 1963). The hysteresis shows that not all the conformation changes are reversed on simply raising the pH. One possibility is that the double-helical regions pass through a pH range where they are metastable, and once they 'melt', at pH_m say, the single-stranded regions can recombine at a higher pH, $pH_m + \Delta pH$, corresponding to the thermodynamic equilibrium point. However, the 'scanning' curves reveal that there is a lag of about 1 pH unit (i.e. $\Delta pH \approx 1$) before the structure present initially at pH 8 is re-formed. It appears that no more than a small fraction of double-helical regions can have these properties because the changes in extinction, e.g. at 260 nm, namely an increase caused by the 'melting' and a corresponding decrease caused by the re-formation of double-helical segment, would also be expected to lag behind one another by about 1 pH unit, and this effect is not observed (e.g. Gould & Simpkins, 1969).

In addition to participating in the classical double-helical structures, both adenine and cytosine residues are able to complex with themselves to form poly(A)·poly(A α H⁺) (e.g. Steiner & Beers, 1959) and poly(C)·poly(C α H⁺) (Hartman & Rich, 1965) which are stable in acidic but not in neutral solutions. Thus hysteresis might arise if rRNA can form oligo(A)·oligo(A α H⁺) or oligo(C)·oligo(C α H⁺) regions in acidic solutions, provided that there exists a pH range when the double-helical segments present initially at pH 7, and either oligo(A)·oligo(A α H⁺) or oligo(C)·oligo(C α H⁺) or both have stability or metastability. At pH 4 or below, when A·U and G·C base pairs 'melt', oligo(A)·oligo(A α H⁺) for example, can have high stability. In this case hysteresis involves different kinds of ordered forms, as was suggested earlier (Cox & Littauer, 1963; Cox, 1963, 1966a). Such a mechanism appears to be more in accord with available information. On this basis there will be a dependence on nucleotide sequence, so that different RNA species would be expected to exhibit hysteresis to different extents (see results for rRNA, tRNA and RNA from turnip-yellow-mosaic virus; Cox, 1966a), and only a fraction of the double-helical segments need be involved, whereas the re-

Table 2. Estimates of the fraction of residues involved in 'hysteresis' and the free energy dissipated ($T\delta d_i S$) on completing a titration cycle

RNA species	Mol fraction of adenine+cytosine residues	Temperature (°C)	$(\Delta H_{HCl})_{max.}$ (see Figs. 1-4)	Minimum % of adenine+cytosine residues involved in hysteresis	$T\delta d_i S$ of total RNA		$T\delta d_i S$ involved in hysteresis	
					(kJ/mol of nucleotide)	(kcal/mol of nucleotide)	(kJ/mol of nucleotide)	(kcal/mol of nucleotide)
<i>E. coli</i> rRNA	0.5	0.1	~0.06	~12	~0.437	~0.104	~3.7	~0.87
		25†	~0	~9	~0.32	~0.075	~3.5	~0.83
		38.5*		~4	~0.22	~0.052	~5.5	~1.3
Rat liver rRNA	0.49		~0.075	~15.3	~0.773	~0.184	~5.0	~1.2
Reticulocyte rRNA	0.45	25	~0.04	~9	~0.32	~0.076	~3.6	~0.85
<i>E. coli</i> rRNA (unfractionated)†	~0.5	25	~0.01	~2	~0.147	~0.035	~7.1	~1.7

* Values of Cox & Littauer (1963).
† Values of Cox (1966a).

mainder are free to 'melt' and re-form reversibly. Moreover, the transition from one ordered form to another may lead to little overall change in the hypochromic effect. The observed dependence of, e.g., ϵ_{260} on pH is consistent with this argument.

Moreover, hysteresis has been demonstrated in the model polynucleotide systems poly(A)·poly(U) and poly(A)·2poly(U) (Cox, 1963; Massoulié, 1968; Clauwaert, 1968; Neumann & Katchalsky, 1970). It was shown that poly(A)·2poly(U) is the metastable form (Neumann & Katchalsky, 1970).

The pattern of 'scanning' curves is also consistent with hysteresis arising from the transition of one ordered form to another. This point is illustrated by utilizing a specific example that serves as a basis for calculations. The example chosen is a hypothetical sequence (see Fig. 5) that was devised (Cox, 1966a) to summarize features of the secondary structure of rRNA from rabbit reticulocytes deduced from spectrophotometric titrations and from an analysis of the hypochromic effect (denaturation at constant pH). The spectral changes and the proton-binding curves can be calculated for this single intramolecular 'domain'. The values so obtained serve as a basis for interpreting the results obtained with rRNA. For simplicity the role of adenine residues is emphasized in the hypothetical scheme. The argument is not affected because the acid-base properties of oligo(A)·oligo(A α H⁺) and oligo(C)·oligo(C α H⁺) are rather similar, although the stabilities of the two complexes differ. The stability of poly(C)·poly(C α H⁺) decreases in solutions more acidic than pH4 and it 'melts' at about pH3 at 20°C (Hartman & Rich, 1965), whereas poly(A)·poly(A α H⁺) remains stable.

Implicit in the hypothetical sequence is the notion that metastability might arise because the nucleation step in the formation of a double-helical region is prevented. Normally segments can explore one another to find a suitable partner, and once the first few base pairs are formed the remainder then snap together. However, if the segments having the potential of interacting with one another are 'frozen' or so constricted that they cannot meet one another, it would appear that nucleation cannot proceed. Thus segments that are potential partners are immobilized either because on titration with acid from pH7 loops 2 and 3 are the constraint, or because on titration with alkali from low pH the oligo(A)·oligo(A α H⁺) loop is the impediment.

In rRNA, each hairpin loop with a particular nucleotide sequence probably occurs no more than once per molecule, i.e. once per 1500 nucleotides (16S RNA) or once per 3000 nucleotides (23S RNA), so that there is little chance of a more distant but closely related segment of nucleotide sequence releasing potential partners locked in metastable forms. Even this small chance is further decreased by

the presence of loops, e.g. loops 1 and 4, which impose further constraints on the polynucleotide chains. Another possible hindrance to nucleation is that the strands in the oligo(A)·oligo(A α H⁺) loop presumably run parallel to one another, as in poly(A)·poly(A α H⁺) (Rich *et al.*, 1961), whereas the segments of loops 1-4 are antiparallel (Fuller *et al.*, 1965).

Calculation of the titration curves of the hypothetical domain

The 'scanning' curves provide a set of observations that test any scheme proposed to explain the hysteresis found on pH-titration of rRNA. The potentiometric titration curves can be calculated (to a first approxi-

mation) for the hypothetical sequence and the aim of this section is to show that the forms of the scanning curves can be reproduced on the basis of our assumption about metastable states. The uptake of acid by the polynucleotide at a particular pH is given by eqn. (2):

$$n_{\text{HCl}} = f_{\text{A(am)}} \alpha_{\text{A(am)}} + f_{\text{A(dh)}} \alpha_{\text{A(dh)}} + f_{\text{A(A}\cdot\text{A)}} \alpha_{\text{A(A}\cdot\text{A)}} + f_{\text{C(am)}} \alpha_{\text{C(am)}} + f_{\text{C(dh)}} \alpha_{\text{C(dh)}} \quad (2)$$

where n_{HCl} is the mol of HCl bound/nucleotide, f_{A} and f_{C} are respectively the mol fractions of adenine and cytosine residues, the subscripts am, dh, and (A·A) denote conformational states, namely the amorphous single-stranded, the classical double-helical and the double-helical form of oligo(A) that is stable when

Fig. 4. Representative scanning curves of *E. coli* rRNA in 0.1 M-NaCl at 0.1°C

(a) Titration from pH 7 to 4.45 and back to pH 7 (protonation was reversible over this pH range; cf. Fig. 7a); (b) titration from pH 7 to 3.70 and back to pH 7 (cf. Figs. 8 and 9a); (c) titration from pH 7 to 3.70 to 5 and back to pH 3.58 (cf. Fig. 9a); (d) titration from pH 7 to 3.58 to 4.88 to 3.58; (e) titration from pH 7 to 3.37 and back to pH 7 (curve IIb) (cf. Fig. 9b); (f) titration from pH 7 to 3.14 to 7.

one strand is partly protonated, i.e. oligo(A)·oligo(A α H⁺) forms; $\alpha_{A(am)}$ etc. and $\alpha_{C(am)}$ etc. are the degrees of protonation of adenine and cytosine in the various conformations for reactions of the type, e.g.:

The equilibrium constant, e.g. $K_{A(am)}$, is given by eqn. (4):

$$\text{pH} = \text{p}K_{A(am)} + \log(1 - \alpha_{A(am)})/\alpha_{A(am)} \quad (4)$$

The electrostatic potential is practically constant over the pH range of interest (Peacocke & Lifson, 1956), and the term $0.43 e\psi/kT$ is included in $\text{p}K_{A(am)}$.

The diagram (Fig. 5) was used to ascertain the conformational state at a particular pH on titration with acid from pH 7 or with alkali from acidic pH values; $f_{A(am)}$, $f_{A(dh)}$ etc. were then derived and values of $\alpha_{A(am)}$ etc. were calculated by means of eqn. (4) from the appropriate $\text{p}K$ value given in Table 1.

Suppose that at a particular pH the number of equivalents of acid bound/nucleotide is $n_{HCl(I)}$ on titration with acid from pH 7 and is $n_{HCl(II)}$ on titration with alkali from acidic solutions. The difference ($n_{HCl(I)} - n_{HCl(II)}$) is obtained by writing eqn. (2) explicitly first for titration with acid (indicated by the subscript I, as in $f_{A(am)I}$ etc.) and then for

titration with alkali (indicated by the subscript II as in $f_{A(am)II}$ etc.) and subtracting one from the other. Thus for a particular pH:

$$\begin{aligned} \Delta n_{HCl(pHx)} &= (n_{HCl(I)} - n_{HCl(II)})_{pHx} \\ &= (f_{A(am)I} - f_{A(am)II}) \alpha_{A(am)} \\ &\quad + (f_{A(dh)I} - f_{A(dh)II}) \alpha_{A(dh)} + \text{etc.} \quad (5) \end{aligned}$$

The calculated potentiometric titration curves for adenine and cytosine residues of the hypothetical sequence are given in Fig. 6. The protonation of cytosine residues also shows hysteresis (Fig. 6c) because the G·C base pairs of loops 2 and 3 that are present on titration from pH 7 to pH 3.7 are prevented from re-forming reversibly, owing to the formation of the metastable oligo(A)·oligo(A α H⁺) loop. The contribution to n_{HCl} is comparable with that of adenine residues (cf. Figs. 6b and 6c) and, depending on the precise nucleotide sequence, could well be the major effect. As a consequence a demonstration of hysteresis on protonation of cytosine residues cannot be taken as evidence for the formation of oligo(C)·oligo(C α H⁺) loops.

With a single 'element' or 'domain' it is possible to move from one boundary curve to another, but it is not possible to reach a point within the loop. Suppose that we double the length of hypothetical sequence by adding another 84 nucleotides, which form loops

Fig. 5. Possible changes in the secondary structure of a polynucleotide on isothermal titration from pH7 to approx. pH3 and back to pH7 (see Cox, 1966a)

The hypothetical sequence has a nucleotide composition similar to rat liver or rabbit reticulocyte rRNA, and the number of A·U and G·C base pairs in the double-helical parts were assigned on the basis of an analysis of the hypochromic effect. (a) Conformation I stable at pH7; (b) protonated forms of conformation I, i.e. adenine and cytosine residues become protonated to an extent that depends on conformation (c). Double-helical parts poorer in G·C base pairs 'melt' at about pH3.75, allowing the formation of acid-stable double-helical regions (II) where the chains are parallel to one another (cf. Rich *et al.*, 1961); lowering the pH further, e.g. to pH3.1, alters the degree of protonation of conformation II, but not the conformation itself. On raising the pH conformation II persists (d) until pH5.5 is reached, where the acid-stable segments 'melt' so permitting conformation I to re-form. Acid-stable segments stabilized by C·(C α H⁺) base pairs cannot be excluded and might also contribute to hysteresis in rRNA. Either conformation I or conformation II or both might be attained because the nucleation step in the formation of the stable double-helical parts is prevented. Oligo(C)·oligo(C α H⁺) regions could also give rise to hysteresis in a similar way. (e) Titration below pH2.75 leads to 'melting' of the G·C-rich loops 1 and 4 (conformation III), which re-form reversibly on raising the pH.

Fig. 6. Potentiometric titration curves for the hypothetical domain (Fig. 5)

The $n_{\text{HCl}} \times 4$ was calculated for the cycle pH7 to 2 and back to pH7 by means of eqn. (5) by using the values of Table 1. (a) Total titration behaviour of a domain on isothermal titration at 25°C. Curve I indicates the value of $n_{\text{HCl}} \times 4$ after starting at pH7. Bringing the solution to pH3.80 does not alter the conformation (cf. Figs. 5a and 5b); loops 2 and 3 'melt' on passing beyond pH3.75, allowing loop A·A-1 to form. Curve II is characteristic of Figs. 5(c) and 5(d). Loops 1 and 4 'melt' on titration to pH2, but re-form reversibly on back-titration. Curve II is followed on back-titration, and loops 2 and 3 do not re-form in passing back through pH3.75 because oligo(A)·oligo(A α H⁺) persists until pH5.50 is attained. The original conformation is re-formed above pH5.50. (b) Contribution of adenine residues; (c) contribution of cytosine residues.

1', 2', 3' and 4' that are very similar to loops 1, 2, 3 and 4 respectively. However, loops 2' and 3' 'melt' at pH3.5 rather than at pH3.75 because, say, each has an extra G·C base pair. We also suppose that 'melting' at pH3.5 leads to an oligo(A)·oligo(A α H⁺) (loop

A·A-2 for simplicity) that 'melts' at pH5.0 because, say, there is one A·A pair less than in loop A·A-1. The potentiometric titration curves calculated for such a polynucleotide are given in Fig. 8.

Suppose that loops 2' and 3' melt at a lower pH

Fig. 7. Scanning curves for the hypothetical cycle (Fig. 5)

(a) Titration from pH 7 to 4 and back-titration to pH 7: curve I (Fig. 6) is followed and is reversible (cf. Figs. 5a and 5b) because there has been no conformational change (i.e. pH 3.75 has not been reached); (b) back-titration from pH 3.25 to 5.2: curve II is followed reversibly because the conformation (Figs. 5c and 5d) remains unchanged; (c) titration from pH 7 to 3.25 to 5.0: curve I is first followed until pH 3.75 is reached (cf. Figs. 5b and 5c) and curve II is subsequently followed because the conformation (Figs. 5c and 5d) remains unchanged; (d) titration from pH 3.25 to 7 to 3.8: to pass from curve II to curve I the pH is brought from pH 3.25 beyond 5.5; curve I is then followed reversibly provided the pH does not fall below pH 3.75.

(say pH 3.5) than loops 2 and 3, because, e.g. each of them possesses an extra A·U base pair; and that loop A·A-2 melts at a higher pH (say pH 5.75) than loop A·A-1, possibly because the run of A·A base pairs is longer; and that $\Delta n_{\text{HCl}}(\text{pH}_x)$ (see eqn. 5) is generally greater for domain 2 (loops 2', 3' and A·A-2) than for domain 1 (loops 2, 3 and A·A-1); then one obtains the calculated curves given in Fig. 10. A new feature is revealed, namely that two scanning curves are possible (cf. Fig. 9). Comparison of the observed scanning curves (Figs. 2 and 4) with those calculated (Figs. 8–10) shows that the main features are similar. It is inferred that the observed hysteresis could arise from cycles of the type illustrated in Fig. 5 although other explanations cannot be excluded.

In rRNA many more than two domains are likely to be present, each having a stability that is different from the others, and the range in the properties of these elements accounts for the shape of the hysteresis loop.

If the intramolecular domain has the form given in Fig. 5 then at approx. pH 5.5 the major contribution to Δn_{HCl} should arise from acid-stable regions [whether oligo(A)·oligo(A α H⁺) or oligo(C)·oligo(C α H⁺)] and those that persist at this pH should be about 50% protonated. Hence $2(\Delta n_{\text{HCl}})_{\text{pH } 5.5}$ should approximate to the fraction of residues participating in acid-stable structures. It appears that for the RNA species mentioned in Table 2, $2(\Delta n_{\text{HCl}})_{\text{pH } 5.5} \approx 0.03$, suggesting that there might be one acid-stable region of 5 base pairs/approx. 330 nucleotides. Thus the number of clusters of adenine or cytosine residues (cf. Fig. 5) required to account for the hysteresis in rRNA is roughly 10 per 16S RNA molecule. Current knowledge of the primary sequence of *E. coli* rRNA allows this possibility (Fellner *et al.*, 1970a,b; Fellner & Ebel, 1970). The main point is that, according to the

Fig. 8. Potentiometric titration curves for two elements

Suppose that the hypothetical sequence (Fig. 5) was extended by another 8 nucleotides that differ in conformation from the original, so that loops 2' and 3' corresponding to loops 2 and 3 'melt' at pH 3.5 and not 3.75; and that loop A·A-2 corresponding to loop A·A-1 [the oligo(A)·oligo(A α H⁺) loop of Fig. 5(c)] 'melts' at pH 5.0 and not 5.50. Curve I is identical with curve I (Fig. 3). Curve IIa refers to the conformational loops 1, A·A-1, 1', 2', 3' and 4', and curve II to the conformational loops 1, A·A-1, 1', A·A-2 and 4'. The differences IIa-I and II-IIa respectively show the transition pH values of loops 2, 3 and A·A-1 and of loops 2', 3' and A·A-2.

scheme given in Fig. 5, hysteresis arises from a few domains and does not necessarily involve all the double-helical segments present at pH 7.

A weakness of the scheme is that there is no direct evidence for oligo(A)·oligo(A α H⁺) or oligo(C)·oligo(C α H⁺) regions in RNA. Both species of acid-stable structure require the polynucleotide chains to

Fig. 9. Scanning curves for the two element system described in Fig. 8

(a) Titration from pH 7 to 3.6 leads to the switch-over from loops 2 and 3 to loop A·A-1 so that curve IIa is followed on back-titration (cf. Fig. 7). (b) Titration from pH 7 to 3 leads to the formation of loops A·A-1 and A·A-2. Back-titration to pH 5.25 switches loop A·A-2 back to loops 2' and 3', but loop A·A-1 persists. However, curve IIa is followed on subsequent titration with acid from pH 5.25.

run parallel to one another, whereas the chains run antiparallel in regions stabilized by A·U and G·C base pairs. If the scheme illustrated in Fig. 5 is correct, rRNA may exist in a form in which two types of double-helical regions coexist in one molecule, one type in which the polynucleotide chains run antiparallel and another type in which the chains run parallel. More direct experiments are needed to test this possibility.

The significance of the hysteresis with respect to the function of rRNA is not clear. Hysteresis could arise

Fig. 10. Scanning curves for another two-element system

In the two-element system described in Fig. 8 loop A·A-2 was less stable than loop A·A-1. We now consider the situation where loop A·A-2 is more stable than loop A·A-1. For clarity $\Delta\eta_{\text{HCl}}$ for element 1 does not equal $\Delta\eta_{\text{HCl}}$ for element 2. Curve IIa is followed after titration to pH 3.6 and back to pH 7 because only domain 1 switches over (loops 2, 3 and A·A-1). Titration from pH 3 to 5.6 leads to another conformational state in which loops 1, 2, 3 and 4 have re-formed, but loop A·A-2 remains stable and prevents loops 2' and 3' from re-forming, so that a new curve Ia is followed on titration from pH 5.6. The differences IIa-I etc. indicate the pH values where the domains switch conformations.

fortuitously as a consequence of the primary sequence. There is no indication that particular features are involved, e.g. ribosomal protein-binding sites or other functional regions.

Boltzmann (1876) pointed out that 'hysteresis' is a memory phenomenon in that the future behaviour of a system depends on its past history, and Ewing (1881a) was aware that hysteresis permitted 'molecular reminiscences'. In this respect rRNA possesses a molecular memory. Memory devices that utilize hysteresis phenomena include ferroelectric and ferromagnetic memory units of electronic computers and of magnetic tape recorders. Everett (1954) suggested that a possible relationship between hysteresis phenomena and short-term memory in higher animals might be worth examining. This point was elaborated by Katchalsky & Oplatka (1966). The phenomenon of hysteresis in rRNA is relevant because other rRNA species have been implicated in memory function (for review see Glassman, 1969). Perhaps the possibility that metastable macromolecular states of high permanence play a role in processes of memorizing cannot be entirely excluded.

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APPENDIX 1

Dissipation of free energy on circulating around any loop of hysteresis

The first law of thermodynamics may be written as:

$$dU = d_e Q - dW \quad (1)$$

where $d_e Q$ is the heat exchanged with the surroundings and dW the work performed by the system on the surroundings. The heat term $d_e Q$ may also be written as $T d_e S$ where $d_e S$ is the entropy taken up from the medium.

The total entropy change of the system, dS , is composed of $d_e S$, taken up from the surroundings, and the entropy, $d_i S$, created by all irreversible processes, i.e.:

$$dS = d_e S + d_i S \quad (2)$$

where:

$$d_i S \geq 0$$

Thus eqn. (1) can be transformed into:

$$dU = T dS - T d_i S - dW \quad (3)$$

Since dU and dS are total differentials, their integral over a cycle vanishes, or at constant T :

$$\oint dU = T \oint dS - T \oint d_i S - \oint dW$$

or

$$0 = -T \oint d_i S - \oint dW$$

so that:

$$-\Delta G = T \oint d_i S = -\oint dW \quad (4)$$

Since $d_i S > 0$, $dW < 0$, which states the physically

evident requirement that to close an irreversible cycle work has to be invested into the system.

Under conditions of constant pressure and temperature, as is the case in our titration, the work is due to change in the number of moles of all components dn_i or $dW = -\sum \mu_i \cdot dn_i$, so that eqn. (4) may be written as:

$$T \oint d_i S = \oint \sum \mu_i \cdot dn_i \quad (5)$$

But $\sum \mu_i \cdot n_i$ is the free energy and its differential is total, so that $d \sum \mu_i \cdot n_i = 0$ and

$$\oint \sum \mu_i \cdot dn_i = -\oint \sum n_i \cdot d\mu_i \quad (6)$$

or

$$T \oint d_i S = -\oint \sum n_i \cdot d\mu_i \quad (7)$$

In the titration the only number of moles changing is that of HCl or $n_i = n_{\text{HCl}}$, and hence $d\mu_i = d\mu_{\text{HCl}}$. Further, $d\mu_{\text{HCl}} = d\mu_{\text{H}} + d\mu_{\text{Cl}}$ and, since Cl^- ions are kept in excess: $d\mu_{\text{Cl}} = 0$ (8)

As usual:

$$\begin{aligned} d\mu_{\text{HCl}} &= d\mu_{\text{H}} = RT \ln a_{\text{H}} = 2.3 RT \log a_{\text{H}} \\ &= 2.303 RT \text{dpH} \end{aligned} \quad (9)$$

Thus the final expression is:

$$T \oint d_i S = -2.303 RT \oint n_{\text{HCl}} \text{dpH} \quad (10)$$

Eqn. (10) holds for both the total hysteresis loop and for any closed loop of scanning curves within the cycle.

APPENDIX 2

Significance of the difference curves ($n_{\text{HCl(I)}} - n_{\text{HCl(II)}}$) against pH

The equivalents of acid bound per nucleotide (n_{HCl}) at a particular pH is $n_{\text{HCl(I)}}$ on titration from pH 7 and is $n_{\text{HCl(II)}}$ on titration with alkali from low pH to pH 7. The difference $n_{\text{HCl(I)}} - n_{\text{HCl(II)}}$ plotted as a function of pH seems to show the pH values where protons are bound or discharged irreversibly, and also reveals the lag in pH between the uptake and release of protons involved in this process. Moreover, the scanning curves expressed as plots of Δn versus pH are particularly useful in revealing the complexity of the hysteresis loop (see the Discussion section of the main paper). In Appendix 1 it was shown that the area under the curve is proportional to the free energy degraded or dissipated during a titration cycle. In addition, the slope of the curve of Δn versus pH is related to the difference in buffering capacities. Buffering capacity is defined as $\beta = \partial n / \partial \text{pH}$. Since:

$$\Delta n_{\text{HCl}} = n_{\text{HCl(I)}} - n_{\text{HCl(II)}} \quad (1)$$

then:

$$\begin{aligned} \frac{\partial \Delta n_{\text{HCl}}}{\partial \text{pH}} &= \frac{\partial n_{\text{HCl(I)}}}{\partial \text{pH}} - \frac{\partial n_{\text{HCl(II)}}}{\partial \text{pH}} \\ &= \beta_{\text{I}} - \beta_{\text{II}} \end{aligned} \quad (2)$$

where β_{I} and β_{II} respectively are the buffering capacities of a particular pH on forward-titration with acid from pH 7 and on back-titration from an acidic pH to pH 7.

The plots of Δn versus pH and also of $\partial \Delta n / \partial \text{pH}$ versus pH are likely to show fine structure because the proton-binding curves themselves are complex. For a simple mixture of nucleotides:

$$n_{\text{HCl}} = \sum C_i K_i / (K_i + H) \quad (3)$$

where C_i is the concentration of the i th group whose dissociation constant is K_i and H is the H^+ ion concentration. By definition:

$$\beta = \partial n_{\text{HCl}} / \partial \text{pH} = \sum \beta_i \quad (4)$$

Eqn. (4) applies when both C_i and K_i are constants. For RNA K_i depends on conformation, which in turn also depends on pH. Thus for those groups involved in secondary structure K_i is no longer constant. Hence:

$$\frac{dn_{\text{HCl}}}{d\text{pH}} = \sum \left[\left(\frac{\partial n_{\text{HCl}}}{\partial K_i} \right)_{C_i, H} \cdot \frac{dK_i}{d\text{pH}_i} + \left(\frac{\partial n_{\text{HCl}}}{\partial \text{pH}} \right)_{K_i, C_i} \right] \quad (5)$$

The term $dK_i/d\text{pH}_i$ denotes the dependence of conformation on pH. In view of the co-operative nature of the transition of the double-helical and single-stranded forms of a polynucleotide, unusually high buffering capacities are to be expected at pH values where conformational changes take place. Theory is not yet sufficiently developed to allow us to calculate *ab initio* the proton-binding curves and the buffering capacity of rRNA.