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A Study of the Alkaline Hydrolysis of Fractionated Reticulocyte Ribosomal Ribonucleic Acid and its Relevance to Secondary Structure

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1. RNA isolated from the sub-units of rabbit reticulocyte ribosomes was hydrolysed by 0·4N-potassium hydroxide at 20°. The probability of main-chain scission was calculated from the number-average chain length, which was obtained from $S_{25,w}$ in 0·01M-phosphate buffer. 2. The fraction, f, of the original secondary structure that the fragments re-formed at neutral pH in 4M-guanidinium chloride, as well as in 0·01M- and 0·1M-phosphate buffer, was derived from changes in extinction over the range 220–310mμ on thermal denaturation. 3. The secondary structure of RNA is regarded as an assembly of hairpin loops each of $2N+b$ residues on average, where N is the number of base-paired residues and b is the number of unpaired residues. 4. If chain scission takes place at random then $2N+b=\log f/\log(1-p)$. 5. For RNA from the smaller sub-unit $2N+b$ was estimated as 25 ± 5 residues, compared with 30 ± 5 residues for the less stable species and 35 ± 5 residues for the more stable species of hairpin loop of RNA from the larger sub-unit.

For several years the secondary structure of RNA has been regarded as an assembly of partly double-helical hairpin loops joined by flexible single-stranded regions (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). Except for the small-angle X-ray-scattering studies by Timasheff, Witz & Luzzatti (1961), most studies of the secondary structure of RNA have been limited to an examination of the gross hydrodynamic properties, to the measurement of the 'helical content', i.e. the fraction of residues in double-helical segments (for review see Spirin, 1963), and to an analysis of the nucleotide composition of the double-helical segments (Felsenfeld & Cantoni, 1964; Fresco, Klotz & Richards, 1963; Cox, 1965, 1966*a*,*b*).

The 'hairpin-loop' model of a polynucleotide may be formulated as L loops, each of $2N+b$ residues on average (where N is the number of base pairs and b is the number of unpaired residues per loop), linked by c residues. The secondary structure of RNA could be more precisely defined if N, b and c were known. Degradation studies appear to offer the possibility of evaluating these parameters (Cox & Kanagalingam, 1967*a*).

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THEORETICAL

The principle of the method was described in the preceding paper (Cox, 1968). Double-helical secondary structure is absent at pH 13 (Cox, 1966*a*,*b*), but hydrolysis of di-esterified phosphate bonds takes place essentially at random over the molecular-weight range of interest (Kaltreider & Scott, 1962). When the solution is neutralized a hairpin loop of i residues may re-form, provided that these residues remain united. The probability of an internucleotide bond remaining intact is $1-p$, where p is the probability of chain scission. The probability that a loop will remain intact is the probability $(1-p)^i$ that a sequence of i residues will stay intact. For the species $2N+b=i$, which forms l_i loops in the intact molecule, the number of loops remaining intact is $(1-p)^i l_i$. Hence the fraction, f_i, of intact loops is:

$$f_i = (1-p)^i \qquad (1)$$

Thus i is the slope of the plot of $\log f_i$ against $\log(1-p)$.

Hydrolysis may also lead to fragments that form damaged or 'frayed' loops which are stabilized by fewer than the original N base pairs, as well as to fortuitously formed hairpin loops which arise from sequences that cannot form either the intact or damaged loops. The fraction, f'_A, of 'frayed' loops is:

$$f'_A = [1-(1-p)^2]\textstyle\sum_{i-2(N-n^0)}^{i}(1-p)^{i-2(N-n)} \qquad (2)$$

where n ($<N$) is the number of base pairs per 'frayed' loop and n^0 is the minimum number of base pairs required to form a stable loop.

The fraction f''_A of loops damaged and unable to form either intact or 'frayed' loops is $1-f_i-f'_A$. A minimum sequence $b''+2n''$ residues is required for hairpin loops to form fortuitously. The fraction f''_A of loops damaged and unable to form 'frayed' loops but big enough to form a loop fortuitously is:

$$f''_A = \{1-(1-p)_i - [1-(1-p)^2]\textstyle\sum_{i-2(N-n')}^{i}(1-p)^{i-2(N-n)}\}(1-p)^{b''+2n''} \quad (3)$$

It is possible that for ribosomal RNA the hairpin loops are heterogeneous in their nucleotide sequence and also possibly in length. Several species of hairpin loops may denature over a particular temperature range so that the fraction, $\bar{f}$, that remain intact after hydrolysis is:

$$\bar{f} = \Sigma f_i l_i \Sigma l_i = (1-p^{\bar{i}}) \quad (4)$$

where $\bar{i}$ is an average value calculated from the plot of $\log \bar{f}$ against $\log(1-p)$.

Evaluation of p. The probability of main-chain scission at time t is equal to the fraction of bonds hydrolysed. The number of bonds hydrolysed is equal to the number of fragments formed, so that if x_n is the number-average chain length of the fragments and x_n^0 refers to the intact molecule then (Tanford, 1961):

$$p = 1/x_n - 1/x_n^0 \quad (5)$$

The values of x_n^0 are about 1500 and 4500 for RNA from the smaller and larger sub-units respectively, so that $1/x_n^0$ is negligible and eqn. (5) reduces to:

$$p = 1/x_n \quad (6)$$

When hydrolysis takes place at random (e.g. Oth & Desreux, 1954; Tanford, 1961):

$$2M_n = M_w \quad (7)$$

where M_n and M_w are the number-average and weight-average molecular weights respectively. Hence p may be obtained from a knowledge of M_w. The procedure adopted was to measure the sedimentation coefficient of the fragments in 0·01 M-phosphate buffer, pH 7. A relation between S^0 and M_w was established by Hall & Doty (1958) for fragments of calf liver ribosomal RNA:

$$S^0_{25,w} = 2{\cdot}1\times10^{-2}M_w^{0{\cdot}49}\,\text{s} \quad (8)$$

Rabbit reticulocyte and calf liver ribosomal RNA have a similar nucleotide composition and the fragments obtained in this work (M_w=15000–100000) were within the range calibrated by Hall & Doty (1958). Thus eqn. (8) may be used to calculate M_w from the sedimentation coefficient. Hence:

$$p = 330\times2/M_w \quad (9)$$

where 330 is taken as the average weight of a nucleotide.

Measurement of $\bar{f}$. The fraction of hairpin loops re-formed on neutralization was obtained from the denaturation spectrum. In solutions such as 4 M-guanidinium chloride the tendency of base residues to stack along a single strand is decreased, but the stability of double-helical structures is scarcely affected (Cox & Kanagalingam, 1967*b*). Provided that the novel species of hairpin loop do not interfere:

$$\bar{f} = (E_{(T_2)}-E_{(T_1)})/(E^0_{(T_2)}-E^0_{(T_1)}) \quad (10)$$

where $E^0_{(T)}$ and $E_{(T)}$ are the extinctions at temperature T ($T_2>T_1$) before and after hydrolysis respectively. The total contribution $\Delta E'_A$, of 'frayed' loops to the denaturation spectrum is:

$$\Delta E'_A = f'_A(\bar{n}/N)(E_{\text{max.}}-E_{(25)}) \quad (11)$$

where $\bar{n}$ is the average value of n, the number of base pairs per frayed loop, and $E_{\text{max.}}-E_{(25)}$ is the total change in E due to double-helical secondary structure. The contribution E''_A to the denaturation spectrum of fortuitously formed loops is:

$$\Delta E''_A = f''_A(\bar{n}_f/N)A(E_{\text{max.}}-E_{(25)}) \quad (12)$$

where $\bar{n}_f$ is the average number of base pairs per loop and A is a factor that takes into account the difference in the denaturation spectrum of intact and fortuitously formed loops.

In solvents such as 0·01 M- or 0·1 M-phosphate buffer a polynucleotide chain reverts to a single-stranded but stacked structure on denaturation (Cox & Kanagalingam, 1967*a*,*b*). At temperature T the extinction E is given by the equation:

$$E = hE_{\text{dh}}+(1-h)E_s \quad (13)$$

where E_s and E_{dh} respectively are the average extinction per residue of base residues in single-stranded but stacked and double-helical conformations respectively (E_{dh} but not E_s being independent of temperature) and h is the fraction of residues in double-helical segments. Hence:

$$h = (E_s-E)/(E_s-E_{\text{dh}}) \quad (14)$$

After hydrolysis for t min. h decreases from h_0 before hydrolysis to h_t and E increases from E_0 to E_t, so that:

$$\bar{f} = \Sigma f_i l_i \Sigma l_i = h_t/h_0 = (E_s-E_t)/(E_s-E_0) \quad (15)$$

Thus E_s is required in order to evaluate f. An approximate value was obtained by studying the hypochromism of RNA after reaction with formaldehyde at 95°. Hydroxymethylation prevents the formation of hairpin loops stabilized by double-helical structure (Stollar & Grossman, 1962; Thomas & Berns, 1962), but does not prevent stacking (Fasman, Lindblow & Grossman, 1964). The increment in E_{260} was found to be the same for polycytidylic acid before and after reaction with formaldehyde (Fasman *et al.* 1964); the increase in E_{260} noted on heating polyadenylic acid was decreased by about 20% (Stevens & Rosenfeld, 1966). Reaction with formaldehyde did not appreciably affect the denaturation spectrum of denatured DNA in mM-sodium phosphate buffer, pH 7, on heating from 50° to 95° (R. A. Cox & K. Kanagalingam, unpublished work). The increments in E_{260} and E_{280} noted for RNA after reaction with 1% formaldehyde at 95° are taken as an indication of changes in E_s as a function of temperature.

EXPERIMENTAL

Chemicals. Guanidinium chloride was prepared from A.R. guanidinium carbonate by neutralization with conc. HCl. The solution was treated with activated charcoal (Norit) to remove coloured impurities. The u.v.-absorbing impurities were destroyed by heating the conc. guanidinium chloride solution to 90°. A.R. formaldehyde was used.

Ribosomes from the immature erythrocytes of anaemic rabbits were isolated as described by Arnstein, Cox, Gould & Potter (1965). The ribosomes were dissociated into sub-units by titration with EDTA (Gould, Arnstein & Cox, 1966). The sub-units were fractionated by zone-centrifuging (Klucis & Gould, 1966) and RNA was then isolated by precipitation as the guanidinium salt (Cox, 1966c).

Spectrophotometry. A Unicam SP.700 spectrophotometer was used for all measurements. The sample-cell holder was replaced by an electrically heated copper block. The temperature was controlled to better than 0·1°.

Ultracentrifuging. A Spinco model E analytical ultracentrifuge fitted with u.v. optics was used. The camera was replaced by a photoelectric cell that scanned the slit, and the movement of the sedimenting species was followed by means of a plot of percentage transmission against distance on an XY recorder.

RESULTS

RNA (40mg.) from the larger ribosomal sub-unit was dissolved in 0·4N-potassium hydroxide (40ml.) at 22°. Samples were removed after 1, 5, 15, 30 and 45min. and neutralized with 0·2M-sodium dihydrogen phosphate made 0·4N with respect to hydrochloric acid. RNA was then precipitated by the addition of 2vol. of ethanol and redissolved in water. Experiments with unfractionated RNA showed that after hydrolysis to fragments of about 2·2S no more than about 5% of the extinction remained in the ethanolic supernatant. After hydrolysis for 45min. the amount of RNA from the larger sub-unit that was precipitated by ethanol and redissolved in water was 93%. RNA (4mg.) from the smaller sub-unit was hydrolysed in the same way and samples were withdrawn after 5, 15, 30 and 45min., neutralized and precipitated by the addition of ethanol. The amount of RNA that was precipitated by ethanol and redissolved in water was 91% after hydrolysis for 45min. For each sample the sedimentation pattern of a dilute solution (0·03g./l.) of RNA in 0·01M-sodium phosphate buffer, pH7, was obtained at 10–15°. Values of $S_{25,w}$ were calculated from the movement of the 50%-concentration point (see Table 1) and the corresponding value of p was calculated by means of eqns. (8) and (9).

The values of p were calculated for both RNA species and are given in Fig. 1 as a function of time in 0·4N-potassium hydroxide. It appears that the probability of hydrolysis of a diesterified phosphate linkage was about the same for both species. Chain scission appeared to take place at random, since p was proportional to time (Fig. 1).

Denaturation studies

RNA from the smaller ribosomal sub-unit. Each sample was neutralized and the denaturation spectrum was then measured in 0·01M-sodium phosphate buffer, pH7, 0·1M-sodium phosphate buffer, pH7, and in 4M-guanidinium chloride in 0·01M-sodium phosphate buffer, pH7. In each case the sample and reference cells contained identical solutions. The sample cell was heated and the difference between the spectrum of the sample and that of the reference solution maintained at 25° was obtained over the range 220–330mμ. In all cases a correction for changes in the concentration of RNA due to the thermal expansion of water was applied.

Table 1. *Values of $S_{25,w}$ (calculated from the movement of the 50%-concentration point) after hydrolysis*

Time of hydrolysis (min.)	$S_{25,w}$ (s) RNA from the smaller sub-unit	$S_{25,w}$ (s) RNA from the larger sub-unit
0	15	28·6
1	—	14·5
5	5·08	7·41
15	4·69	4·45
30	3·98	3·17
45	2·60	2·74

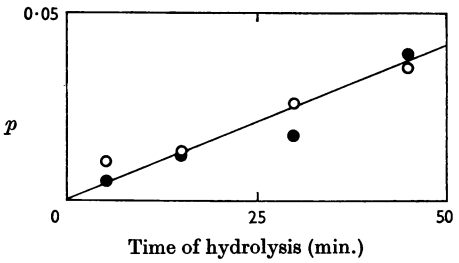

Fig. 1. Probability, p, of main-chain scission calculated by means of eqn. (9) as a function of the period of exposure to 0·4N-KOH. ○, RNA from the smaller sub-unit; ●, RNA from the larger sub-unit.

The denaturation spectra are summarized by the increments in E_{260} and E_{280}. The increments ΔE_{260} and ΔE_{280} observed on heating RNA in 0·1M-sodium phosphate buffer are given in Figs. 2(*a*)–2(*e*). Before hydrolysis E_{260} increased from 1·0 at 25° to 1·27 at 95°. The increment in E_{260} decreased as hydrolysis became more extensive, and after 45min. in 0·4N-potassium hydroxide E_{260} was found to increase from 1·0 at 25° to 1·195 at 95°. The temperature, T_m, at which 50% of the increment in E_{260} was observed was 57° at time of hydrolysis $t=0$ and 51° at $t=45$min. The $\Delta E_{280}/\Delta E_{260}$ ratio increased from 0·66 at $t=0$ to 0·78 at $t=45$min. The increment in E_{280} was only slightly affected by hydrolysis. The increments in E_{260} and E_{280} observed after reaction with formaldehyde (at 95°) are given in the inset Fig. 2(*a*),

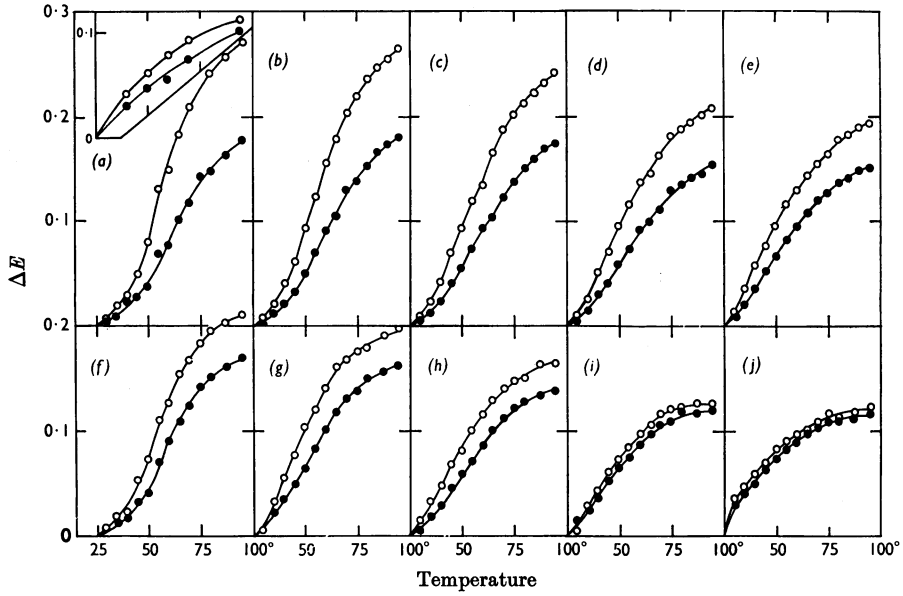

Fig. 2. Effect of hydrolysis on the thermal denaturation spectrum of RNA from the smaller sub-unit. (*a*)–(*e*) respectively refer to the intact molecule and to fragments obtained after hydrolysis for 5, 15, 30 and 45 min.; the solvent was 0·1 M-sodium phosphate buffer, pH 7·2. The inset in (*a*) shows the dependence of ΔE_{260} and ΔE_{280} on temperature found after RNA was heated to 95° in 0·1 M-sodium phosphate buffer, pH 7·2, containing 1% formaldehyde. (*f*)–(*j*), as for (*a*)–(*e*), but the solvent was 4 M-guanidinium chloride in 0·01 M-sodium phosphate buffer, pH 7·2. ○, ΔE_{260}; ●, ΔE_{280} ($E_{260}=1{\cdot}0$ at 25° in each case).

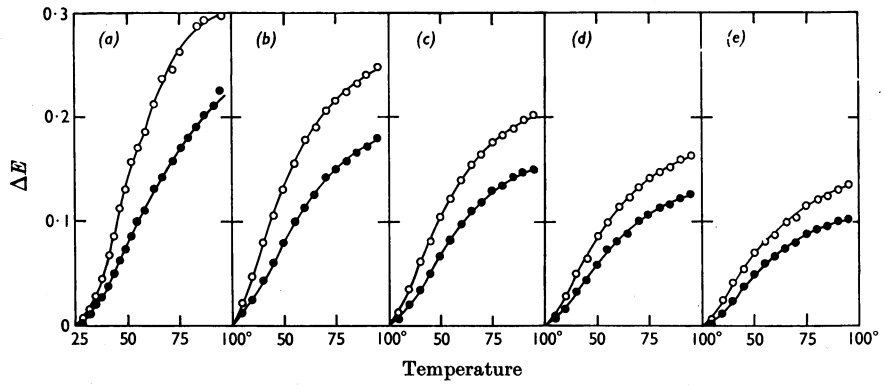

Fig. 3. Effect of hydrolysis on the thermal denaturation spectrum of RNA from the smaller sub-unit in 0·01 M-sodium phosphate buffer, pH 7·2. (*a*)–(*e*) respectively refer to the intact molecule and to the fragments obtained after hydrolysis for 5, 15, 30 and 45 min. ○, ΔE_{260}; ●, ΔE_{280} ($E_{260}=1{\cdot}0$ at 25° in each case).

which shows that although double-helical structure is absent both E_{260} and E_{280} increased reversibly over the range 29–95° by 0·115 ($E_{260}=1{\cdot}0$ at 25° before reaction with formaldehyde) and that the T_m is 47°. Hence the apparently small changes in the spectrum of RNA in 0·1M-phosphate buffer observed on hydrolysis may reflect large changes in the amount of double-helical structure.

It is clear from Figs. 2(*f*)–2(*j*), which summarize the denaturation studies in 4 M-guanidinium chloride, that hydrolysis leads to major changes in double-helical secondary structure. E_{260} increased

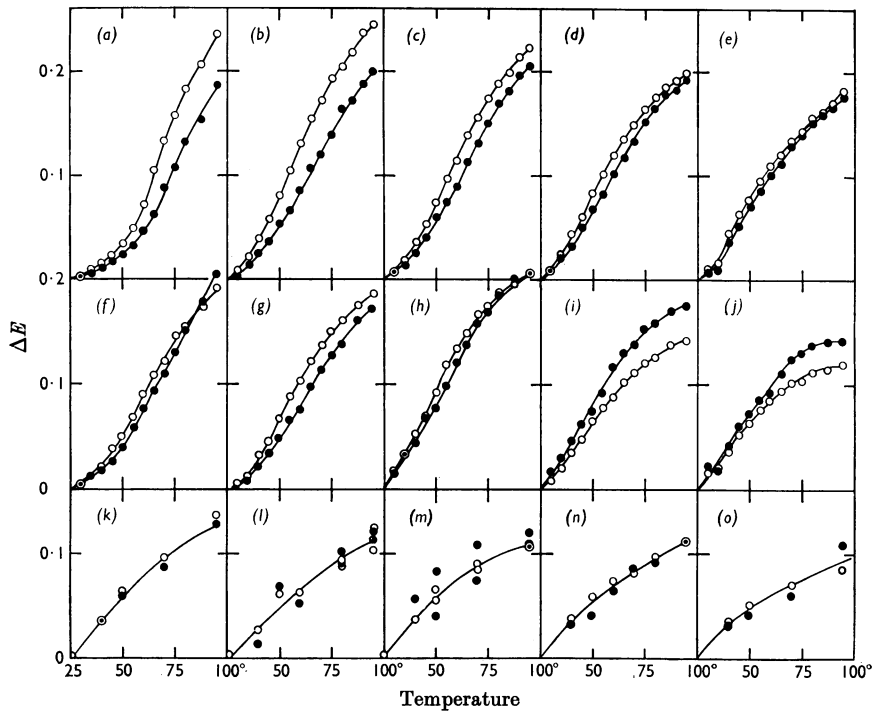

Fig. 4. Effect of hydrolysis on the thermal denaturation spectrum of RNA from the larger ribosomal sub-unit. (*a*)–(*e*) respectively refer to fragments obtained after 0, 5. 15, 30 and 45min. hydrolysis; the solvent was 0·1M-sodium phosphate buffer, pH7·2. (*f*)–(*j*), as for (*a*)–(*e*), but the solvent was 4M-guanidinium chloride in 0·01M-phosphate buffer, pH7·2. (*k*)–(*o*), as for (*a*)–(*e*) after reaction with formaldehyde; the solvent was 0·1M-phosphate buffer, pH7·2, containing 1% formaldehyde. ○, ΔE_{260}; ●, ΔE_{280} ($E_{260}=1{\cdot}0$ at 25° in each case).

from 1·13 at 60° to 1·21 at 95° before hydrolysis. After hydrolysis for 45min. E_{260} increased from 1·098 at 60° to 1·122 at 95°. The total increment in E_{260} was from 1·0 at 25° to 1·21 at 95° before hydrolysis and from 1·0 at 25° to 1·12 after hydrolysis for 45min. T_m was found to decrease from 55° at $t=0$ to 40° at $t=45$min. The $\Delta E_{280}/\Delta E_{260}$ ratio was increased by hydrolysis from 0·81 at $t=0$ to 0·96 at $t=45$min.

When 0·01M-phosphate buffer was the solvent the melting range was shifted to lower temperatures ($T_m=49°$; cf. 57° in 0·1M-phosphate buffer for undegraded RNA), and the progressive loss of double-helical secondary structure on hydrolysis was also apparent (see Fig. 3).

RNA from the larger sub-unit. The effect of hydrolysis on the denaturation spectrum was marked more by an increase in the $\Delta E_{280}/\Delta E_{260}$ ratio than by a decrease in hypochromism. When 0·1M-phosphate buffer was the solvent E_{260} increased from 1·0 at 25° to 1·23 at 95° before hydrolysis (Fig. 4*a*); after hydrolysis for 45min. E_{260} increased from 1·0 at 25° to 1·18 at 95° (Fig. 4*e*). E_{280} increased after hydrolysis for 5, 15 and 30min. (Figs. 4*b*, 4*c* and 4*d*), although T_m decreased from 67° before hydrolysis to 53° at $t=45$min. The $\Delta E_{280}/\Delta E_{260}$ ratio increased from 0·79 at $t=0$ to 1·0 at $t=45$min.

The ability of the base residues to 'stack', as revealed by the dependence of E_{260} and E_{280} on temperature observed after RNA had reacted with formaldehyde, was hardly affected by hydrolysis (Figs. 4*k*–4*o*).

The effect of hydrolysis on double-helical secondary structure was profound (Figs. 4*f*–4*j*), since the structure giving rise to the difference spectrum found above 60° was characterized by an increase in E_{260} from 1·09 at 60° to 1·19 at 95° before hydrolysis but by an increase from 1·085 to 1·12 over the range 60–95° after hydrolysis for 45min. The $\Delta E_{280}/\Delta E_{260}$ ratio increased from 1·08 at $t=0$ to 1·2 at $t=45$. The denaturation spectrum observed after 45min. hydrolysis was found to have a substantial contribution from fragments forming novel hairpin loops, since E_{260} increased from 1·0 at 25° to 1·05 at 50° before hydrolysis and from 1·0 at 25°

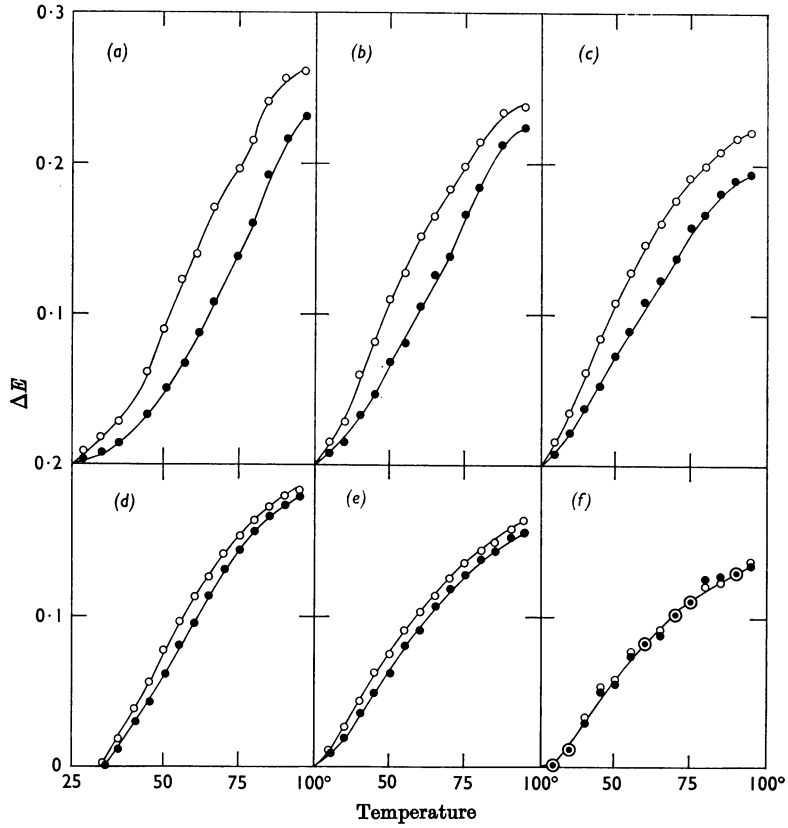

Fig. 5. Effect of hydrolysis on the denaturation spectrum of RNA from the larger sub-unit in 0·01 M-phosphate buffer, pH7·2. (*a*)–(*f*) were observed respectively after hydrolysis for 0, 1, 5, 15, 30 and 45 min. ○, ΔE_{260}; ●, ΔE_{280} ($E_{260}=1{\cdot}0$ at 25° in each case).

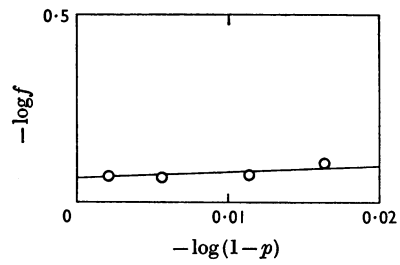

Fig. 6. Estimation of the length of the nucleotide sequence required to give rise to 'stacking'. The ordinate log$f$, i.e. $\log[(E_{(95)}-E_{(25)})_t/(E^0_{(95)}-E^0_{(25)})]$, measures $\Delta f_s/\Delta f^0_s$ where f_s is the fraction of residues in a stacked conformation (cf. eqn. 11), Δf_s refers to the change in f on increasing the temperature from 25° to 95° and Δf^0_s refers to the intact molecule. The observed values of E are given in Figs. 4(*k*)–4(*o*) for RNA that had reacted with formaldehyde.

to 1·065 at 50° after 45 min. hydrolysis; the comparable increments in E_{280} were 0·04 and 0·075 respectively.

The progressive change in the denaturation spectrum on hydrolysis is also shown in Fig. 5, when the solvent was 0·01 M-phosphate buffer.

DISCUSSION

Effect of chain length on the hypochromism of RNA treated with formaldehyde. The tendency of base residues to stack along single strands of a polynucleotide depends on the interaction between adjacent residues (Brahms, Michelson & Van Holde, 1966; Leng & Felsenfeld, 1966), and is not a co-operative phenomenon. The phenomenon is observed with dinucleotides and is insensitive to chain length. The results show that the hypochromism found for RNA treated with formaldehyde scarcely depended on the chain length of the fragments (Figs. 4*k*–4*o*). The slope of the plot of $\log[(E_{(95)}-E_{(25)})_t/E^0_{(95)}-E^0_{(25)})]$ against $\log(1-p)$ was found to lie within the range 2–5 residues (Fig. 6). This result confirms that the formation of base pairs is prevented by the reaction of the base residues

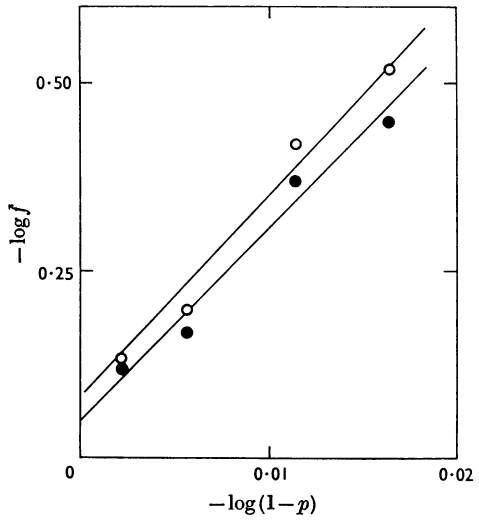

Fig. 7. Plot of $\log f$ against $\log(1-p)$ for RNA from the smaller sub-unit. The solvent was 4 M-guanidinium chloride in 0·01 M-phosphate buffer, pH 7·2. f was calculated by means of eqn. (11) for the range 60–95°. ○, f calculated from E_{260}; ●, f calculated from E_{280}.

with formaldehyde. The increment in extinction observed between 25° and 95° is attributable to base residues 'stacking' along a single strand.

Degradation of RNA from the smaller ribosomal sub-unit. The hypochromism of RNA from the smaller sub-unit is markedly dependent on the size of the fragments. When 4 M-guanidinium chloride was the solvent the hypochromism of the fragments was greater than that of the intact molecule over the range 25–50° due to 'frayed' and fortuitously formed loops but was less above 50°. Values of $\bar{f}$ were calculated by means of eqn. (10) from the extinction over the range 50–95°. The plot of $\log\bar{f}$ against $\log(1-p)$ is given in Fig. 7 and the slope was found to be 25 residues. The average value of p was taken from Fig. 1. An error of $\pm 10\%$ in $S_{25,w}$ would lead to an error of $\pm 20\%$ in $\log(1-p)$ and to an error of ± 5 residues; and an error of $\pm 10\%$ in $\log\bar{f}$ would lead to an error of ± 2 residues for a calculated value of $\bar{\imath}=25$. A value of $\bar{\imath}=25$ is therefore inferred.

Values of $\bar{\imath}$ were also calculated from the denaturation data obtained when phosphate buffer was the solvent. Values of $\bar{f}$ were calculated by means of eqn. (15) from the extinction at 45°, 50° and 60° (0·01 M-sodium phosphate buffer) and at 50°, 60° and 75° (0·1 M-sodium phosphate buffer). The plot of $\log\bar{f}$ against $\log(1-p)$ was linear and had about the same slope as Fig. 7.

As a check the difference:

$$\Delta E_A = (E_{(95)}-E_{(25)})-\bar{f}(E^0_{(95)}-E^0_{(25)}) \qquad (16)$$

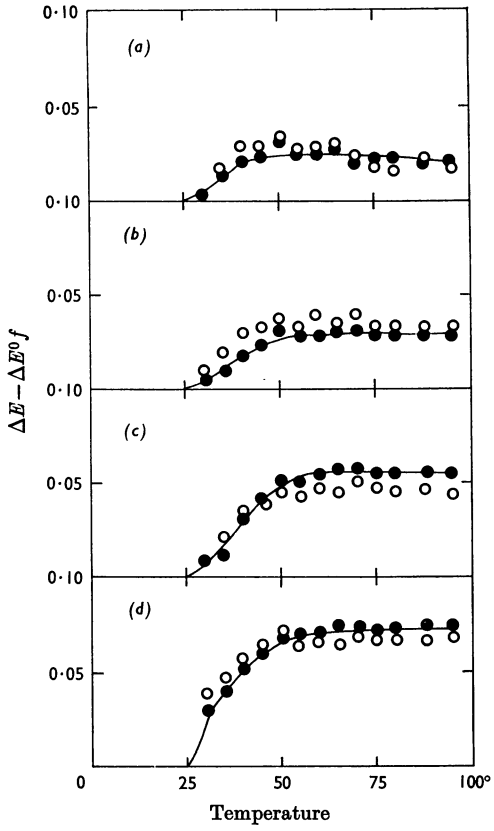

Fig. 8. Check of the value $2N+b=25$ derived for RNA from the smaller sub-unit. $\Delta E-\Delta E^0 f_{2N+b=25}$ measures the difference between observed and calculated values of E (solvent 4 M-guanidinium chloride in 0·01 M-phosphate buffer, pH 7·2) for values of f equal to 0·82, 0·66, 0·44 and 0·30 in (*a*), (*b*), (*c*) and (*d*) respectively. Measurements were made at 5 min. (*a*), 15 min. (*b*), 30 min. (*c*) and 45 min. (*d*). ○, $\Delta E_{260}-f\Delta E^0_{260}$; ●, $\Delta E_{280}-f\Delta E^0_{280}$.

where $\bar{f}$ is the calculated value for $i=25$, was plotted against temperature for each of the hydrolysates (Fig. 8). ΔE_A increased between 25° and 50° but was unchanged between 50° and 95°. This confirms that the hypochromism between 50° and 95° may be accounted for by hairpin loops of $\bar{\imath}=25\pm 5$ residues. The values of ΔE_A found below 50° are accounted for by the novel species of 'frayed' and fortuitously formed loops as given by eqns. (11) and (12). The contribution $\Delta E'_A=0{\cdot}11\Delta E^0$ of 'frayed' loops to ΔE_A was calculated for values of $N=8$, $n^0=4$, $n=6$ and $p=0{\cdot}037$. The contribution $\Delta E''_A=0{\cdot}12$ of loops which formed fortuitously was calculated using the values $A=1$, $b''+n''=15$ and $\bar{n}_f/N=0{\cdot}6$. Hence the calculated value of $\Delta E_A=0{\cdot}23\Delta E^0$ is in good agreement with the value $0{\cdot}2\ \Delta E^0$ observed.

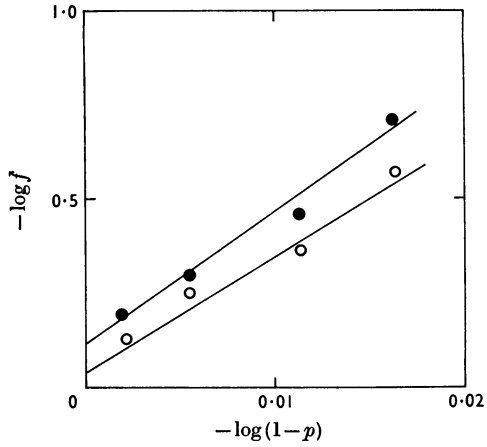

Fig. 9. Calculation of $2N+b$ for RNA from the larger ribosomal sub-unit from values of $\bar{f}$ obtained from the denaturation spectrum of RNA in 4M-guanidinium chloride in 0·01M-phosphate buffer, pH 7·2, by means of eqn. (10) for the temperature range 70–95°. ○, Values derived from E_{260}; ●, values derived from E_{280}.

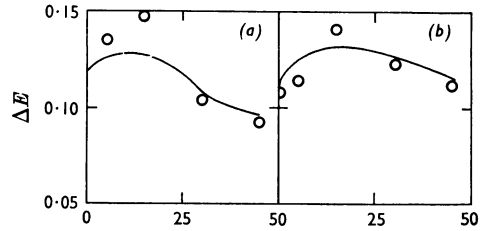

Fig. 10. Comparison of the observed and calculated values of $E_{(70)}-E_{(25)}$ of the hydrolysed fragments: $E_{(70)}-E_{(25)}=f(E^0_{(70)}-E^0_{(25)})+\Delta E'_A+\Delta E''_A$, where $f=30\pm5$; $\Delta E'_A$ was obtained by means of eqn. (11) by using the values of f'_A for a sequence of 25 nucleotides, $\bar{n}/N=0{\cdot}66$, $N=12$, $n^0=4$ and $b=11$ (these values are not critical); $\Delta E''_A$ was calculated by means of eqn. (12) where f''_A is the value of f for a sequence of 25 nucleotides, $A=1$ and $\bar{n}_l/N=0{\cdot}6$. (*a*) ΔE_{260}; (*b*) ΔE_{280} calculated as for (*a*) except that $A=12$.

RNA from the larger ribosomal sub-unit. The melting range of the secondary structure of RNA from the larger sub-unit is broader and the variation in the $\Delta E_{280}/\Delta E_{260}$ ratio is greater than that of RNA from the smaller sub-unit. The $\Delta E_{280}/\Delta E_{260}$ ratio is greatest for secondary structure melting above 70° (solvent 4M-guanidinium chloride), and the length of the nucleotide sequence that must be preserved in order to retain this hypochromism can be estimated unambiguously. Any novel structure that the fragments may form appears to 'melt' below 70°. Values of $\bar{f}$ were calculated from both E_{260} and E_{280} by means of eqn. (10). The value $\bar{\imath}=35\pm7$ residues was obtained from the slope of the plot of $\log\bar{f}$ against $\log(1-p)$ (Fig. 9). The range ±7 residues was calculated for a variation of $\pm20\%$ in $\log(1-p)$. $\bar{f}$ was calculated from the extinction at 60° and 70° (solvent 0·01M-phosphate buffer, pH 7) and 70° and 80° (solvent 0·1M-phosphate buffer, pH 7) and yielded values of $\bar{\imath}$ within the range 32–45 residues.

The estimation of the length of the nucleotide sequence giving rise to the denaturation spectrum where $\Delta E_{280}/\Delta E_{260}<1{\cdot}0$ is complicated by the contribution ΔE_A of 'frayed' and fortuitously formed loops. It is clear that ΔE_A becomes increasingly important as the fragments become smaller because the form of the denaturation spectrum changes drastically. Thus for the intact molecule $\Delta E_{280}/\Delta E_{260}=0{\cdot}88$ over the range 25–70° compared with 1·26 noted for fragments obtained after hydrolysis for 45min., a trend that is also apparent in 0·01M- and 0·1M-phosphate buffer solutions. The observed values of $E_{(70)}-E_{(25)}$ can be accounted for (Fig. 10) by values of ΔE_A calculated by means of eqns. (11) and (12) and a value of $\bar{\imath}=30\pm5$ residues. It is very unlikely that $\bar{\imath}$ is less 20 or more than 35 residues.

General features of secondary structure. The values of $\bar{\imath}$ obtained above taken together with earlier data suggest that the hairpin loop is the main feature of ribosomal RNA. It was noted that when RNA was digested with pancreatic ribonuclease about four out of five scissions were 'hidden' because the fragments were still united by hydrogen bonds (Cox & Kanagalingam, 1967*a*). It was inferred that b is much greater than c. It appears likely that N is smaller than 17 base pairs (Cox, 1966*a*,*b*). Moreover, the fraction h^0 of residues that form base pairs is:

$$h^0=\frac{\sum 2N}{\sum(2N+b+c)} \qquad (17)$$

The basis for measuring h^0 from the denaturation spectrum was discussed in the preceding paper (Cox, 1968). Values of E^0 of 7600 and 7800 respectively were obtained for RNA from the smaller and larger ribosomal sub-units at 25° compared with 9600 and 9800 at 95° (R. A. Cox & H. Gould, unpublished work). The precise value E_{dh} for RNA in the double-helical form is not known but it is likely to be within the range 6000–7000, i.e. within 10% of the value 6600 noted for DNA of widely different base compositions (Chargaff, 1955). For both species of RNA h^0 calculated by eqn. (17) is probably within the range 0·5–0·7. For RNA from the smaller sub-unit

a value of N of 6–8 base pairs, $b \simeq 10$ and $c \simeq 5$ residues would be consistent with the data. The comparable values for RNA from the larger subunit are $N \simeq 8$–16 base pairs, $b \simeq 10$–15 residues and c about 5 residues.

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