Conformation of Nucleic Acids and the Analysis of the Hypochromic Effect

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(Received 10 June 1970)

The spectra of two double-helical RNA species isolated from virus-like particles found in the mycelium of *Penicillium chrysogenum* were measured at about 25°C, and at 95°C after denaturation to a single-stranded form, and compared with the spectrum of the equivalent mixture of nucleotides. For both species the difference spectrum ($\epsilon_{95^\circ\text{C}} - \epsilon_{25^\circ\text{C}}$) when increased by 33% was very similar to the difference spectrum ($\epsilon_{\text{nucleotides}} - \epsilon_{25^\circ\text{C}}$). In contrast it was found for rat liver DNA that there was no simple relation between ($\epsilon_{95^\circ\text{C}} - \epsilon_{25^\circ\text{C}}$) and ($\epsilon_{\text{nucleotides}} - \epsilon_{25^\circ\text{C}}$). These observations were accounted for on the basis of the difference spectra for rA·rU, rG·rC, dA·dT and dG·dC base-pairs derived from model polyribonucleotides and polydeoxyribonucleotides. The difference spectra ($\epsilon_{95^\circ\text{C}} - \epsilon_{25^\circ\text{C}}$) found for rRNA and those calculated from a combination of the difference spectra for rA·rU and rG·rC base-pairs were in good agreement.

The u.v.-absorption spectrum of a nucleic acid or polynucleotide depends on conformation owing to the interaction of base-residues with their neighbours. When a conformational change is well defined, for example when native DNA in neutral solutions is denatured by heating, analysis of the spectral change yields the identity of the base-residues taking part in the transition. Empirical studies support the view that the denaturation spectrum (the difference in the spectrum found on denaturation) is a linear combination of two spectra, namely one for the denaturation of dG·dC base-pairs and the other for the denaturation of dA·dT base-pairs (Felsenfeld & Hirschman, 1965). These reference spectra were calculated by analysing the denaturation spectrum of several species of DNA of different nucleotide composition.

The same principle of the linear combination of two reference spectra, for the denaturation of rA·rU and rG·rC base-pairs, was applied to RNA. Because of the dearth of suitable species of double-helical RNA the reference spectra were obtained with model polynucleotides (e.g. Fresco, Klotz & Richards, 1963).

For neither RNA nor DNA can the reference spectra be considered as established. Different authors report widely different denaturation spectra for the same base-pair [for DNA compare Felsenfeld & Hirschman (1965), Mahler, Kline & Mehrotra (1964), Fresco *et al.* (1963); for RNA compare Fresco *et al.* (1963), Guschlbauer (1966), Cox (1966a,b)].

Potentially the analysis of the denaturation spectrum of a polynucleotide may lead to a knowledge of nucleotide composition and length of double-helical regions and to an estimate of 'helical content', i.e. the fraction of residues located in double-helical regions (Cox, 1966a,b, 1970). Although the method is convenient because it uses small amounts of material and because suitable spectrophotometers are generally available and easy to operate it is limited by the uncertainty in our knowledge of the reference spectra.

The aim of this work is to describe changes in the u.v. spectrum found on denaturing double-helical RNA that suggest a hypothesis for obtaining the reference spectra for both RNA and DNA.

THEORETICAL

Definitions. ϵ_λ is the extinction/g-atom of P of a nucleic acid at wavelength λ . Subscripts, e.g. $\epsilon_{\text{dh}\lambda}$, $\epsilon_{\text{am}\lambda}$ and $\epsilon_{\text{H}\lambda}$, indicate the conformational state of the polynucleotide. The subscripts dh and am refer respectively to the double-helical and amorphous conformations and H denotes the hydrolysis products (i.e. a mixture of nucleotides equivalent in chemical composition with the parent nucleic acid). In practice the amorphous state is considered to be attained when increasing the temperature beyond the range where multihelical conformations are stable leads to a change in ϵ of less than $71\text{ mol}^{-1}\text{cm}^{-1}$.

°C (i.e. ϵ_{260} increases by 1% or less of the value at about 25°C on raising the temperature by 10°C). $\Delta\epsilon_{(rA \cdot rU)\lambda}$ etc. is the difference in ϵ_λ found when a rA·rU base-pair etc. 'melts' to an amorphous state. Hence $\Delta\epsilon_{(rA \cdot rU)} = \epsilon_{am(A \cdot U)\lambda} - \epsilon_{dh(A \cdot U)\lambda}$. This definition applies strictly to a polynucleotide in which all the relative orientations of A·U base-pairs (etc.) occur. For example, in the complex poly rA·poly rU formed between poly rA and poly rU each rA·rU base-pair is so orientated that adenine always has another adenine residue as a neighbour whereas in the alternating co-polymer poly (rA·rU) an adenine residue is flanked by uracil residues. It is assumed that each conformation makes a fixed fractional contribution to the constant $\Delta\epsilon_{rA \cdot rU}$ etc. (for discussion see Felsenfeld & Hirschman, 1965).

$f_{dA \cdot dT}$, $f_{dG \cdot dC}$, $f_{rA \cdot rU}$ and $f_{rG \cdot rC}$ are respectively the mole fractions of dA·dT, dG·dC, rA·rU and rG·rC base-pairs.

For a species of RNA in the form of a perfect double-helix the increase $\Delta\epsilon_\lambda$ found on 'melting' a double-helical segment (concentration a g-atom of P/l) to an amorphous form is given by eqn. (1):

$$(E_{am} - E_{dh})/a = \Delta E_\lambda/a = f_{rA \cdot rU} \cdot \Delta\epsilon_{(rA \cdot rU)\lambda} + f_{rG \cdot rC} \cdot \Delta\epsilon_{(rG \cdot rC)\lambda} \quad (1)$$

This is the linear approximation of Felsenfeld & Sandeen (1962). In a later empirical treatment of the denaturation of DNA Felsenfeld & Hirschman (1965) assumed that the frequency of nearest neighbours was random and so could be calculated from the overall nucleotide composition. According to their analysis a third parameter, $\Delta\epsilon_{\left| \begin{smallmatrix} rA \cdot rU \\ rG \cdot rC \end{smallmatrix} \right|}$ for

RNA, is required in addition to $\Delta\epsilon_{(rA \cdot rU)\lambda}$ and $\Delta\epsilon_{(rG \cdot rC)\lambda}$ to take into account the interactions of neighbouring rA·rU and rG·rC base-pairs. They showed that, provided that the frequency of nearest neighbours is given by the overall nucleotide composition, then:

$$\Delta E_\lambda/a = f_{rA \cdot rU} \cdot \Delta\epsilon_{(rA \cdot rU)\lambda} + f_{rG \cdot rC} \cdot \Delta\epsilon_{(rG \cdot rC)\lambda} + (2K - 1) f_{rA \cdot rU} f_{rG \cdot rC} [\Delta\epsilon_{(rA \cdot rU)\lambda} + \Delta\epsilon_{(rG \cdot rC)\lambda}] \quad (2)$$

where

$$K = \Delta\epsilon_{\left| \begin{smallmatrix} rA \cdot rU \\ rG \cdot rC \end{smallmatrix} \right|} / [\Delta\epsilon_{(rA \cdot rU)\lambda} + \Delta\epsilon_{(rG \cdot rC)\lambda}] \quad (3)$$

Eqn. (2) reduces to eqn. (1) when $K = 0.5$, i.e. when the average difference $\Delta\epsilon_{\left| \begin{smallmatrix} rA \cdot rU \\ rG \cdot rC \end{smallmatrix} \right|}$ found on denatura-

tion of conformations where rA·rU and rG·rC base-pairs are neighbours is the mean of $\Delta\epsilon_{(rA \cdot rU)\lambda}$ (the average for adjacent rA·rU base-pairs) and $\Delta\epsilon_{(rG \cdot rC)\lambda}$ (the average for adjacent rG·rC base-pairs). Experiments show that for DNA (and hence it is likely for RNA) $K \approx 0.5$ and that eqn. (1) satisfactorily accounts for the denaturation spectrum. It is implied that $\Delta\epsilon_{(rA \cdot rU)\lambda}$ etc. may be obtained from model compounds.

On the basis of eqn. (1) the increments in E at 260 nm and 280 nm owing to denaturation are given by eqns. (4) and (5):

$$\Delta E_{260}/a = f_{rA \cdot rU} \cdot \Delta\epsilon_{(rA \cdot rU)260} + f_{rG \cdot rC} \cdot \Delta\epsilon_{(rG \cdot rC)260} \quad (4)$$

$$\Delta E_{280}/a = f_{rA \cdot rU} \cdot \Delta\epsilon_{(rA \cdot rU)280} + f_{rG \cdot rC} \cdot \Delta\epsilon_{(rG \cdot rC)280} \quad (5)$$

However, $\Delta\epsilon_{(rA \cdot rU)280} \approx 0$ (cf. Richards & Simpkins, 1968). Dividing eqn. (4) by eqn. (5) leads to:

$$\Delta E_{260}/\Delta E_{280} = \frac{(f_{rA \cdot rU}/f_{rG \cdot rC})[\Delta\epsilon_{(rA \cdot rU)260}/\Delta\epsilon_{(rG \cdot rC)280}]}{[\Delta\epsilon_{(rG \cdot rC)260}/\Delta\epsilon_{(rG \cdot rC)280}]} \quad (6)$$

For authentic double-helical species (cf. native DNA):

$$f_{rA \cdot rU}/f_{rG \cdot rC} = (A + U)/(G + C) \quad (7)$$

where A, C, G and U are the mole fractions of AMP etc.

Hence a plot of $\Delta E_{260}/\Delta E_{280}$ against $f_{rA \cdot rU}/f_{rG \cdot rC}$ or against $(A + U)/(G + C)$ should be linear, the slope being equal to $\Delta\epsilon_{(rA \cdot rU)260}/\Delta\epsilon_{(rG \cdot rC)280}$ and the intercept yielding $\Delta\epsilon_{(rG \cdot rC)260}/\Delta\epsilon_{(rG \cdot rC)280}$.

The hydrolysis difference spectrum (i.e. the difference in the spectrum of double-helical form and the equivalent mixture of nucleotides) may also be analysed by means of the same principles used to analyse the thermal-denaturation spectrum. Thus eqn. (8) is analogous to eqn. (1):

$$(E_{H\lambda} - E_{dh\lambda})/a = \Delta E_{H\lambda}/a = f_{rA \cdot rU} \cdot \Delta\epsilon_{H(rA \cdot rU)\lambda} + f_{rG \cdot rC} \cdot \Delta\epsilon_{H(rG \cdot rC)\lambda} \quad (8)$$

where

$$\Delta\epsilon_{H(rA \cdot rU)\lambda} = [0.5\epsilon_{AMP(\lambda)} + 0.5\epsilon_{CMP(\lambda)}] - \epsilon_{dh(rA \cdot rU)\lambda}$$

and

$$\Delta\epsilon_{H(rG \cdot rC)\lambda} = [0.5\epsilon_{GMP(\lambda)} + 0.5\epsilon_{CMP(\lambda)}] - \epsilon_{dh(rG \cdot rC)\lambda}$$

Fig. 1. Hydrolysis difference spectra ($\epsilon_H - \epsilon_{dh}$) of model polyribonucleotides. Data of Richards & Simpkins (1968) for poly (rA·rU) (·····), poly A·poly U (----) and mean of ····· and ---- (—, curve 1). Curve (2) was derived from data of Haselkorn & Fox (1965) by subtracting from the spectrum for (0.5mol of CMP+0.5mol of GMP) the spectrum found for poly rG·poly rC (CMP·GMP = 0.98) in 0.1M-phosphate buffer, pH6.8. The complex was synthesized by using an RNA-dependent RNA polymerase (isolated from *Micrococcus lysodeikticus*) with poly rC as template.

Fig. 2. Spectrum of double-helical RNA (38% G+C) isolated from virus-like particles from the mycelium of *P. chrysogenum*. (1) The calculated spectrum of the equivalent mixture of nucleotides (data taken from Pabst circular OR-10); (2) spectrum of double-helical form at 25°C (solvent 1mM- or 0.1M-sodium phosphate buffer, pH7.2); (3) spectrum of single-stranded (probably amorphous) form at 95°C (solvent 1mM- or 10mM-sodium phosphate buffer, pH7.2).

The data for model polyribonucleotides given in Fig. 1 show that at 280nm $\Delta\epsilon_{H(rA·rU)} \ll \Delta\epsilon_{H(rG·rC)}$. Hence eqn. (9), analogous with eqn. (6), may be derived:

$$\frac{\Delta E_{H260}/\Delta E_{H280}}{(f_{rA·rU}/f_{rG·rC})[\Delta\epsilon_{H(rA·rU)260}/\Delta\epsilon_{H(rG·rC)280}] + [\Delta\epsilon_{H(rG·rC)260}/\Delta\epsilon_{H(rG·rC)280}]} \quad (9)$$

Although the eqns. (1)–(9) were derived explicitly for RNA the analogous equations may be written for other polynucleotides that form perfect double helices such as native DNA and RNA–DNA hybrids although the terms such as $\Delta\epsilon_{(rA·rU)\lambda}$, $\Delta\epsilon_{(dA·dU)\lambda}$, $\Delta\epsilon_{(rA·dU)\lambda}$ etc. need not have identical numerical values.

RESULTS

Two species of RNA isolated from the mycelium of *Penicillium chrysogenum* were studied. One species contained 38% G+C and the other 51% G+C and their molecular weights (M_w) calculated from values of $s_{20,w}$ of 7.35 and 12.3S respectively were 0.14×10^6 daltons and 1.2×10^6 daltons (Cox, Kanagalingam & Sutherland, 1970). The spectrum

of each species was measured at both 25°C and 95°C in 1mM-, 10mM- and 0.1M-sodium phosphate buffer, pH7.2. The concentration of phosphate buffer had little effect on the spectrum at either temperature since for either species the variation ($\pm 1\%$) in ϵ was within experimental error. For comparison the spectra calculated for the equivalent mixtures of nucleotides as well as the observed spectra (solvent mM-sodium phosphate buffer, pH7.2) are given in Figs. 2 and 3. The hydrolysis difference spectrum [$(\epsilon_H - \epsilon_{dh})$ against λ] is given in Fig. 4 for each of the RNA species. Excellent agreement was found between the observed values and those calculated by means of eqn. (8) by using the reference spectra given in

Fig. 3. Spectrum of double-helical RNA (51% G+C) isolated from virus-like particles from the mycelium of *P. chrysogenum*. (1) Calculated spectrum of the equivalent mixture of nucleotides (spectrum of nucleotides taken from Pabst Circular OR-10); (2) spectrum of double-helical form at 25°C (solvent 1 mM- or 0.1 M-sodium phosphate buffer, pH 7.2); (3) spectrum of single-stranded (probably amorphous) form at 95°C (solvent 1 mM- or 10 mM-sodium phosphate buffer, pH 7.2).

Fig. 1 and the values of $f_{rA \cdot rU}$ and $f_{rG \cdot rC}$ obtained by chemical analysis. For both RNA species the denaturation spectrum (Fig. 5) had the same form as the hydrolysis difference spectrum (Fig. 4) but differed in magnitude since it was noted that over the range 230–300 nm $1.33(\epsilon_{am\lambda} - \epsilon_{dh\lambda}) = \epsilon_{H\lambda} - \epsilon_{dh\lambda}$.

The plot of $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ against $(A+U)/(G+C)$ is given in Fig. 6. On the basis of the assumption that the nucleotide composition of double-helical regions reflects the overall nucleotide composition data for a number of single-stranded RNA species as well as for double-helical RNA were included in Fig. 6, and all the points fell on a straight line of slope 1.38 and

Fig. 4. Comparison of the observed and calculated hydrolysis difference spectra ($\epsilon_H - \epsilon_{dh}$) of double-helical RNA. (a) Larger species of RNA (51% G+C); (b) smaller species of RNA (38% G+C); —, values calculated from the difference spectra given in Fig. 1 by using the values of $f_{rA \cdot rU}$ and $f_{rG \cdot rC}$ obtained by chemical analysis means of eqn. (8). Δ , Values for $1.33(\epsilon_{am} - \epsilon_{dh})$; $\circ$, observed values (curve 1—curve 2 in Figs. 2 and 3).

which intercepted the ordinate at 0.45 (cf. Cox, 1967). Thus the empirical equation is:

$$\Delta\epsilon_{260}/\Delta\epsilon_{280} = 1.38(A+U)/(G+C) + 0.45 \quad (10)$$

The substitution of the values for $\Delta\epsilon_{(rA \cdot rU)260}$, $\Delta\epsilon_{(rG \cdot rC)260}$ and $\Delta\epsilon_{(rG \cdot rC)280}$ given in Fig. 1 into eqn. (9) yields:

$$\Delta\epsilon_{H260}/\Delta\epsilon_{H280} = 1.38(A+U)/(G+C) + 0.43 \quad (11)$$

The slopes of eqns. (10) and (11) are identical and the intercepts are almost the same, in accord with the observation that the denaturation spectrum and the hydrolysis difference spectrum have the same shape.

DISCUSSION

The hydrolysis difference spectrum observed for the RNA species isolated from virus-like particles are accounted for by the data of Fig. 1. The hydrolysis difference spectrum for A·U base-pairs is reliable since $\epsilon_H - \epsilon_{dh}$ for poly A·poly U and for

Fig. 5. Comparison of the observed and calculated denaturation difference spectra ($\epsilon_{am} - \epsilon_{dh}$) of double-helical RNA. (a) Larger RNA species (51% G+C); (b) smaller RNA species (38% G+C). $\circ$, Observed values (curve 3—curve 2 in Figs. 2 and 3). —, Values calculated by means of eqn. (14) ($m = n = 0.75$) by using the data of Fig. 1: i.e., for (a) $\Delta\epsilon_{\lambda} = 0.49[0.75\Delta\epsilon_{H(rA \cdot rU)\lambda}] + 0.51[0.75\Delta\epsilon_{H(rG \cdot rC)\lambda}]$; for (b) $\Delta\epsilon_{\lambda} = 0.62[0.75\Delta\epsilon_{H(rA \cdot rU)\lambda}] + 0.38[0.75\Delta\epsilon_{H(rG \cdot rC)\lambda}]$.

poly (rA·rU) are known and have the same general features. The corresponding data for rG·rC base-pairs is less certain. Hence the agreement between the observed and calculated curves (Fig. 4) shows that the hydrolysis difference spectrum for G·C base-pairs given in Fig. 1 is substantially correct.

The similarity in the shape of the denaturation difference spectrum and the hydrolysis difference spectrum implies that for both rA·rU and rG·rC base-pairs the denaturation spectrum and hydrolysis spectrum have approximately the same shape, i.e.:

$$\Delta\epsilon_{(rA \cdot rU)\lambda} = m \cdot \Delta\epsilon_{H(rA \cdot rU)\lambda} \quad (12)$$

and

$$\Delta\epsilon_{(rG \cdot rC)\lambda} = n \cdot \Delta\epsilon_{H(rG \cdot rC)\lambda} \quad (13)$$

Hence:

$$\epsilon_{am\lambda} - \epsilon_{dh\lambda} = f_{rA \cdot rU} \cdot m \cdot \Delta\epsilon_{H(rA \cdot rU)\lambda} + f_{rG \cdot rC} \cdot n \cdot \Delta\epsilon_{H(rG \cdot rC)\lambda} \quad (14)$$

Fig. 6. Dependence of $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ on nucleotide composition $(A+U)/(G+C)$. $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ was calculated from the denaturation spectrum (cf. Fig. 5) obtained by heating polyribonucleotides to about 95°C. 1, Poly (G,C) (Fresco *et al.* 1963; Cox, 1966b). 2, rRNA isolated from the larger subparticle of reticulocyte ribosomes (Cox, 1970). 3, 5S rRNA isolated from *E. coli* (Boedtke & Kelling, 1967). 4, rRNA isolated from the smaller subparticle of reticulocyte ribosomes. 5, *E. coli* rRNA (unfractionated) (see Cox, 1966a). 6, Double-helical RNA (51% G+C); data of Fig. 5(a). 7, RNA from tobacco mosaic virus (Fresco *et al.* 1963). 8, Double-helical RNA (38% G+C); data of Fig. 5(b).

when $n = m$:

$$(\epsilon_{am\lambda} - \epsilon_{dh\lambda})/(\epsilon_{H\lambda} - \epsilon_{dh\lambda}) = m \quad (15)$$

The available data (Richards & Simpkins, 1968) show that eqn. (12) is valid and that $m = 0.75$. To fit the observed denaturation spectrum it is necessary to assume that eqn. (13) also applies and that $n = m = 0.75$. The denaturation spectra calculated by this procedure agree with those observed. The calculated reference denaturation spectra are given in Fig. 7.

Application to DNA. In principle the same

hypothesis (cf. eqn. 14) should apply to polydeoxy-ribonucleotides, i.e.:

$$\epsilon_{am} - \epsilon_{dh\lambda} = f_{dA \cdot dT} \cdot m \cdot \Delta\epsilon_{H(dA \cdot dT)\lambda} + f_{dG \cdot dC} \cdot n \cdot \Delta\epsilon_{H(dG \cdot dC)\lambda} \quad (16)$$

In solution the base-pairs of double-helical RNA are tilted to about the same extent as base-pairs of DNA in the A form (Fuller, Wilkins, Wilson & Hamilton, 1965). However, native DNA in solution is in the B form. Thus in solution the relative

Hirschman (1965). Comparable data for RNA are given in Fig. 9(b). It was deduced from Fig. 9(a) that

$$\epsilon_{am} - \epsilon_{dh\lambda} = f_{dA \cdot dT} \cdot 0.55 \Delta\epsilon_{H(dA \cdot dT)\lambda} + f_{dG \cdot dC} \cdot 0.75 \Delta\epsilon_{H(dG \cdot dC)\lambda} \quad (17)$$

positions of the base-pairs of double-helical RNA is not the same as in native DNA. Hence it cannot be assumed that the denaturation spectrum of dG·dC is the same as that of rG·rC even though, for the mononucleotides, the u.v. spectrum is scarcely altered by replacing ribose with deoxyribose. Moreover in the DNA series thymine replaces uracil. The spectrum of thymine is not identical with that of uracil; thymine residues also tend to 'stack' more strongly. Thus it is not surprising that the spectrum of the double-helical complex poly dA·poly dU and of poly rA·poly rU are different (Zmudka, Bollum & Shugar, 1969). The respective denaturation spectra also differ, particularly in the region of 280 nm. The dependence of ϵ_{280} upon % G·C base-pairs for the ribo- and deoxyribo- series are compared in Fig. 8.

Estimates of m and n (eqn. 16) were obtained by plotting values of ϵ_{dh260} , ϵ_{am260} and ϵ_{H260} for DNA as a function of % G+C. The data of Fig. 9a were taken from Mahler *et al.* (1964) and Felsenfeld &

Satisfactory agreement between calculated and observed values was obtained (see Fig. 10).

The denaturation spectrum of dA·dT base-pairs

Fig. 7. Reference denaturation spectra for (1) rA·rU and (2) rG·rC base-pairs. For rA·rU base-pairs $\Delta\epsilon_{\lambda} = 0.75 \Delta\epsilon_{H(rA \cdot rU)\lambda}$; for rG·rC base-pairs $\Delta\epsilon_{\lambda} = 0.75 \Delta\epsilon_{H(rG \cdot rC)\lambda}$. Values of $\Delta\epsilon_{H(rA \cdot rU)\lambda}$ were equated with curve (1), Fig. 1, and values of $\Delta\epsilon_{H(rG \cdot rC)\lambda}$ with curve (2), Fig. 1.

Fig. 8. Effect of denaturation of RNA and DNA on ϵ_{280} . Data for DNA were taken from Felsenfeld & Hirschman (1965). O, Values for RNA (data of Figs. 2 and 3), rA·rU base-pairs (data of Fig. 7) and rG·rC base-pairs (data of Fig. 7).

Fig. 9. Dependence of ϵ_{260} on % G+C. (a) Double-helical DNA. Δ , Data of Mahler *et al.* (1964); the solvent was 0.15M-NaCl-0.015M-sodium citrate, pH 7.0 (values of T_m are quite high in this solvent so that ϵ_{am} is approached on denaturation). $\blacktriangle$, Solvent: 51% methanol [0.15M-NaCl-0.015M-sodium citrate, pH 7.0 (3ml), water (46.0ml), made up to 100ml with methanol]. $\circ$, Data of Felsenfeld & Hirschman (1965); the solvent was 0.01M-NaCl-0.01M-phosphate buffer, pH 7. Solutions were heated until there was no further change in the spectrum with temperature. The value for DNA from *Clostridium welchii* 454 given as $\epsilon_{dh} = 7367$ was omitted as unreliable. $\square$, Data of Inman & Baldwin (1964) for poly dG·poly dC (54% G, 46% C) in 0.028M- Na^+ , pH 7.5; ϵ_{dh} was measured at 82.8°C and ϵ for the denatured form at 93°C. (b) $\bullet$, Double-helical RNA (see Figs. 2 and 3). $\square$, poly A·poly U. $\circ$, Poly (rA·rU) (data of Richards & Simpkins, 1968). Δ , Calculated for rG·rC base-pairs by using the data of Fig. 7 and the value for ϵ_{dh} reported by Haselkorn & Fox (1965). In both (a) and (b) curve (1) refers to values of ϵ_{Hh} , curve (2) to ϵ_{am} (or at 95°C) and curve (3) refers to the double-helical form.

Fig. 10. Comparison of the observed and calculated hydrolysis difference spectrum and denaturation difference spectrum of rat liver DNA. (1) Hydrolysis difference spectrum: —, observed values; Δ , calculated from data of Inman & Baldwin (1964) or of Mahler *et al.* (1964) for poly dG·poly dC, and data of Mahler *et al.* (1964) for poly dA·poly dT. (2) Denaturation spectrum: —, observed values; $\circ$, values calculated on the basis of the equation $\Delta\epsilon = f_{dA \cdot dT} \cdot 0.55\Delta\epsilon_{H(dA \cdot dT)} + f_{dG \cdot dC} \cdot 0.75\Delta\epsilon_{H(dG \cdot dC)}$. In both (1) and (2) the size of the symbols allows for experimental error.

and of dG·dC base-pairs calculated by Felsenfeld & Hirschman (1965) are compared in Figs. 11(a) and 11(b) with the denaturation spectra of model compounds and with the denaturation difference spectra calculated from the hydrolysis difference spectra. The agreement observed provides further evidence for the analysis of Felsenfeld & Hirschman (1965) and also for the use of eqn. (17).

Application to rRNA. In the Theoretical section evidence was presented in favour of the view that the denaturation spectrum of entirely double-helical RNA is governed mainly by the fraction of rA·rU base-pairs and rG·rC base-pairs that 'melt' on heating and is largely independent of nucleotide sequence provided that the frequency with which nearest neighbours occur is close to random. The analysis should also be applicable to partly double-helical RNA species such as rRNA provided that 'stacking' in single-stranded forms makes a minor contribution to hypochromism compared with the contribution of double-helical regions. Although base-pairs located at the ends of short double-helical segments might make a smaller than average contribution to hypochromism 'end effects' will not affect estimates of the nucleotide composition of double-helical secondary structure

Fig. 11. Comparison of the denaturation spectrum for dA·dT and dG·dC base-pairs calculated from the hydrolysis difference spectrum of reference polydeoxyribonucleotides with the reference denaturation spectra obtained by other methods. (a) Data for dA·dT base-pairs etc.: —, $0.55\Delta\epsilon_{H(dA \cdot dT)}$; Δ , values observed on denaturation of poly (dA·dT), data of Mahler *et al.* (1964); ----, denaturation spectrum calculated by Felsenfeld & Hirschman (1965) from the denaturation spectra of a range of DNA species. ∇ , Values calculated by means of a linear approximation (cf. eqn. 1); $\square$, values calculated by using a quadratic approximation (cf. eqn. 2). (b) data for dG·dC base-pairs: —, values calculated from the data of Inman & Baldwin (1964) for poly dG·poly dC by using the equation:

$$\Delta\epsilon_{(dG \cdot dC)\lambda} = 0.75\Delta\epsilon_{H(dG \cdot dC)\lambda} = (0.5\epsilon_{dGMP} + 0.5\epsilon_{dCMP} - \epsilon_{dh})$$

$\circ$, values calculated by Felsenfeld & Hirschman (1965) by using the linear approximation; Δ , calculated values obtained by using the quadratic approximation (Felsenfeld & Hirschman, 1965).

provided that the proportion of rA·rU to rG·rC base-pairs located in terminal positions is roughly the same as in the rest of the molecule. It was suggested earlier (Cox, 1967) for partly double-helical molecules that the nucleotide composition of double-helical regions might reflect the overall

nucleotide composition [i.e. $(A+U)/(G+C) \simeq f_{rA \cdot rU}/f_{rG \cdot rC}$]. In this case the plot of $\Delta\epsilon_{260}/\Delta\epsilon_{280}$ against $(A+U)/(G+C)$ should be consistent with data found for double-helical RNA and the slope and intercepts should yield respectively $\Delta\epsilon_{(rA \cdot rU)260}/\Delta\epsilon_{(rG \cdot rC)280}$ and $\Delta\epsilon_{(rG \cdot rC)260}/\Delta\epsilon_{(rG \cdot rC)280}$ (cf. eqn. 6). It would appear from Fig. 6 that for rRNA the approximation is justified. Furthermore the denaturation spectrum was calculated for the double-helical analogues of rRNA from both the smaller and the larger subparticles of reticulocyte ribosomes and for rRNA from *E. coli* ribosomes. The ratio $(A+U)/(G+C)$ is the same for each of the component strands as for the parent double-helical RNA. In each case it was found that the thermal denaturation spectrum over the range 230–300nm had the same form as that calculated for the double-helical analogue. The difference spectrum observed for rRNA from the subparticle of rabbit reticulocyte ribosomes (57% G+C) was scarcely distinguishable from that of rRNA from *E. coli* ribosomes (55% G+C) and both difference spectra agreed with the difference spectrum calculated by means of eqn. (8) and the reference spectra of Fig. 1 when $f_{rG \cdot rC} = 0.56 \pm 0.2$, but decreased to 72% of $(\epsilon_{am} - \epsilon_{dh})$, implying that no more than 72% of base-residues form base-pairs at 25°C. For *E. coli* rRNA i.r. measurements (Cotter & Gratzer, 1969) also indicate 56% rG·rC base-pairs. The difference spectrum observed for rRNA from the larger subparticle of rabbit reticulocyte ribosomes (69% G+C) agreed with $0.85(\epsilon_{am} - \epsilon_{dh})$ calculated for a double-helical analogue of 69% G+C. For these rRNA species it appears that nucleotide composition of double-

helical segments is a reflection of the overall nucleotide composition.

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