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A Study of the Effects of Reaction with Formaldehyde on some Optical and Physical Properties of Reticulocyte Ribosomes

By R. A. COX
National Institute for Medical Research, Mill Hill, London N.W.7

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1. The optical rotatory dispersion and ultraviolet-absorption spectrum of ribosomal RNA *in situ* appear to be unchanged when the ribosome is dissociated into its RNA and protein moieties. 2. Reaction with 0·05% formaldehyde at 20° for 2hr. 'fixes' ribosomes so that they remain intact in 1% sodium dodecyl sulphate. 3. The RNA moiety of the ribosome undergoes a conformational change when ribosomes in 8% formaldehyde are heated at 70° for 10min. and cooled to 20°. After this treatment no double-helical character can be detected, but neither the sedimentation coefficient nor the morphology of the ribosome determined by electron microscopy is altered. 4. It is concluded that the RNA moiety of reticulocyte ribosomes is freely accessible to formaldehyde.

Formaldehyde has been used in studies of the secondary structure of nucleic acids. Reaction of formaldehyde with the base residues is readily detected spectrophotometrically and prevents the formation of complementary base pairs (for review see Grossman, 1968). Fixation with formaldehyde or glutaraldehyde is a procedure used to preserve the structure of proteins (Quiocho & Richards, 1964). Ribosomes may also be preserved by formaldehyde treatment (Huxley & Zubay, 1960) even in conditions that would otherwise lead to dissociation into subparticles (Bayley, 1964; Spirin, Belitsina & Lerman, 1965).

The aim of the present work is to study in more detail the reaction of formaldehyde with ribosomes. The principal conclusion is that, once the ribosomes are 'fixed' in formaldehyde solutions and the solution is heated to complete the reaction with the base residues, the double-helical structure of the RNA moiety is diminished without alteration of either the sedimentation coefficient or the appearance of the ribosome in the electron microscope. The results also provide further evidence that the spectrum of the RNA moiety does not alter significantly on dissociation from protein. A brief account has been given previously (Cox, 1968).

EXPERIMENTAL

Chemicals. Guanidinium chloride (about 6M) was prepared by neutralizing A.R. guanidinium carbonate with conc. HCl as described by Cox (1966). For a 4M solution $E_{260} < 0{\cdot}05$. A.R. formaldehyde was used without further purification. All other reagents were of A.R. quality. Sodium dodecyl sulphate was recrystallized from aqueous ethanol.

Ribosomal preparations. Reticulocytes were obtained from rabbits that had been given daily intraperitoneal injections of neutralized 2·5% (w/v) phenylhydrazine hydrochloride (0·3ml./kg. body wt.) for 6 or 7 days; the cells were lysed in 5mM-$MgCl_2$ (Schweet, Lamfrom & Allen, 1958). The ribosomes and polyribosome fractions were isolated by differential centrifuging (Arnstein, Cox, Gould & Potter, 1965).

Isolation of RNA. Ribosomal RNA was isolated by precipitation as the guanidinium salt (Cox, 1966).

Determination of protein. The protein content of ribosomes was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Spectrophotometry. The u.v.-absorption spectra and extinctions were measured with a Unicam SP.700 spectrophotometer. The sample-cell holder was an electrically heated copper block that could be maintained at any temperature in the range 25–95° to better than 0·1°. The observed extinctions measured at temperatures other than 20° were corrected for changes in concentration due to the thermal expansion of water.

Ultracentrifuging. MSE low-speed (Major, Mistral and 13) centrifuges, MSE high-speed (40 and Super-Speed 50) ultracentrifuges and the Spinco model L ultracentrifuge were used for all preparative work. The high-speed ultracentrifuges were used for zone centrifuging.

A Spinco model E ultracentrifuge fitted with u.v.-absorption optics was used for analytical work. In the optical path the camera was replaced by a photoelectric cell that scanned the slit, and the movement of the sedimenting species was followed by means of an $X-Y$ recorder. Cells of 12mm. path length were used in all cases. All analytical studies were made at about 20° with ribosomes (50mg./l.) or RNA (25mg./l.).

Optical rotation. Optical rotatory dispersions over the range 220–500nm. were measured with a Bellingham and

Stanley Polarmatic recording spectropolarimeter. Cells of 1 cm. path length were used. The instrument was calibrated with standard sucrose solutions. The extinction at 260 nm. was never more than 1·7 and the measured rotations were directly proportional to concentration. Readings were independent of slit width (1 mm.).

RESULTS

Ultraviolet-absorption spectrum of ribosomes and their constituents

Over the range 230–330 nm. the spectrum of ribosomes appears to be the sum of the separate RNA and protein components. Within experimental error ($\pm 1\%$) the spectrum of ribosomes (see curve I in Fig. 1) was unchanged when the solution was made 1% with respect to sodium dodecyl sulphate to dissociate the RNA from protein (cf. Bonhoeffer & Schachman, 1960). It is inferred that the spectrum of the RNA moiety did not change significantly after the protein was dissociated. The contribution of the protein moiety to the ribosome spectrum is small, so that a small change in the protein spectrum on dissociation of the ribosome into its constituents would not be detected.

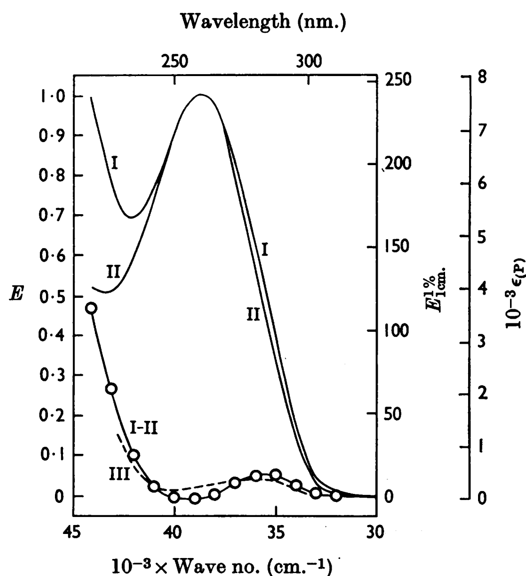


Fig. 1. Comparison of the spectra of reticulocyte ribosomes and their component RNA and protein. Curve I, spectrum of reticulocyte ribosomes in 0·25 M-sucrose–1 mM-$MgCl_2$–25 mM-KCl–50 mM-tris–HCl buffer, pH 7·6; curve II, spectrum of reticulocyte ribosomal RNA in the same buffer as for curve I; curve III, ribosomal protein in 4 M-guanidinium chloride, pH 4·5, diluted with 0·5 vol. of ethanol. The ribosome solution was made 4 M with respect to guanidinium chloride, pH 7, and RNA was precipitated after the addition of acetate buffer, pH 4·5, and 0·5 vol. of ethanol and then cooling to $-12°$. Curve I–II is the calculated curve for ribosomal protein.

The molar extinction of the RNA moiety was obtained by hydrolysing a sample in 0·3 M-potassium hydroxide at 36° for 16 hr. The solution was neutralized, and concentration of nucleotides was calculated from the extinction of the nucleotides (Beavan, Holiday & Johnson, 1955) and the nucleotide composition of RNA (expressed as moles/100 moles of nucleotide: AMP, 18; GMP, 36; UMP, 18; CMP, 28; Gould, Arnstein & Cox, 1965). The RNA/protein ratio was found to be 46:54, in agreement with earlier reports (Edelman, Ts'o & Vinograd, 1960; Blake & Peacocke, 1965). The results are summarized by the equation:

$$\epsilon^{1\%}_{1cm.}\,(\text{ribosomes}) + 0{\cdot}46\,\epsilon^{1\%}_{1cm.}\,(\text{RNA}) + 0{\cdot}54\,\epsilon^{1\%}_{1cm.}\,(\text{protein}) \quad (1)$$

At wavelengths longer than 250 nm. $\epsilon^{1\%}_{1cm.}$ (protein) is very small compared with $\epsilon^{1\%}_{1cm.}$ (RNA): for example at 260 nm. $\epsilon^{1\%}_{1cm.}$ (protein) is 6 and $\epsilon^{1\%}_{1cm.}$ (RNA) is 243. It was calculated that at 260 nm. $\epsilon^{1\%}_{1cm.}$ (ribosomes) is 112.

Optical rotatory dispersion

The polyribosome fraction of reticulocyte ribonucleoprotein was dissolved (80–200 μg./ml.) in 1 mM-magnesium chloride–10 mM-potassium phosphate buffer, pH 7·5. First the u.v.-absorption spectrum and then optical rotatory dispersion were measured (see Fig. 2*a*). The contribution of protein to the optical rotatory dispersion of ribosomes was calculated (Fig. 2*c*) on the basis of the equation:

$$[\alpha]_{\text{ribosomes}} = 0{\cdot}46[\alpha]_{\text{RNA}} + 0{\cdot}54[\alpha]_{\text{protein}} \quad (2)$$

The calculated curve was found to have two minima: one at about 280 nm. and another at about 233 nm., where $[\alpha]_{\text{min.}}$ is 3800°. The calculated curve may not be precise because it is the difference between two quite large numbers. The optical rotatory dispersion of isolated protein was not studied because it is denatured in 4 M-guanidinium chloride (cf. McPhie & Gratzer, 1966).

Reaction of RNA with formaldehyde

Ultraviolet-absorption studies. Formaldehyde (1%) reacts slowly with RNA in 0·1 M-phosphate buffer, pH 7·5, or in 1 mM-magnesium chloride–10 mM-potassium phosphate buffer, pH 7·5, at 25°. However, increasing the temperature leads to the disruption of double-helical secondary structure and to an increase in the rate with which formaldehyde reacts with the base residues. The original

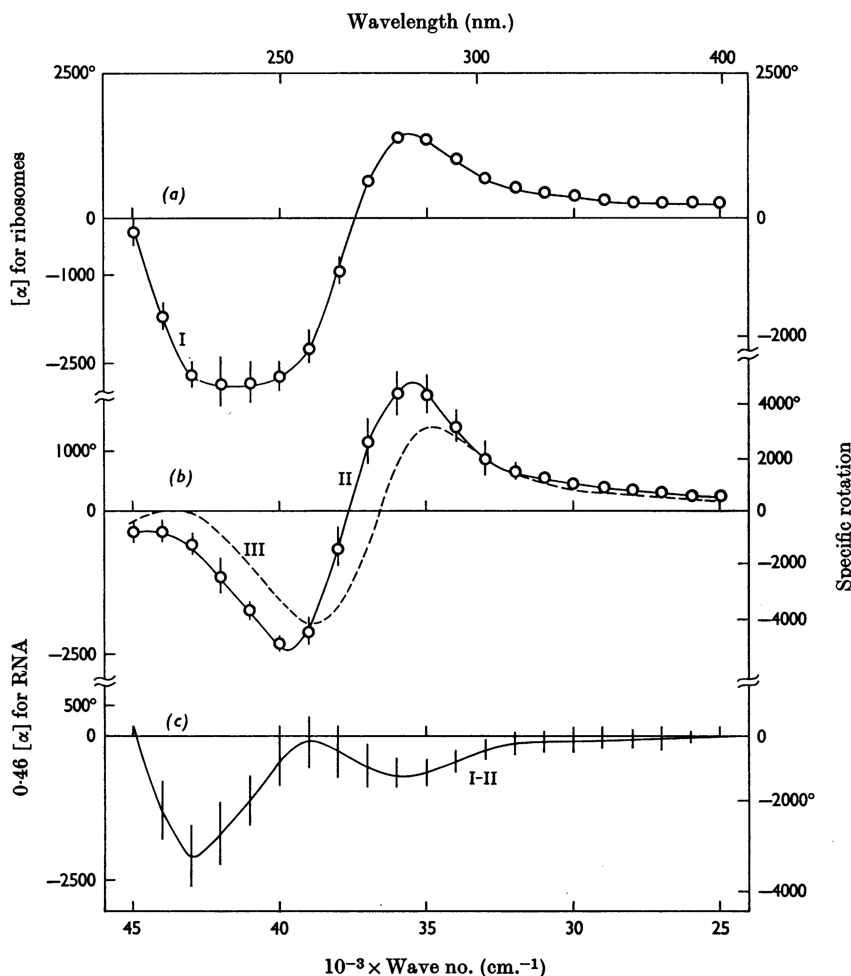


Fig. 2. Optical rotatory dispersion of rabbit reticulocyte ribosomes and their constituents (cf. McPhie & Gratzer, 1966). (*a*) Ribosomes in 1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5. (*b*) RNA in the same buffer as for (*a*); the right-hand ordinate gives the specific rotations and the left-hand ordinate gives values of [α] calculated for the RNA moiety of ribosomes by assuming that ribosomes contain 46% RNA and that [α] is unchanged when RNA combines with protein. Curve II, untreated RNA at 20°; curve III, RNA at 20° after reaction with formaldehyde at high temperatures [RNA (E_{260} 0·78) in 1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5, made 1% with respect to formaldehyde was heated at 90° for 10min. and cooled; the spectrum was measured before and after heating (see Fig. 4*a*)]. In both (*a*) and (*b*) the open circles denote the mean of four independent experiments and the vertical line joins the extreme values, unless the variation is less than the diameter of the circle. (*c*) Calculated values of [α] for protein obtained by subtracting the 0·46[α] for RNA from [α] for ribosomes. The extreme values were calculated and are indicated.

secondary structure is prevented from re-forming on cooling because of chemical modification of the groups involved in the formation of hydrogen bonds between base pairs (e.g. Stollar & Grossman, 1962). The transition from the original partly double-helical structure to a single-stranded form is apparent from measurements of hypochromism and optical rotatory dispersion. When ribosomal RNA was heated in 1% formaldehyde–0·1M-potassium phosphate buffer, pH 7·5, an increment of about 30% in E_{260} was noted on heating from 25° to 95° (curve I in Fig. 3*a*); the temperature, T_m, at which 50% of the increment was found was about 8° lower than when formaldehyde was absent. On cooling from 95° to 25° the extinction fell to about 18% above the original value (curve II in Fig. 3*a*) and increased again reversibly on subsequent heating cycles. Curve II in Fig. 3(*a*) is the ‘melting’

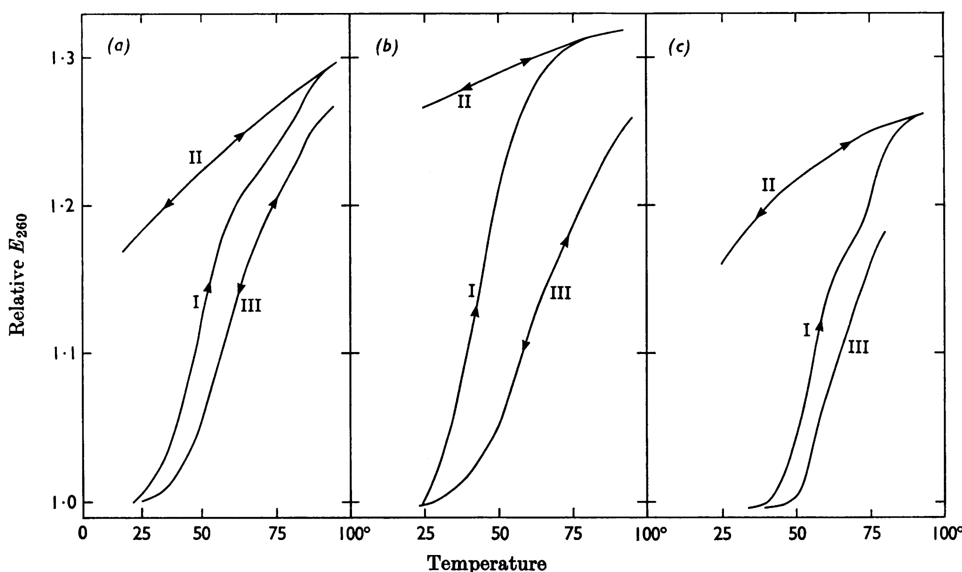


Fig. 3. 'Melting' profile of RNA in the presence and absence of formaldehyde. (*a*) Curve I, dependence of E_{260} of unfractionated reticulocyte ribosomal RNA in 1% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·5, on heating from 25° to 95° for the first time; curve II, profile observed on second and subsequent heating cycles; curve III, profile for RNA in 1 mM-$MgCl_2$–10 mM-potassium phosphate buffer, pH 7·5. (*b*) Curve I, profile of RNA in 10% formaldehyde–0·1 M-potassium phosphate buffer, pH 7·5, observed on heating from 25° to 95° for the first time; curve II, profile found on second and subsequent heating cycles; curve III, RNA in 0·1 M-potassium phosphate buffer, pH 7·5. The decrease in the hypochromism of RNA after reaction with formaldehyde in 10% formaldehyde (curve II in *b*) compared with the hypochromism in 1% formaldehyde (curve II in *a*) is attributed to the property of formaldehyde to behave as a denaturant, i.e. it is to be expected that since 10% formaldehyde decreases T_m of double-helical structures it will also decrease the tendency of base pairs to 'stack' along a single strand. (*c*) 'Melting' profile of reticulocyte ribosomes in the presence and absence of formaldehyde. Curve I, profile of reticulocyte ribosomes in 1% formaldehyde–1 mM-$MgCl_2$–10 mM-potassium phosphate buffer, pH 7·5, heated to 95° for the first time; curve II, profile found on subsequent heating cycles; curve III, profile of ribosomes in 1 mM-$MgCl_2$–10 mM-potassium phosphate buffer, pH 7·5.

profile of the entirely single-stranded polynucleotide.

Both the temperature range over which denaturation took place and the magnitude of the reversible hypochromism over the range 25–95° were affected when the concentration of formaldehyde was increased to 10% (Fig. 3*b*). Thus 50% of the initial increment in E_{260} was obtained at 45° in 10% formaldehyde compared with 52° in 1% formaldehyde, and the hypochromism of the formaldehyde derivative was decreased from 15% to 6% of the original value of E_{260}.

Optical-rotatory-dispersion measurements. When a solution of RNA in 1 mM-magnesium chloride–10 mM-potassium phosphate buffer, pH 7·5, was made 1% with respect to formaldehyde no effect on optical rotatory dispersion was noted. However, after the solution was heated at 95° for 5 min. and cooled the optical rotatory dispersion was appreciably altered (cf. Fig. 2*b*). Thus $[\alpha]_{max.}$ 3000° was found at 286 nm., the Cotton effect at 274 nm. and a minimum at 254·5 nm. The corresponding u.v.-absorption spectrum of the RNA solution before and after reaction with formaldehyde is given in Fig. 4(*a*).

Reaction of ribosomes with formaldehyde

'Fixation' of ribosomes. 'Fixation' occurs rapidly, for example after incubation with 1% formaldehyde for 30 min., since it was noted that after this treatment the sedimentation profile of reticulocyte ribosomes was preserved when the solution was made 1% with respect to sodium dodecyl sulphate whereas in the absence of formaldehyde dissociation into RNA and protein was observed. Thus in this study 'fixed' ribosomes are defined as those that remain as ribosomes in 1% sodium dodecyl sulphate.

(*a*) Mild conditions for the 'fixation' of ribosomes.

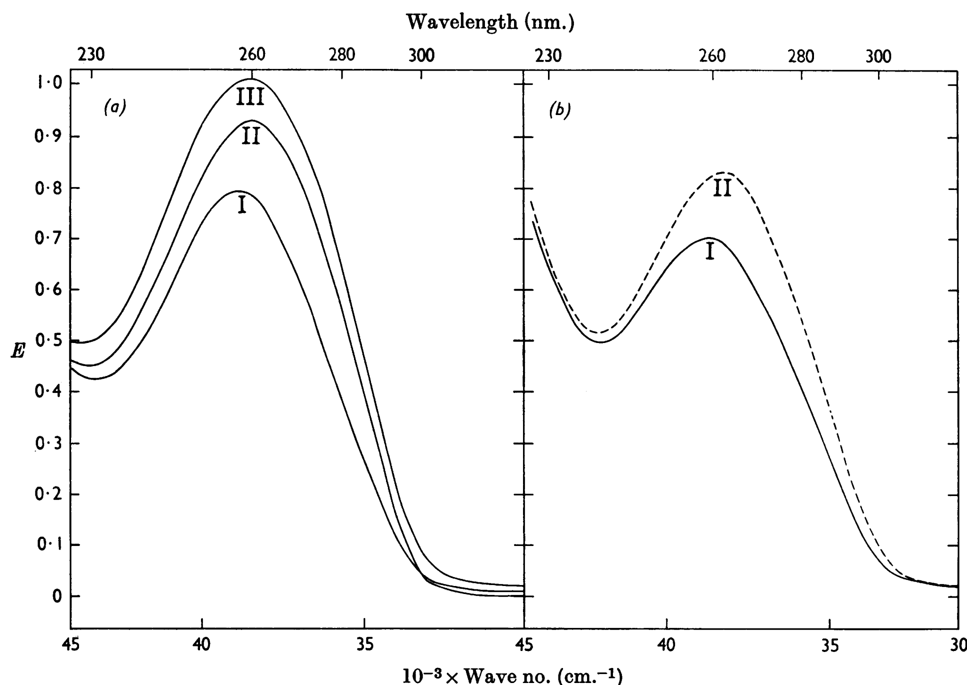


Fig. 4. Spectrum of ribosomal RNA and of ribosomes in the presence and absence of formaldehyde. (*a*) Ribosomal RNA: curve I, RNA in 1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5; curve II, RNA in 1% formaldehyde–1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5, at 25° after heating to 95°; curve III, as for curve II but at 95°. (*b*) Ribosomes in the absence and presence of formaldehyde: curve I, untreated ribosomes in 1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5; curve II, ribosomes (0·3mg./ml.) heated in 8% formaldehyde–1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5, at 70° for 10min., dialysed against buffer at 0° and then diluted fivefold; the composition of the solvent was finally 0·04% formaldehyde–1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5.

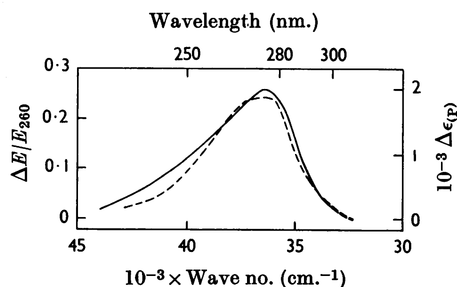


Fig. 5. Comparison of the effect of reaction with formaldehyde on the spectra of RNA and ribosomes. The difference in each spectrum at 20° before and after heating to 95° in formaldehyde was measured. Curve I, difference in the spectrum of RNA [(curve II in Fig. 4*a*–curve I in Fig. 4*a*)/E_{260} curve I in Fig. 4*a*]. Curve II, difference in the spectrum of ribosomes [(curve II in Fig. 4*b* – curve I in Fig. 4*b*)/E_{260} curve I in Fig. 4*b*].

A solution of ribosomes (80μg./ml.) in 1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5, was made 0·05% with respect to formaldehyde. Samples were removed after 1, 5, 20, 75 and 120min. at 20°, made 1% with respect to sodium dodecyl sulphate and cooled, the excess of sodium dodecyl sulphate was removed by centrifuging and the sedimentation pattern was obtained. Dissociation into RNA and protein was observed in those samples exposed to formaldehyde for 20min. or less, an intermediate pattern was obtained after 75min. treatment and the original ribosome pattern was found to be preserved after 120min. (Fig. 6). Fixation was achieved after 75–120min. at 20°. Samples kept at 0° were not 'fixed' after 120min.

(*b*) *Optical properties of 'fixed' ribosomes.* The u.v.-absorption spectrum of ribosomes (curve I in Fig. 1) was apparently not affected when ribosomes were 'fixed' after treatment with 0·05% formaldehyde for 2hr. Little effect was observed after ribosomes in 1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5, were exposed to 1% formaldehyde for 1hr. Treatment with 10% formaldehyde for 90min. led to an increase in E_{260} from 0·67 to 0·69 and in E_{280} from 0·38 to 0·42.

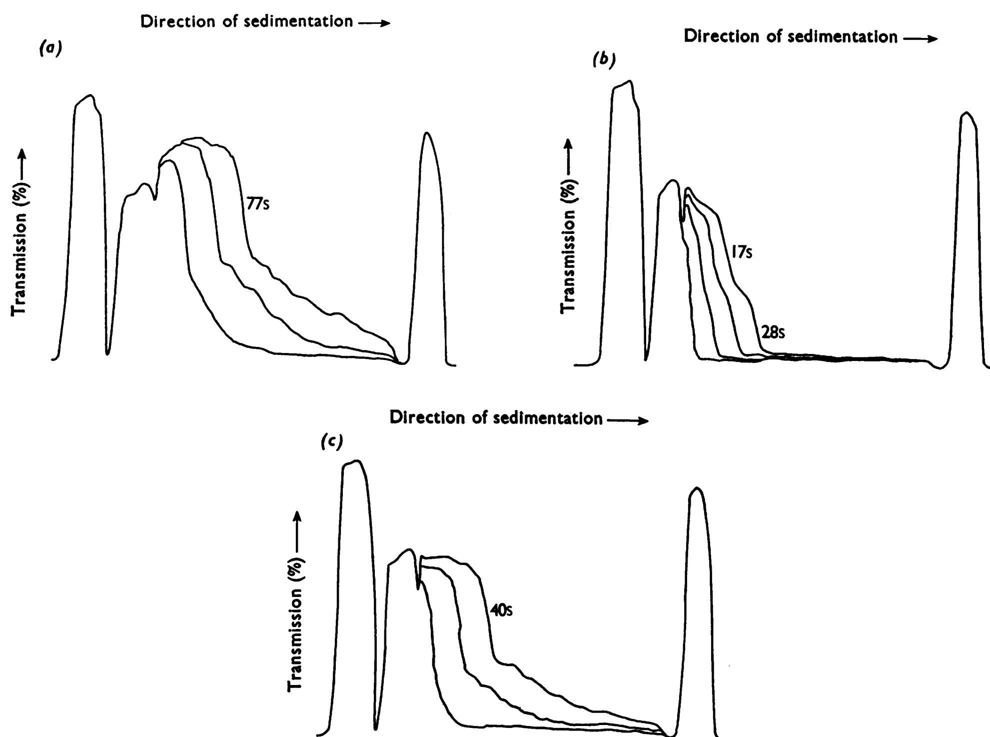


Fig. 6. Effect of formaldehyde fixation on the sedimentation profile of polyribosomes at about 20°. (*a*) Sedimentation profile of the stock polyribosome fraction (about 50 μg./ml.) in 1 mM-$MgCl_2$–10 mM-potassium phosphate buffer, pH 7·5. The percentage transmission of the sedimenting boundary was measured at 4 min. intervals after reaching 33450 rev./min. (Spinco model E ultracentrifuge, analytical D rotor). (*b*) Sedimentation profile of RNA obtained by the addition of 1% sodium dodecyl sulphate to another sample of the ribosome solution described in (*a*). The speed was 33450 rev./min. The interval between tracings was 8 min. Similar profiles were found for samples made 0·05% with respect to formaldehyde at 20° provided that sodium dodecyl sulphate was added after not more than 75 min. (*c*) A sample of the stock solution at 20° was made 0·05% with respect to formaldehyde. Then 2 hr. later the solution was made 1% with respect to sodium dodecyl sulphate. The sedimentation profile was measured at 8 min. intervals after reaching 33450 rev./min. The decreased sedimentation rate is attributed to the interaction of sodium dodecyl sulphate with the protein moiety. When the bulk of the detergent was precipitated as the potassium salt by cooling to 0° and separated by low-speed centrifuging before the analytical run the sedimentation rate was similar to that given in (*a*).

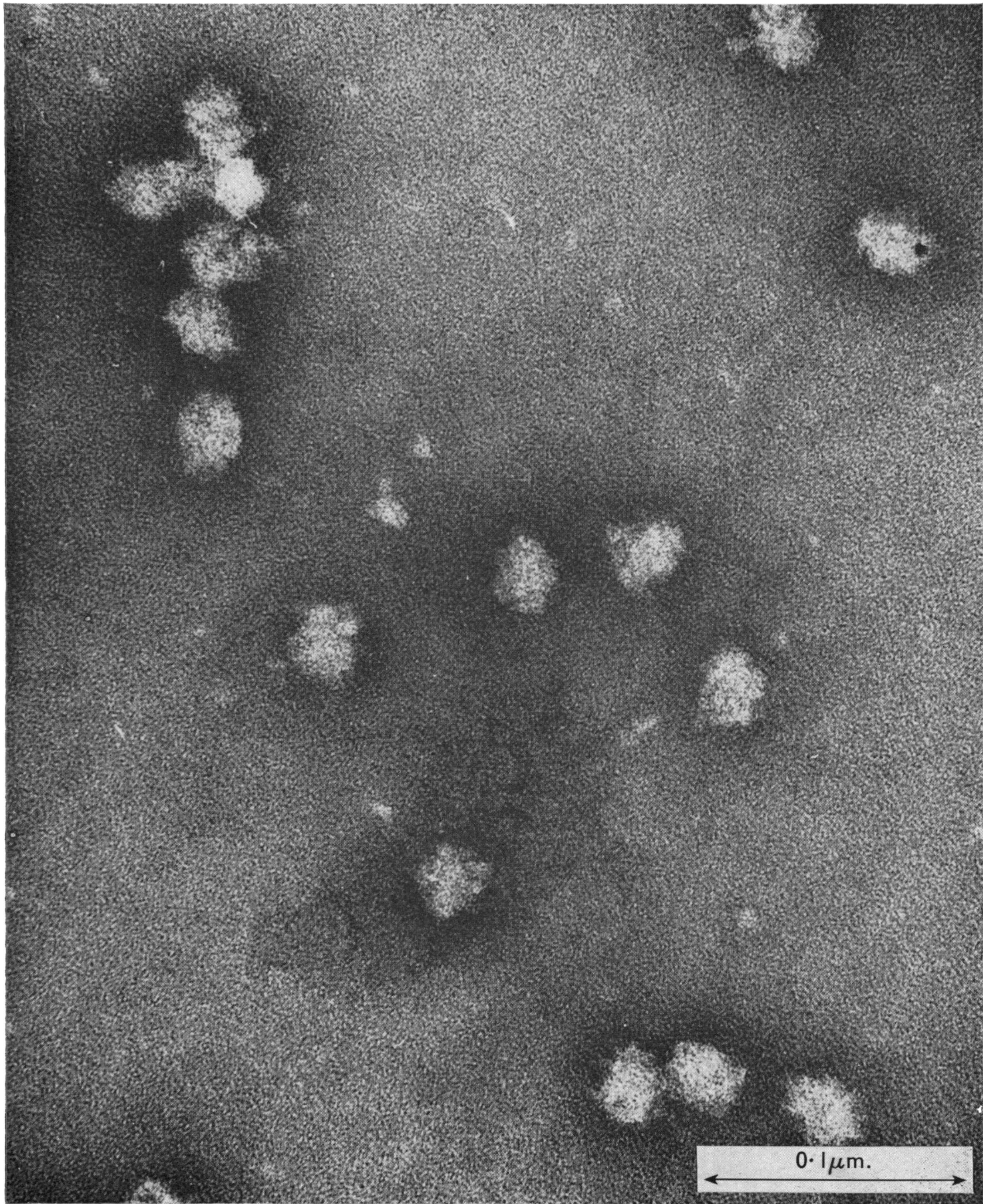
Within experimental error the optical rotatory dispersion was not affected, showing that the secondary structure of both RNA and protein moieties is preserved.

(*c*) Sedimentation properties of 'fixed' ribosomes. The nucleoprotein fraction was separated into a polyribosome and a ribosome fraction by differential centrifuging. The principal species of the ribosome fraction (40–70 μg./ml.) was found to sediment with $S_{20,w}$ 77 S in 1 mM-magnesium chloride–10 mM-potassium phosphate buffer, pH 7·5. After fixation with 0·05–10% formaldehyde the ribosome solution was adjusted to 0·05% formaldehyde–1 mM-magnesium chloride–10 mM-potassium phosphate buffer, pH 7·5. and $S_{20,w}$ was found to be the same as for the untreated sample.

(*d*) Electron microscopy of 'fixed' ribosomes. The polyribosome fraction (50 μg./ml.) was 'fixed' by treatment with 0·05–8% formaldehyde and dialysed where necessary to decrease the formaldehyde concentration to 0·04–1%. Alternatively the polyribosome fraction was fixed by treatment with 2% glutaraldehyde for 30 min. at 20°. The polyribosome fraction was stained with

EXPLANATION OF PLATES 1 AND 2

Electron micrographs of rabbit reticulocyte ribosomes and derivatives (by the late R. C. Valentine). Plate 1: untreated polyribosome fraction (cf. Matthias *et al.* 1964). Plate 2: polyribosome fraction after heating to 70° in 8% formaldehyde solution to diminish the double-helical character of the RNA moiety.



R. A. COX

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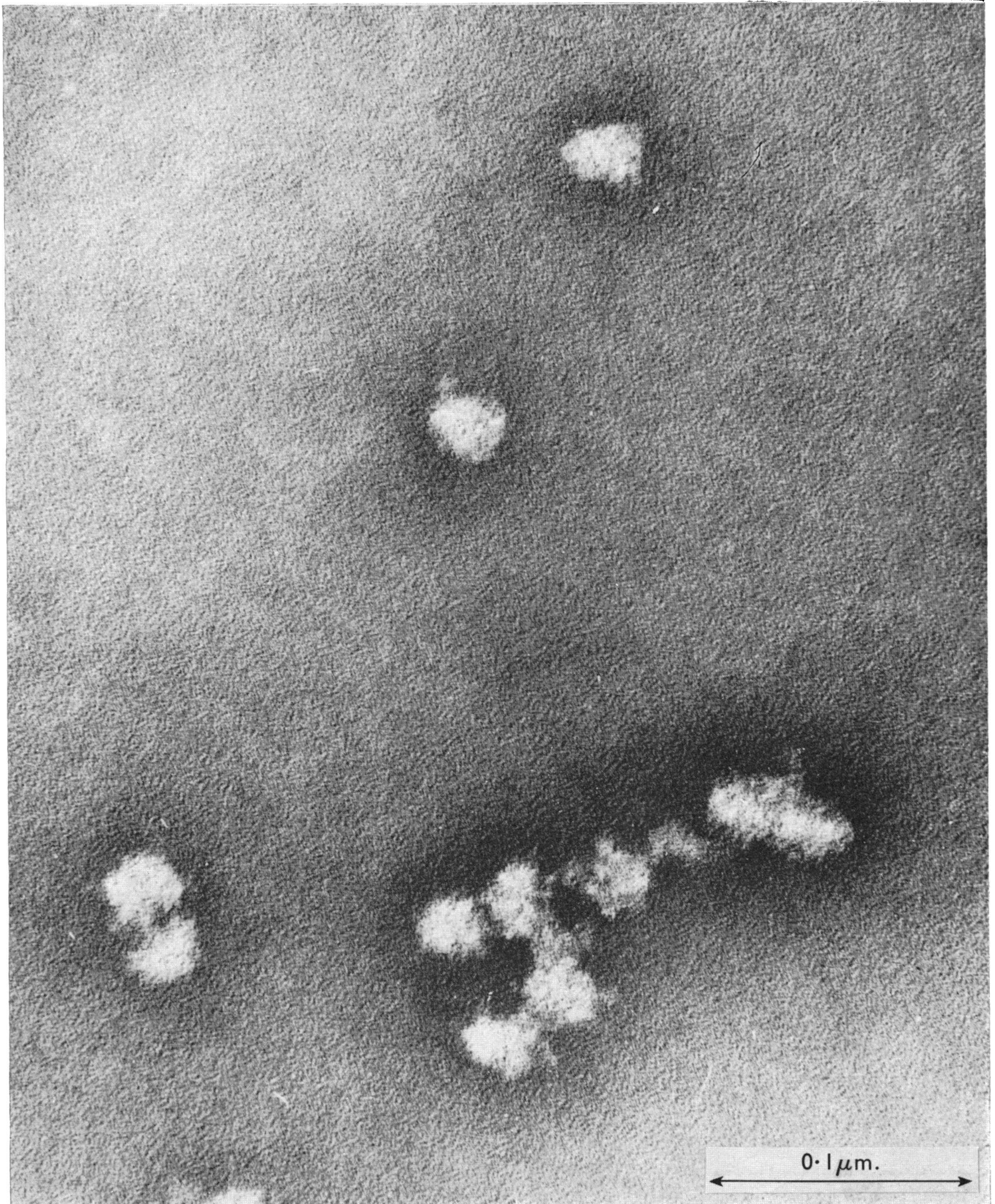

R. A. COX

2% sodium silicotungstate and examined in a Philips EM200 electron microscope (see Plate 1); cf. Matthias, Williamson, Huxley & Page, 1964). Ribosomes that were not 'fixed' were not visible in the electron microscope.

Production of ribonucleoprotein particles in which the RNA moiety is largely single-stranded. (*a*) Ultraviolet-absorption measurements. When ribosomal RNA was heated in 1% formaldehyde solutions an irreversible change in the u.v.-absorption spectrum was noted after heating at 95°. This was attributed to a change from a partly double-helical to a single-stranded conformation. The same transition was observed when ribosomes were heated in 1% formaldehyde–0·1M-potassium phosphate buffer, pH 7·5 (see Fig. 3*c*; cf. Fig. 3*a*). The difference in the spectrum brought about by reaction with formaldehyde was the same for ribosomes as for RNA. In a typical experiment ribosomes were dissolved (3·4mg./ml.) in 1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5. A 2ml. sample was diluted to 20ml. with formaldehyde (final concentration 8%) in the same buffer. Part of the sample was heated at 70° for 10min. and then dialysed overnight at 4° against sufficient buffer to decrease the formaldehyde concentration to 0·04%. The spectra of a control sample not exposed to formaldehyde and of the sample heated in 8% formaldehyde were compared (see Fig. 4*b*). The spectrum of the ribosome fraction was subtracted from that observed after reaction with formaldehyde and $\epsilon_{(P)}$ was calculated on the basis of $\epsilon_{(P)260}$ 7700 for ribosomes as well as for RNA. The difference curve (Fig. 5) was found to be very similar to that calculated from Fig. 4(*a*) for RNA.

The u.v.-absorption spectrum of a base residue is altered after reaction with formaldehyde. In addition the spectrum is altered as a result of the conformational change from a partly double-helical to a single-stranded 'stacked' conformation. The difference in the spectrum of RNA at 20° before and after reaction with formaldehyde is the sum of both these effects. The same difference was found for ribosomes before and after reaction with formaldehyde (see Fig. 5), showing that more than 80% of the base residues of the RNA moiety are as available to formaldehyde as are the base residues of free RNA.

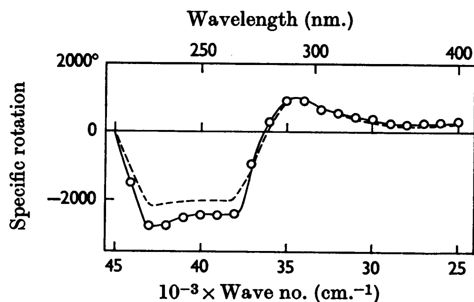

Fig. 7. Optical rotatory dispersion (calculated and observed values) of ribosomes previously heated to 70° in 8% formaldehyde. ○—○, Observed values in 0·04% formaldehyde–1mM-$MgCl_2$–10mM-potassium phosphate buffer, pH 7·5; ----, values calculated by summing 0·46 × curve III in Fig. 2(*b*) and curve I–II in Fig. 2(*c*).

(*b*) Optical rotatory dispersion. Exposure to formaldehyde alone is not sufficient to alter the optical rotatory dispersion of ribosomes. However, a shift in the Cotton effect to longer wavelengths was found after ribosomes were heated at 70° for 10min. in 8% formaldehyde solutions. The optical rotatory dispersion observed (Fig. 7) is close to the calculated curve for a particle of 46% RNA in the single-stranded form and 54% protein in its native conformation. The differences between observed and calculated values of [α] below 260nm. are not considered to be significant. This provides further confirmation that a major conformational change is brought about by heating ribosomes in formaldehyde. It was also shown by X-ray-diffraction studies (by M. Spencer) that after reaction with formaldehyde the double-helical character of the RNA moiety was considerably diminished.

(*c*) Electron microscopy. The polyribosome fraction (60μg./ml.) was dissolved in 8% formaldehyde–1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5. One sample was kept at 0° and another was heated at 70° for 10min. and cooled. An increase of 20% in E_{260} at 20° was observed, and the optical rotatory dispersion was found to be the same as in Fig. 7, confirming that double-helical secondary structure was considerably diminished. Both the heated (Plate 2) and the control unheated (Plate 1) samples were examined in the electron microscope and no major differences were observed.

(*d*) Sedimentation studies. The ribosome fraction (50μg.) was dissolved in 1% formaldehyde–1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5, heated at 95° for 10min. and cooled. The sedimentation pattern was found to be the same before and after heating at 95°, even though the optical properties were considerably changed, as described in Figs. 4(*b*) and 7. In another experiment the ribosome fraction in 8% formaldehyde was first heated at 70° for 10min. and cooled, and the solution then dialysed to decrease the concentration of formaldehyde to 0·04%. The optical properties were measured in 0·04% formaldehyde–1mM-magnesium chloride–10mM-potassium phosphate buffer, pH 7·5, and were found to be identical with those given in Figs. 4(*b*) and 7, showing that

double-helical secondary structure was considerably diminished. It was found both before and after the ribosomes had reacted with formaldehyde that they have $S_{20,w}$ 77s in this solvent. Thus changing the conformation of the RNA moiety does not affect appreciably the sedimentation coefficient of the ribosome. It is inferred that there is no major change in the hydrodynamic volume after the RNA moiety has reacted with formaldehyde.

No systematic study of the effect of formaldehyde on native subparticles was undertaken. However, it was noted that subparticles present in the ribosome fraction were still intact as judged by their sedimentation coefficients ($S_{20,w}$ 40s and 60s respectively) after solutions made 8% with respect to formaldehyde were heated at 70° for 10min. In this respect the behaviour of the subparticles is analogous to that of the intact ribosome.

DISCUSSION

Conformation of RNA within the ribosome. The available evidence suggests that the conformation of the RNA moiety of ribosomes is essentially retained after the removal of the protein. For reticulocyte ribosomes and its derivatives: (*a*) to within experimental error ($\pm 1\%$) the u.v.-absorption spectrum is the sum of the spectra of isolated RNA and protein (see Fig. 1) (cf. Bonhoeffer & Schachman, 1960); (*b*) the contribution of the RNA moiety to the optical rotatory dispersion of ribosomes appears to be the same as that of isolated RNA (see Figs. 2 and 7) (cf. Blake & Peacocke, 1965; McPhie & Gratzer, 1966; Sarkar, Yang & Doty, 1967; Bush & Scheraga, 1967); (*c*) the 'melting' profile of ribosomes has the same general features as that of isolated RNA (cf. curve III in Fig. 3*a* and curve III in Fig. 3*c*; however, the curves are not identical, particularly below 55°; Cox, 1965) as found for yeast RNA and yeast ribosomes (Cotter, McPhie & Gratzer, 1967); (*d*) the reaction of the RNA moiety of ribosomes with formaldehyde is very similar to the reaction of isolated RNA with formaldehyde as judged by changes in the u.v.-absorption spectrum (see Figs. 3 and 4) and in optical rotatory dispersion (see Figs. 2 and 7); (*e*) Furano, Bradley & Childers (1966) suggested, on the basis of dye-binding studies, that within the *Escherichia coli* ribosome the RNA moiety has a largely single-stranded rather than a partly double-helical conformation. Such a particle was obtained when reticulocyte ribosomes were first 'fixed' with and then heated in formaldehyde to complete the reaction because the formaldehyde derivative of RNA is unable to form Watson–Crick base pairs (see, e.g., Stollar & Grossman, 1962) but the ability of the base residues to stack one upon another along the single strand is not impaired (Cox & Kanagalingam, 1968). Thus the optical properties of ribosomes after reaction with formaldehyde at elevated temperatures (Figs. 3, 4 and 7) are those of a particle composed of single-stranded RNA and protein, in contrast with the optical properties of the intact ribosome.

Thus several independent approaches all provide evidence to support the view that the secondary structure of RNA within the ribosome is essentially the same after the removal of protein. Moreover the methods often used for the isolation of ribosomal RNA, e.g. dissociation of RNA and protein with sodium dodecyl sulphate followed by denaturation with phenol (Kurland, 1960), or dissociation of RNA and protein with 4M-guanidinium chloride followed by the precipitation of guanidinium ribonucleate (Cox, 1966), are procedures that do not disrupt double-helical secondary structure. Hence it seems probable that knowledge of the secondary structure of RNA in solution applies also to the ribosomal RNA moiety *in situ*.

Reaction of ribosomes with formaldehyde: 'fixation' of ribosomes. The dissociation of ribosomal RNA from ribosomal protein can be prevented by treatment with 0·05% formaldehyde at 20° for 2hr. (see Fig. 6). The base residues of the RNA moiety react very slowly with formaldehyde under these conditions and become cross-linked only when formaldehyde is present in a very large excess (e.g. Penniston & Doty, 1963*a*,*b*). Formaldehyde is a well-known fixative for proteins, and so it is probable that the fixation of ribosomes arises from the reaction of formaldehyde with ribosomal protein leading to the formation of cross-links between the protein subunits. This suggests that protein–protein interactions are important to the stability of ribosomes. Once ribosomes are 'fixed' they may be heated in formaldehyde to diminish double-helical secondary structure. This change in the conformation of the RNA moiety does not alter the gross morphology of the ribosome, since neither its appearance in the electron microscope nor its sedimentation coefficient are affected. The question arises: how is the ribosome able to accommodate a major change in the conformation of RNA without altering its own gross morphology? The estimate that at least 80% of the base residues of the RNA moiety are as available to formaldehyde as are the base residues of isolated RNA complements previous reports that the RNA chain is freely available to cationic dyes (Furano *et al.* 1966; Miall & Walker, 1967; Cotter *et al.* 1967) and to bivalent cations (Sheard, Miall, Peacocke, Walker & Richards, 1967).

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