4. Particle-size distribution data for *Hevea* latex suggest that *in vivo* the rate of initiation of new rubber particles in the aqueous or other non-rubber phase of latex may be very slow, compared with the rate of incorporation of isopentenyl pyrophosphate into rubber at the surface of existing particles. Experimentally, the conversion of isopentenyl pyrophosphate into rubber in the aqueous phase of latex *in vitro* is so small that it could be accounted for by incomplete removal of rubber during centrifugal separation of the aqueous phase.

5. *Hevea brasiliensis* latex contains a proportion of non-rubber particles bounded by an osmotically sensitive membrane (lutoid particles) which, on disruption, hydrolyse ATP and thereby retard the incorporation of mevalonate into rubber. Disrupted lutoids also hydrolyse isopentenyl pyrophosphate to isopentenol, but possess no detectable isopentenyl pyrophosphate-isomerase activity. Lutoid particles are not necessary for the incorporation of isopentenyl pyrophosphate into rubber *in vitro*.

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REFERENCES

- Agranoff, B. W., Eggerer, H., Henning, U. & Lynen, F. (1960). *J. biol. Chem.* **235**, 326.
 Archer, B. L., Ayrey, G., Cockbain, E. G. & McSweeney, G. P. (1961). *Nature, Lond.*, **189**, 663.
 Baer, E. (1944). *J. Amer. chem. Soc.* **66**, 303.
 Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
 Bloch, K., Chaykin, S., Phillips, A. H. & de Waard, A. (1959). *J. biol. Chem.* **234**, 2595.
 Burrows, S., Grylls, F. S. M. & Harrison, J. S. (1952). *Nature, Lond.*, **170**, 800.
 Chaykin, S., Law, J., Phillips, A. H., Tchen, T. T. & Bloch, K. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 998.
 Childs, C. R. & Bloch, K. (1962). *J. biol. Chem.* **237**, 61.
 Clark, W. M. & Lubs, H. A. (1916). *J. biol. Chem.* **25**, 479.
 Cramer, F. & Böhm, W. (1959). *Angew. Chem.* **71**, 775.
 Dauben, W. G., Reid, J. C. & Yankwich, P. E. (1947). *Analyt. Chem.* **19**, 828.
 de Waard, A., Phillips, A. H. & Bloch, K. (1959). *J. Amer. chem. Soc.* **81**, 2913.
 de Waard, A. & Popják, G. (1959). *Biochem. J.* **73**, 410.
 Eggerer, H. & Lynen, F. (1960). *Liebigs Ann.* **630**, 58.
 Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.
 Henning, U., Möslin, E. M. & Lynen, F. (1959). *Arch. Biochem. Biophys.* **83**, 259.
 Homans, L. N. S. & van Gils, G. E. (1948). *Proc. 2nd Rubb. Technol. Conf., London*, p. 292.
 Kekwick, R. G. O., Archer, B. L., Barnard, D., Higgins, G. M. C., McSweeney, G. P. & Moore, C. G. (1959). *Nature, Lond.*, **184**, 268.
 Lynen, F., Eggerer, H., Henning, U. & Kessel, I. (1958). *Angew. Chem.* **70**, 738.
 Lynen, F. & Henning, U. (1960). *Angew. Chem.* **72**, 82.
 Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
 Mertz, E. T. & Owen, C. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 204.
 Park, R. B. & Bonner, J. (1958). *J. biol. Chem.* **233**, 340.
 Ruinen, J. (1950). *Arch. Rubbercult. Ned.-Ind.* **27**, 255.
 Schoon, Th. G. F. & Phoa, K. L. (1956). *Arch. Rubbercult. Ned.-Ind.* **33**, 195.
 Tchen, T. T. (1957). *J. Amer. chem. Soc.* **79**, 6345.
 Tchen, T. T. (1958). *J. biol. Chem.* **233**, 1100.
 Tunncliffe, M. E. (1954). *I.R.I. Trans.* **30**, 97.
 van den Tempel, M. (1952). *I.R.I. Trans.* **28**, 303.
 Witting, L. A. & Porter, J. W. (1959). *J. biol. Chem.* **234**, 2841.
 Yuan, C. & Bloch, K. (1959). *J. biol. Chem.* **234**, 2605.

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The Isolation, Characterization and Acid-Base Properties of Ribonucleic Acid from Rabbit-Reticulocyte Ribosomes

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The importance of ribonucleic acids in protein biosynthesis is now well established (Chantrenne, 1961). The incorporation of amino acid residues into peptide linkage in specific sequence appears to be under the control of RNA, ranging from intermediate to high molecular weight (Otaka, Mitsui & Osawa, 1962; Ishihama, Mizuno, Takai, Otaka & Osawa, 1962; Monier, Naono, Hayes, Hayes &

Gros, 1962). The bulk of the ribosomal RNA differs from 'messenger' RNA in nucleotide composition (Volkin & Astrachan, 1956*a, b*; Volkin, Astrachan & Countryman, 1956), rate of metabolic turnover (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961) and metabolic activity in cell-free systems (Nirenberg & Matthaei, 1961; Wood & Berg, 1962). Further, DNA and 'messenger' RNA form hybrid

helical conformations (Hall & Spiegelman, 1961; Bautz & Hall, 1962), a phenomenon that is possible only with polynucleotides having complementary sequences (Marmur & Lane, 1960; Doty, Marmur, Eigner & Schildkraut, 1960; Schildkraut, Marmur & Doty, 1961; Marmur, Rownd & Schildkraut, 1963). On the other hand, ribosomal RNA from *Escherichia coli* hybridizes with only a very small fraction of DNA (Schildkraut, Marmur, Fresco & Doty, 1961; Yankofsky & Spiegelman, 1962). Pronounced differences in nucleotide sequence may also be deduced from comparative studies of the acid-base properties of RNA and DNA (Cox, 1961, 1963a).

Since reticulocytes synthesize mainly one protein, haemoglobin, and would therefore be expected to contain relatively homogeneous ribosomes and ribosomal RNA, the present work has concentrated on the isolation of RNA from reticulocyte ribosomes in good yield (cf. Cox & Arnstein, 1962) and on a study of its physicochemical properties. It is shown that RNA may be isolated from rabbit-reticulocyte ribosomes in more than 90% yield and that whatever the method of isolation the ribosomal RNA consists of an approximately equimolar mixture of two species having sedimentation coefficients, $S_{20, w}$, of 16.8s and 29.8s. In contrast with denatured (single-stranded) DNA, the acid-base properties of reticulocyte-ribosomal RNA agree with a nucleotide sequence favouring the formation of relatively-stable ordered domains which include the majority of cytosine and (an equivalent number of the complementary) guanine residues. Also, there appear to be occasional sequences rich in adenine residues, which may form ordered domains in acidic but not in neutral solution.

MATERIALS AND METHODS

Chemicals. Guanidinium chloride (about 6M) was prepared by neutralization of AnalaR guanidinium carbonate with conc. hydrochloric acid.

Bentonite (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) was equilibrated with buffer (5 mM-MgCl₂ in 1 mM-potassium phosphate, pH 7) at 4°, and a fraction sedimenting within the range 6000–15000g for 20 min. at 20° was retained.

Pancreatic ribonuclease was obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany.

Sodium dodecyl sulphate was recrystallized from aqueous ethanol. Solutions (1%, w/v) were stabilized by traces of butanol.

Phenol was redistilled immediately before use. Polyadenylic acid (poly-A) and polyuridylic acid (poly-U) were given by Professor P. Doty. The complex poly-(A + U) was formed by the interaction of equimolar poly-A and poly-U in 0.1 M-sodium phosphate, pH 7. Oligoguanylic acid was a gift of Dr A. M. Michelson.

Ribosomal preparations. Reticulocytes were obtained from rabbits that had been given daily intraperitoneal

injections of neutralized 2.5% (w/v) phenylhydrazine hydrochloride (0.3 ml./kg.) for 6 or 7 days; the cells were lysed in 5 mM-MgCl₂, and ribosomes were isolated by differential centrifuging (Schweet, Lamfrom & Allen, 1958).

Analyses. Nitrogen was determined by the Dumas method. Phosphorus was determined gravimetrically as ammonium phosphomolybdate after dry combustion.

Ultracentrifuging. A Spinco model E ultracentrifuge equipped with ultraviolet optics was used in sedimentation studies of RNA (about 0.04 g./l.) in 50 mM-phosphate buffer, pH 7. Photographs were taken at 4 min. intervals after the rotor (the temperature of which was about 20°) reached full speed (42040 rev./min.). The optical density of the photographic film was measured with a double-beam recording microdensitometer (model E12 Mk III B; Joyce, Loeb and Co. Ltd., Newcastle upon Tyne). The sedimentation coefficients were corrected for temperature and viscosity to 20° in water, and for density, and are expressed in Svedberg units, *s* (i.e. $S_{20, w} \times 10^{13}$ sec.). A Spinco model L or MSE ultracentrifuge was used for the preparation of cell fractions.

Spectrophotometry. Absorption spectra and extinctions were measured with a Unicam SP. 500 spectrophotometer fitted with thermospacers. The cell holder was an electrically-heated copper block (Cox, 1963a). The observed extinctions measured at temperatures other than 20° were corrected for the thermal expansion of water.

Measurement of pH. The electrometric-titration technique has been described by Cox & Littauer (1963). A model 39A E.I.L. pH-meter (Electronic Instruments Ltd., Richmond, Surrey) accurate to better than ± 0.005 pH unit was used in the spectrophotometric studies. Electrodes covering the range pH 0–14 were used for both electrometric and spectrophotometric studies.

RESULTS

Isolation of ribonucleic acid by precipitation as the guanidinium salt

A suspension of ribosomal particles (10 mg./ml.) in medium A [sucrose (0.25 M), potassium chloride (25 mM), magnesium chloride (5 mM), tris (50 mM), pH 7.6] was mixed at 0° with 5 vol. of 5 M-guanidinium chloride containing EDTA (33 mM), pH 4.5–5.0. After 30 min. the RNA that was precipitated was isolated by centrifuging (3000 rev./min.), and washed successively with 6 M-guanidinium chloride, 0.2 M-EDTA, pH 7, and ethanol.

The product was extracted several times with water (or with dilute neutral salt solutions such as 10 mM-tris, 2 mM-EDTA, or 1 mM-potassium phosphate), which contained bentonite (1 mg./ml.) to inhibit traces of ribonucleases that were present (Brownhill, Jones & Stacey, 1959; Singer & Fraenkel-Conrat, 1961), until dissolution was complete (Table 1). A pellet of aggregated bentonite and residual protein was generally found after this treatment. The RNA was converted into the potassium salt by passing through a sterile column (10 cm. $\times$ 2 cm. diam.) of a cation-exchange resin (Amberlite IR-120 or Amberlite IRC-50; chromato-

graphic grade; K^+ form) at pH 4–7. Residual bentonite was removed by centrifuging (12000 rev./min. at 0°). A N:P ratio of 1.72 was found for the potassium salt of RNA compared with a theoretical value of 1.76 (Wallace & Ts'O, 1961). Ribonucleoprotein, RNA and ribosomal protein (which was recovered by dialysis of the guanidinium chloride supernatant against water) were characterized by their ultraviolet-absorption spectra (Fig. 1).

The sedimentation pattern (Fig. 2a) of a dilute solution of the guanidinium salt of RNA (0.04

mg./ml.) was unchanged by treatment with up to 0.2M-EDTA and only two components ($S_{20,w} = 16.8s$ and $29.8s$) were present. The amount of the slower-sedimenting component was about 25–35 %, and about 50–60 % after ion-exchange chromatography (Fig. 2b). After this step the sedimentation pattern was unchanged by incubation at 37° for

Table 1. *Dissolution of the guanidinium salt of ribonucleic acid by repeated extraction with water*

Experimental details are given in the text. The amount of RNA dissolved at each step was maximum after about 30 min.

Extraction no.	E_{260}	
	Reticulocyte RNA	Liver RNA
1	0.03	0.069
2	13.75	40.183
3	0.00	24.60
4	—	4.66
5	—	1.20

Fig. 1. Ultraviolet-absorption spectra of: (a) ribosomes in 0.1M-sodium phosphate, pH 7; (b) RNA (guanidinium salt) in 0.1M-sodium phosphate, pH 7; (c) protein fraction in water; (d) water-insoluble protein fraction (about 50% of total) in dilute acetic acid, pH 3.

Fig. 2. Sedimentation patterns of RNA in 0.1M-sodium phosphate at about 20° . The microdensitometer tracings which measure the optical density of the photographic film are presented. The direction of sedimentation is from left to right. In each case the interval between exposures was 4 min. (a) Guanidinium salt of RNA. (b) Potassium salt of RNA, prepared by ion-exchange chromatography. (c) RNA from untreated ribosomes. (d) RNA from the same batch of ribosomes as (c) but pretreated with ribonuclease (see Arnstein, 1961). (e) RNA from ribosomes before preincubation. (f) RNA from ribosomes preincubated according to the method of Nirenberg & Matthaei (1961).

2 hr. (cf. Boedtke, Möller & Klemperer, 1962) in the presence or absence of bentonite, or by dialysis at 4° for 24 hr. against buffer [sucrose (0.25M), potassium chloride (25 mM), magnesium chloride (5 mM), tris (20 mM)].

Satisfactory results were obtained with washed and unwashed ribosomes since the extraneous protein was soluble in 4M-guanidinium chloride. No change in the sedimentation pattern, except possibly a sharpening of the boundaries, was found when the isolation was carried out at -18° or when guanidinium chloride was slowly added. In one experiment guanidinium chloride (3 ml.) was allowed to diffuse at -18° into a suspension of ribosomes in a mixture of medium A (0.4 ml.) and glycerol (0.6 ml.). Guanidinium chloride solution (6M) containing magnesium chloride (5 mM) was also added slowly (3 ml. at the rate of 5 μ l./min.) to a suspension of ribosomes in glycerol. The yield of RNA was about 95 %.

The critical steps in this method are, first, the precipitation of RNA, secondly, the dissolution of the precipitate, and thirdly the inhibition and removal of ribonucleases. The precipitation of the purified potassium salt from 4M-guanidinium chloride solutions depended on pH, the concentration of Mg²⁺ ions and temperature. Precipitation occurred at 0° at pH 5.5 or below (Fig. 3a), or at pH 7 when the concentration of Mg²⁺ ions was greater than 0.5 mM (Fig. 3b) or when 0.5-1.0 vol. of ethanol was added. Recoveries of 85 % were obtained at 0° (Table 2) and of about 95 % at -18.5°. The concentration of RNA was generally greater than 0.5 mg./ml.; no precipitate was given by concentrations less than 0.1 mg./ml. The solubility of the precipitate in water was the same in all cases. When protein was present the precipitation of RNA was less consistent but was always achieved by the addition of ethanol. No major differences in the sedimentation pattern were observed for fractions having different solubilities in guanidinium chloride.

Frequently, more than one extraction with water was necessary for the dissolution of the guanidinium salt (cf. Tashiro, Shimidzu, Inouye & Kakiuchi, 1960). As shown in Table 1, most of the product dissolved on the second or third extraction with water.

Stability. In 4M-guanidinium chloride RNA was stable indefinitely, as judged by its sedimentation behaviour whether in solution or suspension. In water the guanidinium salt of RNA was stable at 0°, or below, but not at 20° unless bentonite (or other ribonuclease inhibitor) were present. The stability of RNA at 20° was improved after zone centrifuging in a sucrose gradient at 4° (R. A. Cox, unpublished work). The addition of bentonite and its subsequent removal by centrifuging was re-

Table 2. *Yield of ribonucleic acid isolated from ribosomes by precipitation as the guanidinium salt*

Blood was taken from an anaemic rabbit about 23 hr. after injection with 0.5 mc of [³²P]phosphate, and reticulocyte ribosomes were isolated. RNA was precipitated out when guanidinium chloride was added to a suspension of the ribosomes. The precipitate was washed with guanidinium chloride, dissolved in 10 mM-EDTA (5 ml.), and then precipitated with ethanol (15 ml.) and potassium acetate (100 mg.). A sample (0.1 ml.) of the aqueous solution was evaporated on a glass slide (2 cm.²) and the radioactivity was measured. The radioactivity of the combined guanidinium chloride solutions (25 ml.) and of the combined ethanol solutions (35 ml.) were measured in the same way. The total radioactivities are given.

Fraction	Radioactivity		Percentage yield (from $E_{280} : E_{260}$)
	(counts/min.)	(% of total)	
Guanidinium chloride	20800	10.7	—
Ethanol (after washing RNA)	5600	2.9	—
RNA (in aqueous solution)	167000	86.5	85-90

Fig. 3. Precipitation of purified RNA from guanidinium chloride solutions (4M). (a) The effect of pH in the absence of magnesium chloride. (b) The effect of magnesium chloride at pH 7.0. O, Extinction of supernatant at 0°; ●, extinction of supernatant at -20°; Δ, extinction of precipitated RNA redissolved in 0.1M-sodium phosphate, pH 7; x, total RNA (sum of ● plus Δ).

peated several times but did not lead to a completely stable product. After ion-exchange chromatography, as described above, however, RNA solutions were stable at 37° for 2 hr.

Effects of previous history of ribosomes. The sedimentation pattern (Fig. 2d) of RNA isolated from ribosomes pretreated with ribonuclease according to the method described by Arnstein (1961) was broader than that of the control (Fig. 2c), although two species with $S_{20,w}^0$ values of 16·8s and 29·8s were still present. RNA isolated from preincubated ribosomes prepared by the method of Nirenberg & Matthaei (1961) was generally similar in sedimentation behaviour to that of the control (Figs. 2e and 2f).

Ribosomes isolated from the cell sap by centrifuging at 105000g for 1 hr. were characterized in medium A by sedimentation coefficients of 80s, 100s and higher (Arnstein & Cox, 1963). Residual ribosomes (80s) sedimented from the cell sap on centrifuging at 105000g for a further hour. After the first ultracentrifuging step the cell sap was brought to pH 5·15 with N-acetic acid to precipitate residual ribosomes and activating enzymes; the precipitate was then redissolved in medium A; the residual ribosomes were then isolated by ultracentrifuging. RNA was isolated from both the bulk and the residual ribosomes and the same sedimentation pattern was found in both cases (cf. Fig. 2a).

Isolation of ribonucleic acid by sodium dodecyl sulphate treatment

In a typical experiment ribosomes (0·10 mg.) were added to 1% (w/v) sodium dodecyl sulphate (1 ml.) at 20° and the solution was made 0·1M with respect to phosphate buffer, pH 7. No species with $S_{20,w}$ greater than 8s were observed on centrifuging. Improved results were obtained when the solution was cooled immediately after the addition of ribosomes in order to remove protein by coprecipitation with the sodium dodecyl sulphate, or when the solution was immediately subjected to cation-exchange chromatography at 4°. The sedimentation pattern (Fig. 4) showed two distinct boundaries and was similar to that of RNA prepared by the guanidinium chloride method. The slower-sedimenting species ($S_{20,w} = 17s$) accounted for two-thirds of the ultraviolet absorption and the remaining one-third was associated with the faster-sedimenting species ($S_{20,w} = 30s$). RNA was obtained by the guanidinium method from the same batch of ribosomes and gave a sedimentation pattern almost identical with that shown in Fig. 2a.

Isolation of ribonucleic acid with phenol

A washed ribosomal pellet (43 mg.), water (4 ml.) and aq. 90% (w/v) phenol (4 ml.) were emulsified at 0° in a Dounce homogenizer. Aq.

45% (w/v) phenol (6 ml.) was added to the emulsion which was then shaken at 4° for about 18 hr. The emulsion was broken by centrifuging at 12000 rev./min. for 15 min. at 4°. The aqueous phase was separated off. The phenol phase was extracted with water and the water phase with phenol. The aqueous phases were combined and shaken with phenol, and traces of phenol were removed by extraction with peroxide-free ether. The yield of RNA was 6·3 mg. Three distinct boundaries ($S_{20,w}$ values of about 4s, 17s and 30s) were observed on ultracentrifuging. The sedimentation pattern is given in Fig. 5a. Further extraction of the phenol phase with water yielded 1 mg. of RNA, which was polydisperse (Fig. 5c). Similar results were obtained for ribosomes that were first suspended in sodium deoxycholate (0·5%) for a few minutes, diluted with 10 vol. of medium A, reisolated by centrifuging and then treated with phenol as described above.

The presence of bentonite (5 mg./ml.) in the aqueous phase throughout the isolation did not improve the yield or materially affect the sedimentation pattern.

In a parallel experiment, ribosomes (about 50 mg.) were suspended in buffer (4 ml. of medium A), and water (10 ml.) containing bentonite (5 mg./ml.) and aq. 90% (w/v) phenol (10 ml.) were added. The emulsion was shaken at room temperature for about 18 hr. The aqueous phase was extracted three times with an equal volume of phenol, and then freed from phenol by extraction with ether. The yield of RNA was about 10 mg. The presence of two major components ($S_{20,w} = 17s$ and 29s) was deduced from the sedimentation pattern (Fig. 5b).

In one experiment it was found that the yield of RNA increased from about 24% to about 60% when the temperature of the phenol extraction was increased from 4° to 60° or when EDTA (33 mM) was added to the aqueous phase. These procedures led to a polydisperse product (cf. Fig. 5c).

Fig. 4. Sedimentation pattern of RNA isolated by treatment with sodium dodecyl sulphate.

Fig. 5. Sedimentation patterns of RNA isolated by extraction with phenol-water mixtures. (a) Extraction at 0°. (b) Extraction at 20°. (c) Second extraction of phenol layer with water.

Fig. 6. Dependence of the ultraviolet-absorption spectrum of RNA on pH and temperature. Curve I (○), pH 7; curve II (●), pH 4.01; curve III (△), pH 3.60; curve IV (○), pH 3.01.

The sedimentation pattern of RNA isolated by these procedures varied from preparation to preparation, in accordance with the observations of Hall & Doty (1959).

Spectrophotometric-titration studies

The spectrum of RNA from rabbit reticulocytes in, for example, 0.1M-sodium phosphate depended on pH and temperature. The results are expressed in Fig. 6 as relative values of the extinction at 260 mμ. When neutral solutions were heated at from 28° to 87°, the extinction increased by about 24%. This took place in two steps, A-B and B-C, with a marked inflexion at about 62° (point B). Identical values were found at pH 4.40. The

extinction at 260 mμ, E_{260} , decreased by about 2% at 22° when the pH was brought to pH 4.01. Increasing the temperature at this pH led to a regular increase in E_{260} . Comparison of the curves at pH 7 and pH 4.01 (curve II) suggests that the region corresponding to A-B (curve I) was displaced towards a lower temperature range whereas the region B-C was hardly affected. The extinction at 25° increased by about 4% on acidification to pH 3.60, and on heating at this pH it increased further by about 20% over the range 28–85° (curve III). The extinction at pH 3.0 at 25° was about 11% higher than at pH 7 and increased regularly by 13% over the range 25–85° (curve IV).

The changes in the spectrum of RNA at 22° on titration to pH 2.5 are summarized in Fig. 7, where the $E_{280}:E_{260}$ ratio, and relative values of E_{260} (Fig. 7b), are plotted as a function of pH. When RNA was titrated with acid in 3 mM-sodium chloride the $E_{280}:E_{260}$ ratio increased over the range pH 3.5–5.5. The extinction at 260 mμ was greater by 7% at pH 3 than at pH 6.10, and the increase was found to take place over the range pH 3.5–4.5 where values of $E_{280}:E_{260}$ increased most rapidly.

The spectrophotometric-titration curves depended on the concentration of added salt. The same limiting value of $E_{280}:E_{260}$ was attained in 3 mM-sodium chloride made 5 mM with respect to sodium acetate (curve II of Fig. 7a) as in 3 mM-sodium chloride although the changes in the spectrum occurred over a lower pH range, and 50% of the total increase in $E_{280}:E_{260}$ was observed at pH 4.0. Increasing the salt concentration to 0.1M with respect to Na^+ ions (80 mM-sodium chloride made 20 mM with respect to sodium acetate) or to 50 mM-sodium dihydrogen phosphate led to a suppression of ionization, as measured by the $E_{280}:E_{260}$ ratio, since the mid-point of the titration curve (curve III of Fig. 7a) was displaced to pH 3.4 (cf. pH 3.25 for native DNA and pH 4.0 for denatured DNA; Cox, 1961), and the limiting value was approached at about 2.4. The extinction at 260 mμ increased

regularly by about 16 % over the range pH 4.5–2.5 (curve III of Fig. 7b).

RNA (0.63 mg./ml.) was also titrated electrometrically with acid (10 mM-hydrochloric acid) from pH 7 to pH 3.5 (curve I) and then back-titrated with alkali (10 mM-sodium hydroxide) to pH 7 (curve II). Although curves I and II were repeated on subsequent titration cycles they were not identical, and in general the number of equivalents of acid bound by RNA at a particular pH was greater on back-titration with alkali (curve II). The differences (curve II – curve I) on forward- and back-titration in the numbers of equivalents of acid bound/4 g.atoms of P over the range pH 3.5–7.0 are given (Fig. 7c).

Solutions of RNA in 50 mM-sodium phosphate were titrated with alkali. The changes in the spectrum are summarized as the changes in the

$E_{230}:E_{260}$ ratio (Fig. 8a), in the $E_{270}:E_{260}$ ratio (Fig. 8b) and in the E_{260} (Fig. 8c). The major changes occurred over the range pH 10–11.7. The ionization of uracil residues appears to be measured by the $E_{230}:E_{260}$ ratio, whereas the ionization of guanine residues appears to be measured by the $E_{270}:E_{260}$ ratio. Thus on titration of polyuridylic acid with alkali the $E_{230}:E_{260}$ ratio increased from 0.245 to 0.990, whereas the $E_{270}:E_{260}$ ratio varied between 0.793 and 0.806, the mean value being 0.80. Titration of the complex poly-(A + U) with alkali led to an increase in the $E_{230}:E_{260}$ ratio from 0.340 to 0.575, whereas the $E_{270}:E_{260}$ ratio was 0.767 over the range pH 8.42–10.56 and 0.738 over the range pH 10.76–12.00. The change over the range pH 10.56–10.76, over which E_{260} increased by 20 %, is attributed to changes in the spectrum due to the transition from poly-(A + U) to poly-A plus poly-U. When oligoguanylic acid was

Fig. 7. Spectrophotometric- and electrometric-titration studies of rabbit-reticulocyte RNA. (a) The dependence of the $E_{260}:E_{280}$ ratio on pH at 20°: curve I (Δ), 3 mM-NaCl; curve II ($\blacktriangle$), 3 mM-NaCl made 5 mM with respect to sodium acetate; curve III ($\circ$ and $\bullet$), 80 mM-NaCl made 20 mM with respect to sodium acetate. (b) The dependence of E_{260} on pH at 20°: curves I, II and III are as described for (a). (c) Electrometric titration of RNA at 25° in 0.1 M-NaCl; the Figure gives the difference in the equivalents of acid bound/4 g.-atoms of P on titration from pH 7 to pH 3.6 and on titration from pH 3.6 to pH 7.0.

Fig. 8. Spectrophotometric-titration studies of RNA at 20° in 50 mM-sodium phosphate.

titrated with alkali, the $E_{230}:E_{260}$ ratio was constant at 0.424 over the range pH 8.80–11.80. The extinction at 260 m μ increased by 14% over the range pH 9.5–11.5. The changes in the spectrum of RNA on titration with alkali are entirely consistent with the view (Cox, 1961; Cox & Littauer, 1963) that, over the range pH 8–10, ionization of guanine and uracil residues (pK 9.70 in 0.2M-sodium chloride; Warner & Breslow, 1958) is suppressed, owing to secondary structure, and that increasing the pH still further leads to a co-operative transition to the unfolded form of RNA as denoted by the increase in E_{260} .

DISCUSSION

Isolation of ribosomal ribonucleic acid

Current methods for the isolation of cytoplasmic RNA from mammalian cells are based chiefly on either extraction with phenol–water mixtures (Kirby, 1956; Colter & Brown, 1956; Laskov, Margoliash, Littauer & Eisenberg, 1959) or on the dissociation of protein from RNA by detergents, followed by denaturation of the protein (Hall & Doty, 1958), e.g. by extraction with chloroform–pentanol mixtures (Ts'o & Squires, 1959) or with phenol–water mixtures (Hall & Doty, 1959). These general methods yield infectious RNA from viruses (Gierer & Schramm, 1956; Fraenkel-Conrat, Singer & Williams, 1957; Colter, Bird, Moyer & Brown, 1957). Concentrated sodium chloride solutions have also been used to precipitate RNA from the cytoplasmic fraction (Tashiro *et al.* 1960). Guanidinium chloride (2M) was used by Volkin & Carter (1951) and by Grinnan & Mosher (1951) for the isolation of cytoplasmic RNA which was of moderate molecular weight, and by Reichmann & Stace-Smith (1959) and by Kawade (1960) for the isolation of RNA from plant viruses. The observation by Sela, Anfinsen & Harrington (1957) that pancreatic ribonuclease is inactive in 4M-guanidinium chloride encouraged us to persevere with this reagent. The method of isolating RNA from ribonucleoprotein particles by precipitation as the guanidinium salt described in the present paper gives essentially quantitative yields of a product that has sedimentation properties similar to those of RNA obtained by extraction with phenol or by treatment with sodium dodecyl sulphate. The quality of the RNA, however, as judged by the amount of the faster-sedimenting component and the sharpness of the boundaries, compared favourably with that of RNA obtained by the other methods. The method is probably generally applicable to ribonucleoprotein particles, since ribosomal RNA was also isolated by precipitation as the guanidinium salt (R. A. Cox, unpublished work) from guinea-pig-reticulocyte ribosomes, rabbit-

liver ribosomes and ribosomes from *Bacillus cereus*. In each case two components were observed: those of *B. cereus* RNA had $S_{20,w}$ values of 17s and 24s (cf. *E. coli* RNA; Littauer & Eisenberg, 1959; Kurland, 1960), and those from other sources had $S_{20,w}$ values of about 17s and 30s (cf. Colter & Brown, 1956; Timasheff, Brown, Colter & Davies, 1958; Hall & Doty, 1959). In addition it appears to be useful also for the isolation of the ribosomal protein.

Physicochemical properties of ribosomal ribonucleic acid

Sedimentation studies. The sedimentation properties of RNA isolated from rabbit-reticulocyte ribosomes depended on neither the ability of the nucleoprotein particles to form higher aggregates nor on the extent to which ribosomal particles function in the incorporation of labelled amino acids into peptide linkage [cf. RNA from normal (Fig. 2a) and preincubated (Fig. 2e) ribosomes (Arnstein, Cox & Hunt, 1962)]. These observations are in agreement with the view that both aggregation of ribosomes and their metabolic activity are attributable to 'messenger' RNA rather than to differences in the bulk of the ribosomal RNA. Hence, if the RNA of the ribosomes (80 s) of rabbit reticulocytes were present as a continuous thread its molecular weight would be 2×10^6 (Dintzis, Borsook & Vinograd, 1958). However, whatever the method of isolation, RNA was a mixture of two species differing in sedimentation coefficient. It is estimated that the molecular weights of these species are about 0.5×10^6 and about 1.5×10^6 on the basis of the relation between molecular weight and sedimentation coefficient derived by Hall & Doty (1959) and Kurland (1960). One molecule of each could account for the total ribosomal RNA, as suggested by Hall & Doty (1959) for calf-liver ribosomal RNA. If so, the ultraviolet extinction of the 16.8s and 29.8s components would be in the ratio 1:3, a value that was approached in several cases (cf. Bock, 1961). However, the variability in this ratio, e.g. when the nucleic acid was isolated in parallel experiments from the same batch of ribosomes by different procedures (cf. Figs. 2a and 4), indicates that the faster-sedimenting species is converted into the slower, in agreement with the observations of McCarthy & Aronson (1961) and Huppert & Pelmont (1962). Thus the possibility remains that the RNA molecule is present within the ribosome as one continuous thread which is unavoidably damaged during isolation (cf. Boedtke *et al.* 1962).

Acid-base properties. The properties of isolated ribonucleic acids have been widely studied since it was first proposed by Doty and his co-workers (Doty, Boedtke, Fresco, Hall & Haselkorn, 1959a;

Doty, Boedtker, Fresco, Haselkorn & Litt, 1959*b*; Fresco, Alberts & Doty, 1960), that this polynucleotide has a well defined secondary structure in neutral salt solutions. The optical properties (Hall & Doty, 1958, 1959; Doty *et al.* 1959*a, b*), X-ray diffraction patterns (Fuller, 1961; Spencer, Fuller, Wilkins & Brown, 1962), mass per unit length (Timasheff, Witz & Luzzatti, 1961), hydrodynamic properties (Doty *et al.* 1959*a, b*; Boedtker, 1959, 1960; Spirin, 1960, 1961*a, b*; Cox & Littauer, 1959, 1962; Cox, 1960; Littauer, 1960) and acid-base properties (Cox & Littauer, 1959, 1963; Cox, 1961, 1962, 1963*a*) all support the concept of Doty *et al.* (1959*a, b*) that the polynucleotide chain may fold on itself to form helical domains stabilized by hydrogen bonds formed between complementary base residues. Specific nucleotide sequences were shown not to be a requirement for the formation of secondary structure (Doty *et al.* 1959*a, b*; Fresco & Alberts, 1960; Fresco *et al.* 1960), since random copolynucleotides (Ortiz & Ochoa, 1958) formed by co-polymerization of adenylic acid, guanylic acid, uridylic acid and cytidylic acid have a marked capacity for forming ordered domains (Doty *et al.* 1959*a, b*; Breslow & Warner, 1959; Steiner, 1960; Lipsett, Heppel & Bradley, 1961; Cox, 1963*b*).

However, detailed studies of the acid-base properties of *E. coli* ribosomal RNA (Cox, 1961; Cox & Littauer, 1963) and rat-liver ribosomal RNA (Cox, 1961, 1962, 1963*a*) revealed properties that suggested that in these cases secondary structure does not arise from a fortuitous mating of base residues arranged in a near-to-random sequence.

First, it was noticed that the pH-titration curve was different on forward- and back-titration from pH 7 to pH 3 to pH 7 (Littauer & Eisenberg, 1959; Cox & Littauer, 1959, 1963; Cox, 1961, 1962), and that the two curves were accurately reproduced on successive cycles in a manner that is characteristic of hysteresis loops (cf. Fig. 6*b*). Similar behaviour was found (Cox, 1962, 1963*a*) on titration of poly-(A + U), the complex formed between polyadenylic acid and polyuridylic acid. The hysteresis phenomenon can be explained if titration to pH 3 partly disrupts the secondary structure which is stable at pH 7, and enables adenine residues, for example, to interact with each other to form stable ordered domains which impede the return to the structure stable at pH 7. In accordance with this view the value of E_{260} of *E. coli* ribosomal RNA in 0.1M-sodium chloride, at about pH 3.0, increased by about 14% over the range 25–80°; even in 1 mM-sodium chloride, at pH 3.0, an increase in E_{260} of about 5% was found over the range 40–70°, indicating the presence of stable domains at this pH. RNA from rabbit-reticulocyte ribosomes has similar properties (Fig. 6). The explanation proposed for the hysteresis requires that the nucleotide

sequence of ribosomal RNA allows the adenine nucleotides to interact to form ordered domains that are stable in acidic but not in neutral solutions. It is relevant that the biosynthetic co-polynucleotide poly-AGUC, which has a random nucleotide sequence (Ortiz & Ochoa, 1958), does not appear to form stable domains in acidic solutions, since at pH 2.80 E_{260} was independent of temperature (R. A. Cox, unpublished work).

Secondly, increasing the temperature led to shifts in the pH-titration curve which would be predicted on the basis of the assumption that raising the temperature increases the sensitivity of helical domains to rupture in acidic solutions (Cox & Littauer, 1963). Native but not denatured DNA has similar titration properties (Cox & Peacocke, 1957; Cavalieri & Rosenberg, 1957).

Thirdly, the dependence on pH of the $E_{280}:E_{260}$ ratio, which measures principally the ionization of cytosine residues, was similar for both *E. coli* ribosomal RNA and native DNA, and distinct from the dependence noted for poly-AGUC and denatured DNA (Cox, 1961) (cf. Fig. 7*a*).

The change in the acid-base properties of *E. coli* RNA with temperature, taken together with the spectrophotometric-titration findings, suggest that most of the cytosine residues are present at pH 7 in ordered domains that are stable both to acid and to heat to an extent approaching that of native DNA. The acid-base properties of RNA from rabbit-reticulocyte ribosomes are similar to those of RNA from *E. coli* ribosomes except that slightly more extreme pH values, in either acidic or alkaline solutions, or higher temperatures, have to be attained to disrupt secondary structure.

The temperature ranges over which the secondary structure of both *E. coli* and rabbit-reticulocyte ribosomal RNA 'melt' out are compared in Fig. 8. The 'melting' ranges for poly-AGUC and RNA from turnip-yellow-mosaic virus are included for comparison. It is apparent that there is a tendency for the 'melting' range to depend on the probable proportions of A-U to G-C base pairs estimated from the nucleotide composition, as would be expected if, at pH 7, ordered domains were stabilized by interaction between complementary base pairs (cf. Spirin, 1960, 1961*a, b*; Fresco *et al.* 1960). The minor differences in the acid-base properties of *E. coli* and rabbit-reticulocyte ribosomal RNA which reflect the stability of secondary structure are therefore attributed to the difference in nucleotide composition.

Ribosomal RNA is not homogeneous with respect to molecular weight. However, the nucleotide composition of the two components of *E. coli* ribosomal RNA have been shown to be similar (Spahr & Tissières, 1959), and the hydrodynamic properties respond in a similar manner (Cox &

Littauer, 1962) to changes in ionic strength, suggesting that the secondary structures have similar stabilities.

In conclusion, it is inferred that the nucleotide sequence of ribosomal RNA differs significantly from random. Sequences that include the majority of cytosine residues are such as to favour the formation of helical domains that are relatively stable to acid, alkali, heat and, as shown previously by viscosity measurements on *E. coli* RNA, to exposure to low ionic strengths (Cox, 1960; Cox & Littauer, 1962). Also, there appear to be occasional sequences rich in adenine residues which form stable domains in acidic solutions. Fig. 9 indicates that domains comparable in stability and in adenine content with those formed by a co-polymer of adenylic acid (60%) and uridylic (40%) acid could be present in both *E. coli* and rabbit-reticulocyte RNA.

There is evidence for inhomogeneities in the distribution of base residues along the DNA molecule (Shapiro & Chargaff, 1960; Burton, 1960; Petersen, 1961; Geiduschek, 1962; Simha & Zimmerman, 1962). This trend is apparently more extreme with ribosomal RNA. In contrast, turnip-yellow-mosaic-virus RNA which may act as a 'messenger' in protein synthesis (Ofengand & Haselkorn, 1961) has a small 'hysteresis loop' (the pH-titration curve pH 7 to pH 3 differed from

the curve pH 3 to pH 7 by no more than 0.06 equiv./4 g. atoms of P at 25°), providing a further indication that the nucleotide sequence of ribosomal RNA might differ significantly from that of 'messenger' RNA.

SUMMARY

1. RNA was isolated from rabbit-reticulocyte ribosomes in better than 90% yield by precipitation as the guanidinium salt.

2. After precipitation of the RNA, ribosomal protein was recovered from the guanidinium chloride solution by dialysis.

3. Ribosomal RNA was found to be an approximately equimolar mixture of two species having sedimentation coefficients, $S_{20,w}$, of 16.8s and 29.8s.

4. The quality of the RNA, as judged by the proportion of the faster-sedimenting component and the sharpness of the boundaries, compared favourably with that of RNA isolated by extraction with aqueous phenol or sodium lauryl sulphate.

5. The sedimentation pattern of RNA did not depend on the extent to which the ribosome preparations function in the incorporation of labelled amino acids into peptide linkage, but pretreatment of ribosomes with ribonuclease led to polydisperse preparations of RNA.

6. Reticulocyte RNA was titrated electrometrically and spectrophotometrically. The results were consistent with the formation in neutral salt solutions of a stable secondary structure which was determined by a non-random nucleotide sequence.

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REFERENCES

- Arnstein, H. R. V. (1961). *Biochem. J.* **81**, 24P.
 Arnstein, H. R. V. & Cox, R. A. (1963). *Biochem. J.* **88**, 27P.
 Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1962). *Nature, Lond.*, **194**, 1042.
 Bautz, E. K. F. & Hall, B. D. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 400.
 Bock, R. (1961). *Colloques internationaux du Centre National de la Recherche Scientifique*, no. 106, *Acides Ribonucléiques et Polyphosphates; Structure, Synthèse et Fonctions*, Strasbourg, p. 120. Paris: Centre National de la Recherche Scientifique.
 Boedtker, H. (1959). *Biochim. biophys. Acta*, **32**, 519.
 Boedtker, H. (1960). *J. molec. Biol.* **2**, 171.
 Boedtker, H., Möller, W. & Klemperer, E. (1962). *Nature, Lond.*, **194**, 444.
 Brenner, S., Jacob, F. & Meselson, M. (1961). *Nature, Lond.*, **190**, 576.
 Breslow, E. & Warner, R. C. (1959). *Fed. Proc.* **18**, 197.

Fig. 9. Comparison of the 'melting' curves of RNA from different sources. The vertical line indicates the 'melting' range and the interval between horizontal markers denotes the range of temperature over which E_{260} increased by 5% of the value at about 25°. O denotes the temperature at which E_{260} increased by 50% of the total increment.

- Brownhill, T., Jones, A. S. & Stacey, M. (1959). *Biochem. J.* **73**, 438.
- Burton, K. (1960). *Biochem. J.* **77**, 547.
- Cavalieri, L. F. & Rosenberg, B. (1957). *J. Amer. chem. Soc.* **79**, 5352.
- Chantrenne, H. (1961). *The Biosynthesis of Proteins*, p. 66. London: Pergamon Press Ltd.
- Colter, J. S., Bird, H. H., Moyer, A. W. & Brown, R. A. (1957). *Virology*, **4**, 522.
- Colter, J. C. & Brown, J. A. (1956). *Science*, **124**, 1077.
- Cox, R. A. (1960). *J. Polym. Sci.* **47**, 441.
- Cox, R. A. (1961). *Colloques internationaux du Centre National de la Recherche Scientifique*, no. 106, *Acides Ribonucléiques et Polyphosphates: Structure, Synthèse et Fonctions*, Strasbourg, pp. 92 and 135. Paris: Centre National de la Recherche Scientifique.
- Cox, R. A. (1962). *Biochem. J.* **84**, 103f.
- Cox, R. A. (1963a). *Biochim. biophys. Acta*, **68**, 401.
- Cox, R. A. (1963b). *Biochim. biophys. Acta*, **72**, 203.
- Cox, R. A. & Arnstein, H. R. V. (1962). *Biochem. J.* **83**, 4f.
- Cox, R. A. & Littauer, U. Z. (1959). *Nature, Lond.*, **184**, 818.
- Cox, R. A. & Littauer, U. Z. (1962). *Biochim. biophys. Acta*, **61**, 197.
- Cox, R. A. & Littauer, U. Z. (1963). *Biochim. biophys. Acta*, **78**, 188.
- Cox, R. A. & Peacocke, A. R. (1957). *J. chem. Soc.* p. 4724.
- Dintzis, H. M., Borsook, H. & Vinograd, J. (1958). In *Microsomal Particles and Protein Synthesis*, p. 95. Ed. by Roberts, R. B. London: Pergamon Press Ltd.
- Doty, P., Boedtker, H., Fresco, J. R., Hall, B. D. & Haselkorn, R. (1959a). *Ann. N.Y. Acad. Sci.* **81**, 693.
- Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R. & Litt, M. (1959b). *Proc. nat. Acad. Sci., Wash.*, **45**, 482.
- Doty, P., Marmur, J., Eigner, J. & Schildkraut, C. L. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 453.
- Fraenkel-Conrat, H., Singer, B. & Williams, R. C. (1957). *Biochim. biophys. Acta*, **25**, 87.
- Fresco, J. R. & Alberts, B. M. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 311.
- Fresco, J. R., Alberts, B. M. & Doty, P. (1960). *Nature, Lond.*, **188**, 98.
- Fuller, W. (1961). *J. molec. Biol.* **3**, 175.
- Geiduschek, E. P. (1962). *J. molec. Biol.* **4**, 467.
- Gierer, A. & Schramm, G. (1956). *Nature, Lond.*, **177**, 702.
- Grinnan, E. L. & Mosher, W. A. (1951). *J. biol. Chem.* **191**, 719.
- Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Risebrough, R. W. & Watson, J. D. (1961). *Nature, Lond.*, **190**, 581.
- Hall, B. D. & Doty, P. (1958). In *Microsomal Particles and Protein Synthesis*, p. 27. Ed. by Roberts, R. B. London: Pergamon Press Ltd.
- Hall, B. D. & Doty, P. (1959). *J. molec. Biol.* **1**, 111.
- Hall, B. D. & Spiegelman, S. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 137.
- Huppert, J. & Pelmont, J. (1962). *Arch. Biochem. Biophys.* **98**, 214.
- Ishihama, A., Mizuno, N., Takai, M., Otaka, E. & Osawa, S. (1962). *J. molec. Biol.* **5**, 251.
- Kawade, Y. (1960). *Virus, Osaka*, **10**, 29; cited in *Chem. Abstr.* **54**, 250 (35b).
- Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
- Kurland, C. G. (1960). *J. molec. Biol.* **2**, 83.
- Laskov, R., Margoliash, E., Littauer, U. Z. & Eisenberg, H. (1959). *Biochim. biophys. Acta*, **33**, 257.
- Lipsett, M. N., Heppel, L. A. & Bradley, D. F. (1961). *J. biol. Chem.* **236**, 857.
- Littauer, U. Z. (1960). In *Symposium on Protein Biosynthesis*, p. 143. Ed. by R. J. C. Harris. New York: Academic Press Inc.
- Littauer, U. Z. & Eisenberg, H. (1959). *Biochim. biophys. Acta*, **32**, 320.
- McCarthy, B. J. & Aronson, A. I. (1961). *Biophys. J.* **1**, 227.
- Marmur, J. & Lane, D. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 453.
- Marmur, J., Rownd, R. & Schildkraut, C. L. (1963). In *Progress in Nucleic Acid Research*, p. 231. Ed. by Davidson, J. N. & Cohn, W. E. New York: Academic Press Inc.
- Monier, R., Naono, S., Hayes, D., Hayes, F. & Gros, F. (1962). *J. molec. Biol.* **5**, 311.
- Nirenberg, M. W. & Matthaei, J. H. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1588.
- Ofengand, J. & Haselkorn, R. (1961). *Biochem. biophys. Res. Commun.* **6**, 469.
- Ortiz, P. J. & Ochoa, S. (1958). *J. biol. Chem.* **234**, 1208.
- Otaka, E., Mitsui, H. & Osawa, S. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 425.
- Petersen, G. B. (1961). *Biochim. biophys. Acta*, **51**, 212.
- Reichmann, M. E. & Stace-Smith, R. (1959). *Virology*, **9**, 710.
- Schildkraut, C. L., Marmur, J. & Doty, P. (1961). *J. molec. Biol.* **3**, 595.
- Schildkraut, C. L., Marmur, J., Fresco, J. R. & Doty, P. (1961). *J. biol. Chem.* **236**, 2.
- Schweet, R., Lamfrom, H. & Allen, E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 1029.
- Sela, M., Anfinsen, C. B. & Harrington, W. F. (1957). *Biochim. biophys. Acta*, **26**, 506.
- Shapiro, H. S. & Chargaff, E. (1960). *Nature, Lond.*, **188**, 62.
- Simha, N. & Zimmerman, J. M. (1962). *J. theoret. Biol.* **2**, 87.
- Singer, B. & Fraenkel-Conrat, H. (1961). *Virology*, **14**, 59.
- Spahr, P. F. & Tissières, A. (1959). *J. molec. Biol.* **1**, 257.
- Spencer, M., Fuller, W., Wilkins, M. H. F. & Brown, G. L. (1962). *Nature, Lond.*, **194**, 1014.
- Spirin, A. S. (1960). *J. molec. Biol.* **2**, 436.
- Spirin, A. S. (1961a). *Biokhimiya*, **26**, 454.
- Spirin, A. S. (1961b). *Colloques Internationaux du Centre National de la Recherche Scientifique*, no. 106, *Acides Ribonucléiques et Polyphosphates: Structure, Synthèse et Fonctions*, Strasbourg, p. 74. Paris: Centre National de la Recherche Scientifique.
- Steiner, R. F. (1960). *J. biol. Chem.* **235**, 2946.
- Tashiro, Y., Shimidzu, H., Inouye, A. & Kakiuchi, K. (1960). *Biochim. biophys. Acta*, **43**, 544.
- Timasheff, S. N., Brown, R. A., Colter, J. S. & Davies, M. (1958). *Biochim. biophys. Acta*, **27**, 662.
- Timasheff, S. N., Witz, J. & Luzzatti, V. (1961). *Biophys. J.* **1**, 525.

- Ts'O, P. O. P. & Squires, R. (1959). *Fed. Proc.* **18**, 341.
Volkin, E. & Astrachan, L. (1956a). *Virology*, **2**, 149.
Volkin, E. & Astrachan, L. (1956b). *Biochim. biophys. Acta*, **29**, 544.
Volkin, E., Astrachan, L. & Countryman, J. L. (1956). *Virology*, **6**, 545.
Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1516.
Wallace, J. M. & Ts'O, P. O. P. (1961). *Biochem. biophys. Res. Commun.* **5**, 125.
Warner, R. C. & Breslow, E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **9**, 157.
Wood, W. B. & Berg, P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 94.
Yankofsky, S. K. & Spiegelman, S. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1466.

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Avidin

1. THE USE OF [¹⁴C]BIOTIN FOR KINETIC STUDIES AND FOR ASSAY

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The recent demonstration of the role of biotin in a number of carboxylation reactions has led to a renewed interest in the coenzyme function of this vitamin. Avidin, the biotin-binding protein from egg white, has been found to inhibit many of the carboxylating systems and has been widely used in the characterization of biotin-containing enzymes. Although avidin has been considerably purified and its mode of action studied in some detail (Fraenkel-Conrat, Snell & Ducay, 1952), pure preparations have not been conveniently available and the nature of the biotin-binding site has remained obscure, so that reinvestigation of this protein appeared desirable. The assay of avidin has hitherto been based on the sensitive but time-consuming microbiological assay of biotin. A rapid method employing radioactive biotin has therefore been devised. Preliminary kinetic measurements were made on the reaction of avidin with biotin to establish the best conditions for the determination and these limited results are also presented here.

MATERIALS AND METHODS

[¹⁴C]Carbonyl chloride. ¹⁴CO (1 mc; 0.17 m-mole) was supplied by The Radiochemical Centre, Amersham, Bucks., in a tube with a break seal. This tube was sealed to a male B14 joint to which a test tube containing chlorine could be attached. The desired partial pressure of dry redistilled chlorine (0.41 m-mole) was transferred to this test tube on a vacuum line. The chlorine was frozen out with liquid air, and dry nitrogen admitted to the apparatus. The cooled chlorine tube was quickly attached to the CO tube, evacuated (10⁻³ mm.) through a side arm and sealed off. The seal was broken with a glass-covered steel ball that had

been retained by a magnet in a second side arm. The apparatus was irradiated for 90 min. with nine 60 w tungsten lamps. The ¹⁴COCl₂ and excess of Cl₂ were condensed in the test tube with liquid air and transferred to a vacuum line. The condensed gases were distilled into a tube containing amalgamated copper turnings and allowed to react for 1 hr. to remove all excess of chlorine. The ¹⁴COCl₂ was distilled back to the original tube together with a small amount of mercury which formed a mirror. This tube was cooled to -78° at which temperature the carbonyl chloride could be distilled into a third tube, cooled in liquid air. Dry nitrogen was admitted to the apparatus, the tube was removed from the vacuum line and the ¹⁴COCl₂ was dissolved in 1 ml. of ice-cold anhydrous chloroform.

D-[2',-¹⁴C]Biotin. The sulphate of δ-(3,4-diaminothiophan-2-yl)pentanoic acid was prepared from D-biotin by hydrolysis with baryta (Hofmann, Melville & Du Vigneaud, 1941) and 150 mg. was esterified with methanolic HCl. The methyl ester dihydrochloride was converted quantitatively into the free diamino ester by running a solution in anhydrous methanol through a column (6 cm. × 0.8 cm.) of Dowex 2 (OH⁻ form; 200 mesh) in anhydrous methanol. The methanolic solution was evaporated to dryness *in vacuo*. The gum was redissolved in 1–2 ml. of anhydrous chloroform and again evaporated to dryness *in vacuo*. This was repeated once more. The diamino ester (80 mg.; 0.42 m-mole) began to crystallize on the walls of the flask and was used immediately to avoid losses by self-condensation. It was dissolved in 2 ml. of chloroform and added to the solution of ¹⁴COCl₂ described above. After standing overnight the solution was transferred to a flask, evaporated to dryness and the biotin methyl ester hydrolysed with 1.5 ml. of 1.6N-NaOH. After warming (at 40°) a cloudy solution was obtained which was kept for 3 hr. at room temperature before the slight precipitate was centrifuged down. The supernatant and washings were concentrated to 1–2 ml. and conc. HCl (0.3 ml.) was added slowly. The biotin crystallized and was filtered and washed with water. It was recrystallized from hot water and freed from a minor radioactive impurity by chromatography on a column (1.6 cm. × 20 cm.) of Dowex 1 (formate form; 200–400 mesh) in aq. 50% (v/v) ethanol. The biotin was eluted as a single sharp

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