Synthesis of Ribonucleic Acid in Normal Bone *in vitro*

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1. The incorporation of [2-¹⁴C]uridine into nucleic acids of bone cells was studied in rat and pig trabecular-bone fragments surviving *in vitro*. 2. The rapid uptake of uridine into trichloroacetic acid-soluble material, and its subsequent incorporation into a crude nucleic acid fraction of bone or purified RNA extracted from isolated bone cells, was proportional to uridine concentration in the incubation medium over a range 0.5–20.0 μ M. 3. During continued exposure to radioactive uridine, bulk RNA became labelled in a curvilinear fashion. Radioactivity rapidly entered nuclear RNA, which approached its maximum specific activity by 2 hr. of incubation; cytoplasmic RNA, and particularly microsomal RNA, was more slowly labelled. The kinetics of labelling and rapid decline of the nuclear/microsomal specific activity ratio were consistent with a precursor-product relationship. 4. Bulk RNA preparations were resolved by zonal centrifugation in sucrose density gradients into components with approximate sedimentation coefficients 28s, 18s and 4s. 5. Rapidly labelled RNA, predominantly nuclear in location, demonstrated a polydisperse sedimentation pattern that did not conform to the major types of stable cellular RNA. Material of highest specific activity, sedimenting in the 4–18s region and insoluble in 10% (w/v) sodium chloride, rapidly achieved its maximum activity during continued exposure to radioactive precursor and decayed equally rapidly during 'chase' incubation, exhibiting an average half-life of 4.3 hr. 6. Ribosomal 28s and 18s RNA were of lower specific activity, which increased linearly for at least 6 hr. in the continued presence of radioactive uridine. There was persistent but variable incorporation into ribosomal RNA during 'chase' incubation despite rapid decline in total radioactivity of the acid-soluble pool containing RNA precursors.

The activity of fragments of trabecular bone surviving *in vitro* and the nature of several specific metabolic processes that characterize this tissue preparation have been reported previously from this Laboratory (Borle, Nichols & Nichols, 1960*a,b*; Vaes & Nichols, 1961; Flanagan & Nichols, 1962, 1964). Several functions unique to bone and perhaps to other closely related tissues, including the biosynthesis of collagen (Flanagan & Nichols, 1962, 1964), the production of metabolic acids, citrate and lactate (Nichols, 1963), and collagenolytic activity (Woods & Nichols, 1965), as well as the derangements seen in human hyperparathyroidism (Flanagan & Nichols, 1965) and osteoporosis (Griffith, Nichols, Asher & Flanagan, 1965), have been examined in this system. The large number of morphological (Heller, McLean & Bloom, 1950) and metabolic (see Nichols, Flanagan & Woods, 1965) changes in bone after parathyroid hormone treatment, and the recent inference that at least one of the biological effects of the hormone, namely the delayed hypercalcaemic response, is dependent on

the formation of new RNA (Rasmussen, Arnaud & Hawker, 1964; Tashjian, 1965) has pointed to the need for a direct examination of RNA synthesis in bone.

It is the purpose of the present paper to describe the normal pattern of RNA synthesis in surviving bone fragments *in vitro*, and the characteristics of labelling and subcellular localization of its components. In particular it is shown below that a species of RNA present both in nuclei and microsomes demonstrates kinetic and sedimentation properties consistent with those of m-RNA* and exhibits a half-life of approx. 4 hr.

MATERIALS AND METHODS

Chemicals. Uridine (crystalline A grade) was supplied by Calbiochem, Los Angeles, Calif., U.S.A. [2-¹⁴C]Uridine (sp. activity 30 μ C/ μ mole) was obtained from New England

* Abbreviations: m-RNA, messenger RNA; r-RNA, ribosomal RNA; t-RNA, transfer RNA.

Nuclear Corp., Boston, Mass., U.S.A. Dextran (average mol. wt. 144000) was purchased from R. K. Laros Co., Bethlehem, Pa., U.S.A. Deoxyribonuclease (type I, once-crystallized) from Worthington Biochemical Corp., Freehold, N.J., U.S.A., was assayed before use and shown to be free of ribonuclease activity by its failure to release acid-soluble u.v.-absorbing products from RNA after 30 min. incubation at 37°. DNA (calf thymus type I, highly polymerized sodium salt) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and yeast RNA from Worthington Biochemical Corp. Phenol was purchased from Fisher Scientific Co., Pittsburgh, Pa., U.S.A., as a colourless liquid and was not purified before use. All other chemicals were reagent grade from commercial sources.

Animals and tissue preparation. Male albino rats, weighing 150–180 g., obtained from Charles River Laboratories, Wilmington, Mass., U.S.A., and Camm Research Institute, Wayne, N.J., U.S.A., were maintained on Lab-Blox (Allied Mills Inc., Chicago, Ill., U.S.A.) and tap water. The animals were killed by decapitation and metaphyseal bone from the upper tibia and lower femur was prepared as described by Borle *et al.* (1960a). Piglets aged 3–4 weeks, obtained from Harvard University Animal Research Centre, were killed by stunning and exsanguination. Knee joints were excised, chilled and split longitudinally to expose the trabecular bone of distal femur and proximal tibia. Epiphyses were readily shelled out of encasing articular cartilage by blunt dissection, epiphyseal cartilage was cut away and the trabecular bone removed by curettage. All bone was finely diced, collected in ice-cold Krebs–Ringer bicarbonate medium (Cohen, 1957), containing 11 mm-glucose, pH 7.4, and washed vigorously with three changes of medium. The yield was approx. 250 mg./rat or 25–30 g./pig.

Incubation. Weighed quantities of minced bone were placed in Erlenmeyer flasks containing Krebs–Ringer bicarbonate medium (see above) (10 ml./g. of bone) plus [2-¹⁴C]uridine (0.30 μ C to 0.60 μ C/ml.). Incubations were carried out in a Dubnoff metabolic incubator at 37° and 80 oscillations/min. under an atmosphere of O₂ + CO₂ (95:5) for periods ranging from 20 min. to 6 hr. In 'chase' experiments, after a 1 hr. labelling period *in vitro*, incubation medium was decanted, the bone rapidly chilled and washed with two changes of ice-cold Krebs–Ringer bicarbonate medium (see above) containing 100-fold concentration (1 mM) of non-radioactive uridine ('chase' medium). A portion of the tissue pool was retained as the zero-time sample, and the remainder was divided into equal portions, each of which was blotted, weighed and reincubated in 'chase' medium (10 ml./g.) for intervals up to 6 hr.

Cell isolation, homogenization and fractionation. At the end of the incubation the medium was decanted and the flask contents were rapidly chilled and washed with two changes of the appropriate iced (Krebs–Ringer bicarbonate or 'chase') medium. All subsequent operations were performed at 2–4°. Bone cells were isolated by the method of Nichols *et al.* (1965) with minor modifications. Briefly, bone fragments were hand-ground for 3 min. in a mortar containing 5 vol. of 0.15 M-NaCl–1.25 mM-CaCl₂–1.25 mM-phosphate buffer, pH 7.4. Grinding medium was decanted and the operation repeated twice more. Calcified debris was allowed to settle in the decanted medium for 20 min., leaving the released cells in the supernatant. Cells were then harvested by centrifugation at 750 g for 15 min.

Approx. 44% of total bone DNA is recovered by this means and there is essentially no contamination by bone matrix. Cells were resuspended in a measured volume of 0.25 M-sucrose–1 mM-MgCl₂–10 mM-tris buffer, pH 7.4 (sucrose medium), and equilibrated with N₂ at 800 lb./in.² in a chilled high-pressure chamber (Artisan Industries Inc., Waltham, Mass., U.S.A.). Cell disruption was accomplished by intracellular cavitation of N₂ on release to atmospheric pressure (Hunter & Commerford, 1961).

Nuclei were isolated by a modification of the method of Allfrey, Littau & Mirsky (1964). Cell homogenates were layered over 0.5 vol. of a barrier solution consisting of 30% (w/v) dextran in sucrose medium, pH 6.8, and nuclei were sedimented by centrifugation at 750 g for 15 min. The supernatant cytoplasmic fraction was removed; the interface material, consisting largely of cell debris and nuclei with adherent cytoplasmic tags, was discarded along with the dextran solution. The tubes were wiped dry, and the nuclei rinsed and resuspended in sucrose medium. Microsomes derived from crude cytoplasm by centrifugation of the post-mitochondrial (15000 g for 20 min.) supernatant for 2 hr. at 105000 g in the Spinco L-HV centrifuge (no. 40 rotor), were resuspended in sucrose medium.

Preparation of RNA. When total uridine incorporation was measured in preliminary experiments, metabolic activity was stopped at the end of incubation by plunging the flasks into ice and adding an equal volume of cold 20% (w/v) trichloroacetic acid. The cold-acid extract was discarded and residual bone fragments were treated by the method of Schneider (1957) to isolate a nucleic acid fraction. Total incorporated nucleic acid radioactivity was related to the cell content of the tissue and expressed as counts/min./mg. of DNA.

In all other experiments, bone cells were isolated and homogenized before further treatment. A sample of cell homogenate was removed for simultaneous nucleic acid fractionation by the procedure of Fleck & Munro (1962). Homogenates or organelle suspensions were then made 0.5% with respect to sodium dodecyl sulphate and shaken for 10 min. An equal volume of 90% phenol–0.1% 8-hydroxyquinoline was added and the mixture shaken at 4° for 30 min. After centrifugation at 1200 g for 20 min. the aqueous and interface material were removed and re-extracted for 15 min. with 0.5 vol. of phenol. The mixture was again centrifuged and the aqueous phase carefully removed, made 0.25 M with respect to sodium acetate, pH 5.3, then 75% (v/v) with ethanol, and the solution stored overnight at –25°. The resultant precipitate was collected by centrifugation, suspended in 0.02 M-MgSO₄–0.02 M-tris buffer, pH 7.4, and treated with deoxyribonuclease (10 μ g./ml.) for 20 min. at room temperature with occasional stirring. The solution, considerably cleared and reduced in viscosity, was then made 0.5% with respect to sodium dodecyl sulphate, and residual protein was removed by extraction with 0.5 vol. of phenol for 15 min. The mixture was centrifuged and the aqueous supernatant phase treated as before. After 90 min. at –25° the nucleic acid precipitate was collected by centrifugation, dissolved in 0.25 M-sodium acetate, pH 5.3, and made 2 M with respect to potassium acetate. Deoxyribonucleotides were removed by the procedure of Hiatt (1962). The final ethanol-washed precipitate of purified RNA was dissolved in a small volume of 0.25 M-sodium acetate, pH 5.3.

In several experiments low-molecular-weight RNA was

separated by treatment with 10% (w/v) NaCl at 4° for 24–36 hr. (Crestfield, Smith & Allen, 1955). In these, sedimented high-molecular-weight RNA was washed twice with ethanol and dissolved in acetate buffer. Salt-soluble RNA was precipitated from the separated extract by addition of 3 vol. of ethanol, similarly washed and dissolved.

Final RNA solutions were stored at –25° until further use. Ultraviolet-extinction measurements demonstrated a single homogeneous peak at 258 m μ , with an E_{260}/E_{280} ratio (1.9–2.0) characteristic of nucleic acid. No deoxyribose could be detected by the diphenylamine method (Dische, 1930). RNA was estimated quantitatively on the basis of an extinction coefficient $E_{1\text{cm}}^{1\%}$ 224 at 258 m μ . RNA recoveries were consistently 70% based on simultaneous quantitative assays of cell homogenates. In several experiments, attempts were made to improve the recovery of RNA by extracting the initial two phenolic residues with an equal volume of 1 mM-MgCl₂–10 mM-tris buffer at 60° for 5 min. Only small additional amounts (range 5–15%) of RNA of essentially the same specific activity as the cold-extracted material were recovered by this means. In addition, the extinction pattern of this material on sedimentation analysis was unpredictably varied, presumably indicating degradation, and the hot-phenol extraction procedure was not pursued.

Sucrose-density-gradient centrifugation. Linear 5–20% (w/v) sucrose gradients in 0.25 M-sodium acetate, pH 5.3, were prepared with the device described by Wallach & Kamat (1964). A solution containing 100–500 μ g. of RNA in 0.2 ml. of acetate buffer was layered over 4.8 ml. and centrifuged at 4° for 3½ hr. at 39 000 rev./min. in the Spinco model L-HV centrifuge (SW 39 rotor). At the end of the run the bottom of the tube was punctured with a no. 25 gauge hypodermic needle. Successive 3-drop fractions were collected, diluted by the addition of 1.0 ml. of distilled water and the extinction and total radioactivity of each were measured.

Measurement of radioactivity. All counting was done in an automatic liquid-scintillation spectrometer (Packard Tri-Carb model 314E, machine efficiency for ¹⁴C 70–72%). Aqueous samples were added to 15 ml. of scintillation fluid consisting of 7 g. of 2,5-diphenyloxazole, 50 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 50 g. of naphthalene/l. of 1,4-dioxan. Acid extracts of bone or cell homogenates were counted in Bray's (1960) solution. Samples were corrected for quench by internal standardization, recoveries ranging from 90 to 97%. Diluted sucrose gradient fractions (1.2 ml.) were consistently 93% recoverable and quenching was monitored by the two-channel discriminator ratio method.

Chemical procedures. Ultraviolet-extinction measurements were done in a Zeiss model PMQII spectrophotometer. RNA was measured by the orcinol method (Mejbaum, 1939) after extraction by the procedure of Fleck & Munro (1962). DNA was measured by the diphenylamine method (Dische, 1930) after extraction by the procedure of Schneider (1957).

RESULTS

The feasibility of examining RNA synthesis *in vitro* in freshly isolated trabecular bone was demonstrated in preliminary experiments, of which an example is shown in Fig. 1. The incorporation

Fig. 1. Incorporation of [¹⁴C]uridine into the nucleic acid fraction of trabecular bone. Pig bone fragments (approx. 250 mg./flask) were incubated in duplicate in Krebs–Ringer bicarbonate medium containing [¹⁴C]uridine (sp. activity 30 μ C/ μ mole, 0.15 μ C/ml.). Metabolic activity was stopped at the indicated times by chilling and addition of an equal volume of cold 20% (w/v) trichloroacetic acid. Total radioactivity in the nucleic acid extract is related to DNA as an index of cell number.

of [¹⁴C]uridine into the nucleic acid fraction of incubated bone fragments was rapidly established at a linear rate and continued for at least 4 hr. The observation that the process was even more rapid during the initial few minutes suggested that exogenous nucleoside readily gained access to the intracellular precursor pool, where it underwent the required conversion for entry into nucleic acid. These findings are in good agreement with radioautographic evidence in rabbit bone 'pulse'-labelled *in vivo* (Owen, 1967).

Although little is known of the mechanism of entry of exogenous nucleoside into the intracellular pool in mammalian tissues (Graham & Rake, 1963), it has been suggested that this may be a diffusion-related process, since the initial rapid uptake into acid-soluble precursor material has been shown to be concentration-dependent in HeLa cells (Feinendengen, Bond & Hughes, 1961). To ascertain whether a similar concentration-dependence could be shown in the present experiments, cells isolated from bone slices that had been incubated over a range of uridine concentration from 0.5 to 20 μ M were homogenized. The total radioactivity present in a cold-trichloroacetic acid-soluble precursor pool

Fig. 2. Dependence of uridine incorporation on uridine concentration in the medium. (a) 4 hr. incorporation of radioactivity into total nucleic acid. Pig trabecular-bone fragments (250 mg./flask) were incubated in triplicate in Krebs-Ringer bicarbonate medium containing [^{14}C]uridine ($0.30\text{ m}\mu\text{C}-0.15\text{ }\mu\text{C}/\text{ml.}$) adjusted to the indicated concentrations by dilution with non-radioactive uridine. Nucleic acid total radioactivity, calculated as in Fig. 1, is corrected for initial uridine sp. activity (range $0.3-30.0\text{ }\mu\text{C}/\mu\text{mole}$). Vertical bars indicate $\pm$ s.e.m. (b) Specific activity of purified RNA as a function of external uridine concentration. Rat metaphyseal bone fragments were incubated for 4 hr. in Krebs-Ringer bicarbonate medium containing [^{14}C]uridine at constant sp. activity ($30\text{ }\mu\text{C}/\mu\text{mole}$) over a range of concentrations $0.15-0.60\text{ }\mu\text{C}/\text{ml.}$ ($5-20\text{ }\mu\text{M}$). Purified RNA was isolated from cell homogenates. The straight line was fitted by the method of least squares and has a slope $b\ 1.64 \times 10^{-4}$, coefficient of correlation $r\ 0.94$, and y -intercept $16.9 \pm \text{s.e.m. } 11.5$, which is not significantly different from zero ($t\ 1.47$, $P > 0.1$).

at the end of 1 hr. proved to be directly related to the uridine uptake/mg. of incubated bone, which in turn reflected the initial medium concentration of uridine ($n\ 16$, $r\ 0.7$, $P < 0.01$). Moreover, in six experiments of longer duration (up to 4 hr.), soluble radioactivity remained at an average $95 (\pm \text{s.d. } 18)\%$ of the 1 hr. value, indicating maximum labelling and relative stability of this acid-soluble pool. In keeping with increased labelling of the precursor pool at higher concentrations of uridine in the medium, the 4 hr. radioactivity incorporated into total nucleic acid (Fig. 2a) and the specific activity of purified RNA isolated from cell homogenates (Fig. 2b) were similarly proportional to external uridine concentration.

Pattern of labelling of bulk RNA. The rise in specific activity of purified bulk RNA extracted from isolated bone-cell homogenates demonstrated a characteristic time-course *in vitro*. The mean results shown in Fig. 3 are representative of individual experiments. In each case there was a rapid initial rise in specific activity followed by a slower increase beyond 2 hr. of incubation. The

curvilinear nature of this function suggested that the labelling of bulk RNA is a composite process divisible into at least two kinetic components or compartments.

The labelling pattern of component RNA species was further examined after their separation by zonal centrifugation in sucrose density gradients. By this means, RNA extracted from bone cells could be clearly resolved into three major components that demonstrated sedimentation coefficients similar to those reported for other mammalian tissues (Petermann, 1964). Approximate values were derived by analogy with liver RNA as follows. The three extinction and radioactivity peaks of purified bone RNA were coincident (Fig. 4a) after incubation for 4 hr. in the presence of [^{14}C]uridine. In the experiment shown in Fig. 4(b), $40\text{ }\mu\text{g.}$ of such highly labelled bone RNA was added to $500\text{ }\mu\text{g.}$ of simultaneously prepared non-radioactive rat liver RNA. When this mixture was sedimented in a 5–20% (w/v) sucrose gradient, the radioactivity (bone) and extinction (liver) peaks were coincident. Values of 28s, 18s and 4s, which have been reported

Fig. 3. Specific activity of bulk RNA as a function of incubation time. Rat metaphyseal-bone fragments (2-3 g./flask) were incubated in the presence of [^{14}C]uridine (0.3 $\mu\text{C}/\text{ml.}$). Cells were isolated at the indicated times and RNA was prepared from homogenates. Numbers in parentheses indicate the numbers of experiments and vertical bars the $\pm$ S.E.M.

previously for liver (Hall & Doty, 1959), and which correspond to the two components of r-RNA and t-RNA respectively, were assigned on this basis. Moreover, the mass ratio of 28s to 18s r-RNA, calculated from the areas under the respective peaks, consistently ranged from 2.2 to 2.4, and is in good agreement with similar values reported for undegraded r-RNA of rat liver (Stæhelin, Wettstein, Oura & Noll, 1964; Trakatellis, Axelrod & Montjar, 1964) and Ehrlich ascites-tumour cells (Huppert & Pelmont, 1962).

In view of the rapidity of labelling of the trace amounts of RNA sedimenting in the 4-18s area (see below), and to characterize the kinetics of labelling of this material without confusion due to the relatively large amounts of radioactivity in adjacent 4s and lighter material (presumably t-RNA and other soluble products), the reliability of using the average 4-18s specific activity was examined. Several preparations of radioactive bone RNA were fractionated by treatment with 10% (w/v) sodium chloride (Crestfield *et al.* 1955) before comparison by sedimentation analysis. As shown in the example (Fig. 5), 28s and 18s components from the starting material (Fig. 5a) could be quantitatively recovered in the salt-precipitable

Fig. 4. Derivation of sedimentation coefficients of bone RNA. (a) Rat metaphyseal-bone fragments were incubated for 4 hr. in Krebs-Ringer bicarbonate medium containing [^{14}C]uridine (0.3 $\mu\text{C}/\text{ml.}$). Purified RNA prepared from the cell homogenate was layered over a linear 5-20% (w/v) sucrose gradient and centrifuged as described in the text. Sedimentation is towards the left: ●, E_{258} ; ○, radioactivity. (b) Radioactive bone RNA (40 $\mu\text{g.}$) from (a), together with 500 $\mu\text{g.}$ of non-radioactive rat liver RNA prepared simultaneously, were layered over a linear 5-20% (w/v) sucrose gradient and sedimented together as described in the text: ●, E_{258} ; ○, radioactivity.

Fig. 5. Effect of salt fractionation. Rat metaphyseal-bone fragments were incubated for 1 hr. as in Fig. 3 and bulk RNA (a) was prepared from the cell homogenate. A portion was treated with 10% (w/v) NaCl as described in the Materials and Methods section and separated into salt-precipitable (b) and salt-soluble (c) fractions. Each was applied to a linear 5–20% (w/v) sucrose gradient and centrifuged as described in the text. ●, E_{258} ; ○, radioactivity. Specific activity profiles of bulk (▲) and salt-precipitated (△) RNA are shown in (d).

fraction (Fig. 5b) and their specific activities were unchanged (Fig. 5d). The 4s extinction peak was eliminated, together with a portion of the radioactivity that previously had been sedimenting in that area (compare Figs. 5b and 5a); both could be recovered in the salt-soluble fraction (Fig. 5c). Specific-activity profiles (Fig. 5d), other than showing a slight displacement of the fractions < 18s after salt precipitation, were essentially similar. When average specific activities (ratio of total counts/min. to total extinction) were calculated for each of the areas indicated by the horizontal bar (see Figs. 5a and 5b), no significant difference in specific activity of the 4–18s component of bulk RNA compared with its salt-precipitated < 18s counterpart could be demonstrated in 15 paired salt-fractionated RNA preparations (mean difference 0.03%, $t=0.462$, $P>0.7$). It was thus considered justifiable in subsequent experiments to characterize the kinetic behaviour of 4–18s RNA in terms of its average specific activity calculated from the ratio of total radioactivity to total extinction without further purification.

Sedimentation analysis of the labelling pattern of bulk bone RNA during incubation for 6 hr. is

shown in Fig. 6, and the mean specific-activity results for all experiments in Fig. 7. Significant labelling, demonstrable as early as 30 min., did not conform to the major RNA components (Fig. 6a). This early labelled RNA sedimented in a heterogeneous fashion, indicating a spectrum of molecular sizes and configurations. The material of highest specific activity sedimented in the region 4–18s and comprised approx. 60% of the total radioactivity. By 2 hr. 4–18s RNA was labelled to a maximum (Figs. 6c and 7) and there was little change in specific activity thereafter. A continued rise in the specific activities of 28s and 18s RNA occurred with time, although 4–18s material remained relatively more highly labelled. The radioactivity pattern, which initially was polydisperse, progressively conformed to the extinction pattern representing the bulk of stable RNA (Figs. 6d and 6e). In all cases the labelling of 18s RNA was greater than that of 28s RNA, the ratio of their specific activities declining from 2.7 (30 min.) to 1.4 (6 hr.). This time-course of labelling *in vitro* was qualitatively similar to observations made in several mammalian systems both *in vitro* (Darnell, Penman, Scherrer & Becker, 1963; Cline, 1966) and

Fig. 6. Sedimentation analysis of bulk bone RNA isolated at various times during continuous labelling with [^{14}C]uridine. Rat metaphyseal-bone fragments were incubated as in Fig. 3 for (a) 0.5 hr., (b) 1 hr., (c) 2 hr., (d) 4 hr. and (e) 6 hr. RNA prepared from cell homogenates was layered over linear 5–20% (w/v) sucrose gradients and centrifuged as described in the text. Specific-activity profiles recorded in the lower panels were derived from the ratio of counts/min. to E_{258} for each fraction. ●, E_{258} ; ○, radioactivity; ▲, specific activity.

Fig. 7. Kinetics of labelling of component RNA species. Cell homogenates were analysed as in Fig. 6. Specific activities of 28s and 18s RNA are the values at the respective extinction peaks. Average specific activity of the 4–18s region was calculated in each instance from the ratio of counts/min. to E_{258} for the area indicated by the horizontal bar. Numbers in parentheses indicate the numbers of experiments and vertical bars the $\pm$ s.e.m.

in vivo (Staehelin *et al.* 1964; Trakatellis *et al.* 1964; Posner & Fanburg, 1966). The findings indicate the presence of a rapidly labelled polydisperse component, the kinetic behaviour of which is distinctly different from that of the bulk of cellular RNA.

Pattern of labelling of subcellular fractions. The subcellular location of this rapidly labelled component was sought by examining the time-interrelations of nuclear RNA and cytoplasmic RNA synthesis. Bone-cell homogenates were separated into nuclear and cytoplasmic fractions by sedimentation through a dextran barrier (Allfrey *et al.* 1964). Isolated nuclei examined by light microscopy and phase-contrast microscopy consisted of single spherical nuclei with preserved chromatin structure and nucleoli, and were free from adherent cytoplasmic contaminants as judged by differential staining with aceto-orcein–fast green (Kurnick & Ris, 1948).

RNA labelling patterns of dextran-purified nuclear and crude cytoplasmic extracts were compared in preliminary experiments. Nuclear RNA showed an initial rapid rise, followed by a later slower rise, in specific activity. Crude cytoplasmic RNA was consistently lower and approached nuclear specific activity by 4 hr. Under these conditions the nuclear/cytoplasmic specific-activity ratio in seven experiments was greatest at 2 hr. (1.40 ± 0.07 s.e.m.) and declined subsequently towards unity in keeping with the expected precursor-product relationship.

Fig. 8. Kinetics of labelling of subcellular fractions. Nuclei were isolated from cell homogenates by sedimentation through a dextran barrier. Microsomes were obtained from crude cytoplasm by centrifugation of the 15000g supernatant at 105000g for 2 hr. Nuclear (●) and microsomal (○) RNA (a) were prepared as described in the text. ▲, Nuclear/microsomal specific-activity ratio (b).

RNA extracted from crude cytoplasm would be expected to include highly labelled 4s and lighter material (see Fig. 5). Since the bulk of cytoplasmic RNA is ribosomal (Hogeboom & Schneider, 1955), the inclusion of small quantities of such highly labelled material would yield a specific-activity determination for cytoplasmic RNA that might not be characteristic of its major components. Therefore a microsomal fraction was isolated from crude cytoplasm by differential centrifugation. In the experiments shown in Fig. 8, the time-courses of labelling of nuclear RNA and microsomal RNA were distinctly different. Whereas the specific activity of nuclear RNA rose rapidly, approaching a maximum by 2 hr., that of microsomal RNA rose in a continuous fashion throughout. The rapid labelling to a maximum of nuclear RNA, the delayed appearance of radioactivity in microsomal RNA and the rapid decline of the ratio of their specific activities towards unity (Fig. 8b) are compatible with the view summarized by Prescott (1964) that all RNA is of nuclear origin. The present results correspond to observations made in several mammalian tissues including bone (Owen, 1967), and the initially high nuclear/microsomal ratio (Fig. 8b) is of the same order of magnitude as

that reported for brain *in vivo* (Adams, 1966) and L-cells *in vitro* (Ennis, 1966).

Sedimentation analysis demonstrated that the 2 hr. and 4 hr. nuclear radioactivity patterns (Figs. 9a and 9b) were similar and largely polydisperse, with material of highest specific activity in the 4–18s region. As with bulk nuclear RNA, specific activity of the 4–18s component remained essentially constant during this time (Fig. 9c). The 28s extinction peak contained only a small proportion of the total radioactivity, which did not change significantly between 2 and 4 hr. (12% and 14% respectively). In contrast, although the early labelling pattern of microsomal RNA was also polydisperse, with high 4–18s specific activity (Fig. 9d), by 4 hr. (Fig. 9e) the pattern more closely resembled that of bulk cellular RNA (see Fig. 6d) in that ribosomal radioactivity and extinction profiles had become coincident, the 28s component containing 35% of the total radioactivity. Whereas microsomal 4–18s radioactivity comprised only a small proportion (less than 25%) of the total microsomal radioactivity, its specific activity was persistently higher than that of the major constituent 28s r-RNA and 18s r-RNA (Fig. 9f). The similarity of microsomal RNA and bulk cellular

Fig. 9. Sedimentation analysis of nuclear RNA and microsomal RNA. Nuclear RNA (upper panels) and microsomal RNA (lower panels) from one of the experiments depicted in Fig. 8 were prepared at 2 hr. (*a* and *d*) and 4 hr. (*b* and *e*) of incubation. Each was layered over a linear 5–20% (w/v) sucrose gradient, centrifuged and fractionated as described in the text. ●, E_{258} ; ○, radioactivity; specific activity at 2 hr. (△) and 4 hr. (▲).

RNA patterns by 4 hr. is in keeping with the predominantly cytoplasmic location of 28s r-RNA and 18s r-RNA. Thus it seems likely that the pattern of labelling of bulk homogenate RNA represents a composite picture, and that the relative contributions of early labelled (mostly nuclear) polydisperse RNA and ribosomal RNA change with time.

'Chase' experiments. To explore the kinetic behaviour of the rapidly labelled RNA further, several 'chase' experiments were performed with both rat and pig trabecular bone. After an initial 1 hr. 'pulse'-labelling period in the presence of [^{14}C]uridine, by which time 4–18s RNA labelling was approx. 75% complete (see Fig. 7), incubation medium was replaced by one containing a 100-fold higher concentration of non-radioactive uridine. Reincubation in this medium led to a sharp fall in the total radioactivity of the intracellular trichloroacetic acid-soluble precursor pool (Fig. 10). Although the major part of the change occurred rapidly, there was a continued exponential decline to a low of 20% by 6 hr.

The difficulty of establishing conditions of true 'chase' in mammalian cells either *in vivo* or *in vitro* has been extensively discussed (Feinendengen *et al.* 1961; Graham & Rake, 1963; Prescott, 1964; Watts, 1964) and is further illustrated in the present

experiments. The decay of acid-soluble radioactivity was accompanied by continued but variable incorporation of precursor into some RNA components (see Fig. 11 below) of lower specific activity (e.g. 28s r-RNA). Further, such continued incorporation has been shown *in vivo* (Owen, 1967) to represent an increasing proportion of the remaining soluble pool. Taken together these findings support the view that acid-soluble precursor undergoes a time-dependent change of form or 'compartmentation', rendering it relatively unavailable to external manipulations while proceeding sequentially along the pathway to RNA synthesis.

Serial sedimentation analyses of the 6 hr. 'chase' pattern of bulk RNA are shown in Fig. 11. The early polydisperse radioactivity profile underwent several changes.

First, RNA of maximum specific activity in the 4–18s region decayed rapidly in a simple exponential fashion (Fig. 12), with a rate constant $k = 0.177 \text{ hr.}^{-1}$ calculated by the method of least squares. The results of several similar experiments (Table 1) permitted the calculation of an average rate constant $k = 0.161 (\pm 0.016 \text{ s.e.m.}) \text{ hr.}^{-1}$, corresponding to an average half-life of 4.3 hr.

Secondly, definitive labelling of 28s and 18s

components was noted by 2 hr. (Fig. 11c) and the distribution of radioactivity became progressively more uniform with the passage of time so that, eventually, radioactivity and extinction profiles

coincided (Figs. 11d and 11e) much as in the continued presence of radioactive uridine. In the experiment illustrated, the 28s component showed a slight rise in specific activity from 0 to 6 hr. (400–600 counts/min./ E_{258} unit). Although the direction was generally the same, the extent of this specific-activity change proved quite variable in several additional experiments (Table 2), probably indicating continuing incorporation from an incompletely diluted precursor pool into the vast, relatively poorly labelled, pool of r-RNA (Watts, 1964).

Thirdly, the overlapping of radioactivity in the 4–18s and 18s regions, quite extensive at first (Fig. 11a), was gradually eliminated as a result of rapid 4–18s decay on the one hand, and definition of an 18s radioactivity peak on the other. The specific-activity time-course of 18s r-RNA, although generally of a decay nature (Table 2), again proved quite variable, reflecting the different relative contributions to total radioactivity in this area from each of the two component processes. The calculation of a decay rate for 18s r-RNA was therefore not considered to be meaningful in these experiments.

DISCUSSION

The attractive messenger hypothesis of Jacob & Monod (1961) requires the participation of cellular RNA species in the transfer of inherent genetic information to ribosomal sites of protein synthesis with the consequent elaboration of specific proteins

Fig. 10. Radioactivity of acid-soluble fractions at various times during 'chase' incubation. Pooled trabecular bone (approx. 15g. of rat bone or 25g. of pig bone) was incubated for 1 hr. in medium containing [14 C]uridine, then washed, divided into equal portions and reincubated in chase medium. Portions of cell homogenates were made 10% (w/v) with respect to cold trichloroacetic acid. Total acid-soluble radioactivity/ μ g. of DNA is expressed as a percentage of the initial value. Numbers in parentheses indicate the numbers of experiments and vertical bars the $\pm$ S.E.M.

Fig. 11. Sedimentation analysis of 'pulse'-labelled RNA isolated at various times during 6 hr. 'chase' incubation. Rat trabecular bone (15g.) was incubated for 1 hr. in medium containing [14 C]uridine (0.3 μ C/ml.). A portion was retained as the zero-time sample (a), and equal portions of the remainder were reincubated in non-radioactive medium containing 1 mM-uridine for (b) 1 hr., (c) 2 hr., (d) 4 hr. and (e) 6 hr. Bulk RNA was prepared from cell homogenates, centrifuged and fractionated as described in the text. $\bullet$, E_{258} ; $\circ$, radioactivity; $\blacktriangle$, specific activity.

Fig. 12. Decay of 4-18s RNA during 'chase' incubation. Average specific activities were calculated for each of the areas indicated by the horizontal bars in Fig. 11 and have been plotted on a logarithmic-linear scale. The slope b was calculated by the method of least squares and the rate constant k from the relationship $k=b/\log e$.

Table 1. Decay of 4-18s RNA during 'chase' incubation

Trabecular-bone fragments derived from rat (R1-4) and pig (P1-4) were incubated for 1 hr. in the presence of $[2-^{14}\text{C}]$ uridine ($0.25\text{--}0.60\mu\text{C}/\text{ml.}$) followed by 6 hr. 'chase' incubation with non-radioactive uridine as described in the Materials and Methods section and in the legend to Fig. 11. In each case the rate constant k of 4-18s specific-activity decay was calculated as described in Fig. 12.

Expt.	Initial sp. activity 4-18s RNA (counts/min./ E_{258})	k (hr. $^{-1}$)	r
R1	4282	-0.177	-0.98
R2	3647	-0.170	-0.88
R3	3621	-0.157	-0.96
R4	3677	-0.259	-0.98
P1	9945	-0.133	-0.96
P2	8635	-0.115	-0.91
P3	4449	-0.166	-0.98
P4	4456	-0.114	-0.98
Mean $\pm$ S.E.M.		-0.161 $\pm$ 0.016	

whose primary structures were encoded in DNA. These functional messenger molecules, representing only a few per cent of total cellular RNA, have been

assigned the following characteristics: high metabolic lability with rapid turnover, heterogeneity of molecular weights, formation of ribosomal complexes (polyribosomes), stimulation of amino acid incorporation in ribosomal systems *in vitro*, base composition reflecting that of homologous DNA and therefore capable of forming molecular hybrids when extensively annealed (Moldave, 1965).

The present experiments have been largely concerned with an examination of the labelling kinetics and sedimentation properties of component RNA species isolated from cell homogenates and subcellular fractions of bone. The detailed conditions of RNA extraction (temperature, buffers, detergents, ribonuclease inhibitors, deproteinizing agents etc.) are of paramount importance in determining the yield and physical characteristics of the final product (Kirby, 1964). The procedure followed in the present experiments resulted in uniform recovery, and was similar to those (Barondes, Dingman & Sporn, 1962; Hiatt, 1962; Mach & Vassalli, 1965) that have permitted the extraction from other tissues of a rapidly labelled component exhibiting several of the characteristics of m-RNA.

Sedimentation analysis demonstrates that the pattern of labelling at early incubation time is distinctly different from the extinction pattern representing the bulk of stable cellular RNA. Early labelled RNA is polydisperse, indicating a heterogeneity of molecular weights, and may be demonstrated both in nuclei and microsomes, although their specific activities differ. An increasing body of evidence supports the view that this polydisperse material, in particular the highly labelled trace quantities of RNA sedimenting in the 4-18s trough and to some extent overlapping with 18s r-RNA, consists of a spectrum of m-RNA molecules of various sizes (Staehelin *et al.* 1964; Trakatellis *et al.* 1964; Mach & Vassalli, 1965). The functional significance of this overlap and apparent association between rapidly labelled and r-RNA has been extensively investigated in several tissues, and, although in part attributable to m-RNA length, the additional possibility of covalent links or other intimate association has been proposed (DiGirolamo, Henshaw & Hiatt, 1964; Henshaw, Revel & Hiatt, 1965; McConkey & Hopkins, 1965; Hadjiolov, 1966).

Clearly, not all rapidly labelled RNA is m-RNA, and indeed in some mammalian cells much of the early labelled nuclear RNA consists of ribosomal precursors. We have been unable to demonstrate a rapidly labelled high-molecular-weight component such as that shown by Hiatt (1962) in liver nuclei and by Penman, Smith & Holtzman (1966) in HeLa nuclei, although it is conceivable that under other conditions of extraction similar material may be

Table 2. *Specific-activity changes in ribosomal RNA during 'chase' incubation*

Ribosomal RNA (18s and 28s) components from each of the eight experiments in Table 1 were obtained at the indicated times of 'chase' incubation. Specific activities (counts/min./ E_{258}) of peak fractions are expressed as percentages of the initial (0 hr. of 'chase') values. Mean values are given $\pm$ s.e.m. with the numbers of experiments in parentheses.

RNA component	'Chase' incubation (hr.)	% of initial sp. activity					
		0.5	1	2	3	4	6
18s	...	79 (2)	82 $\pm$ 5 (8)	79 $\pm$ 6 (8)	80 $\pm$ 6 (4)	69 $\pm$ 5 (8)	61 $\pm$ 8 (4)
28s	...	112 (2)	100 $\pm$ 5 (8)	102 $\pm$ 6 (8)	100 $\pm$ 1 (4)	105 $\pm$ 10 (8)	128 $\pm$ 20 (4)

demonstrable in bone cells. On the other hand, these cells, derived from the growing ends of long bones, are not in a state of exponential growth (cf. HeLa-cell studies of Penman *et al.* 1966), hence r-RNA synthesis may well be limited and the non-appearance of '45s type' r-RNA precursors entirely reasonable. In addition, there have been many demonstrations in tumour cells (Watson & Ralph, 1966; Hadjiolov, Venkow & Dolapchiev, 1965; Roberts, 1965) that the synthesis of rapidly labelled high-molecular-weight RNA is modified after transfer of the cells to a new environment. The turnover of r-RNA (Hirsch & Hiatt, 1966) and template activity of rapidly labelled RNA (DiGirolamo, DiGirolamo, Gaetani & Spadoni, 1966) in liver have also been shown to be influenced by variations in nutritional state. However, it seems unlikely that the absence of '45s-type' RNA in the present studies can be explained on a nutritional shift-down basis, for, although the incubation conditions *in vitro* (simple buffered salt media without added serum or other factors) are probably quite different from those which obtain *in vivo*, studies in progress of bone RNA 'pulse'-labelled in intact animals indicate that the patterns of labelling are essentially similar under both conditions. The suggestion that at least some rapidly labelled 4-18s RNA may represent breakdown products of larger molecular species (Galibert, Larsen, Lelong & Boiron, 1965; Henshaw *et al.* 1965) cannot be excluded by these experiments.

Many recent reports have emphasized the wide range of m-RNA stabilities in different living forms. In bacteria, m-RNA is characterized by a half-life of a few minutes (Levinthal, Keynan & Higa, 1962). In animal cells, on the other hand, the turnover rate is much lower and varies considerably among different tissues. Indeed, evidence is accumulating that several m-RNA species of different stabilities may exist even in the same cell (see review by Singer & Leder, 1966). In the present experiments, the highly labelled 4-18s material rapidly achieves its maximum specific activity during continued exposure to radioactive precursor or, conversely,

rapidly decays during 'chase' incubation. The average 4.3 hr. half-life of this component is in broad agreement with average m-RNA half-lives estimated for a host of mammalian tissues and tumour cells by techniques of pulse-labelling, decay of either polysomes or synthetic capacity for specific proteins after actinomycin (see Singer & Leder, 1966). Moreover, this value agrees closely with the half-lives that have been derived for collagen m-RNA in the mouse fibroblast (Bloom, Goldberg & Green, 1965) and embryonic chick tibia (Jeffrey, 1965). If the rapidly labelled polydisperse material we have demonstrated in bone cells does in part perform a messenger function, not only is the average half-life compatible, but also the range of sedimentation velocities would include that expected for collagen m-RNA. If the smallest collagen sub-unit is a peptide of about 250 amino acids, as proposed by Gallop (1964), or at the other extreme is a complete α -chain of mol.wt. 120 000 (Robertson, 1964), the required RNA template (mol.wt. 2.3×10^5 or 9.7×10^5 respectively) would have an approximate sedimentation coefficient derived by the formulation of Hall & Doty (1958) of 9s or 18s respectively, which is well within the present demonstrated range.

In all cases, both during continuous labelling and during 'chase' incubation, 18s RNA was of higher specific activity than 28s RNA, as has been found in liver (DiGirolamo *et al.* 1964; Revel & Hiatt, 1964; Staehelin *et al.* 1964; Trakatellis *et al.* 1964; Hadjiolov, 1966), brain (Jacob, Samec, Stevenin, Garel & Mandel, 1967) and HeLa cells (Penman *et al.* 1966; Zimmerman & Holler, 1967). That this rapid labelling of 18s relative to 28s r-RNA is more apparent than real has been discussed by Staehelin *et al.* (1964). Taken at face value, the hierarchy of specific activities and their change during 'chase' incubation would seem to suggest a precursor-product relationship: 4-18s $\rightarrow$ 18s $\rightarrow$ 28s. Although such an arrangement has not been entirely excluded, it has been made unlikely by the demonstration in liver (Staehelin *et al.* 1964) and HeLa cells (Penman *et al.* 1966; Zimmerman & Holler,

1967) that during the course of r-RNA synthesis the two varieties (18s and 28s) appear simultaneously even after short labelling periods.

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