

# METABOLISM OF RIBOSOMAL PRECURSOR RIBONUCLEIC ACID IN KIDNEY

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## ABSTRACT

The labile precursors of ribosomal RNA in mouse kidney are preserved when nuclei rapidly isolated after sieving through multiple screens are swollen and cleansed in the presence of an RNase inhibitor before digestion with DNase and phenol extraction. The kinetics of nucleolar labeling analyzed on polyacrylamide gels show that 36S RNA is the major intermediate product in the catabolism of the original 45S RNA precursor to 32S RNA, from which 28S RNA is derived. Each kidney nucleus contains about 200–600 molecules of 45S RNA; the turnover time of the 45S pool is about  $3 \pm 2$  min. Compared with HeLa cells, kidney nuclei have a different major intermediate product and a much smaller and more rapidly turning-over pool of ribosomal precursor RNA.

In mammalian cells, the two major ribosomal RNA (rRNA)<sup>1</sup> species, 18S and 28S, derive from a common 45S precursor RNA (1–5) that is synthesized and methylated in the nucleolus (6–9). 45S RNA is subsequently cleaved by a nonconservative process into intermediate species, including a relatively long-lived 32S RNA component, which undergo further scission ultimately to become the 28S and 18S RNA (10–16).

Since most of the evidence for the processing of the precursors of rRNA (r-pre-RNA) has come from experiments with HeLa cells and mouse L cells (14, 16), it is now of particular interest to compare the data obtained in those cultured cells with r-pre-RNA metabolism in whole mammalian organs. Previous work has considered certain aspects of r-pre-RNA metabolism in normal and

regenerating mouse kidney and rat liver (17–19). We report here newer findings in mouse kidney, obtained in part as a consequence of a method of rapidly isolating nuclei from mouse kidney under circumstances in which the highly labile precursors of rRNA are preserved. Kinetic data on incorporation of uridine-<sup>3</sup>H and methyl-methionine-<sup>3</sup>H suggest that kidney nuclei contain a much smaller and more rapidly turning-over pool of r-pre-RNA than HeLa cells.

## MATERIALS AND METHODS

### *Specimens*

Kidneys were removed from decapitated young adult Charles River mice (40–50 days, 30–35 g), maintained on a diet of Purina rat pellets, and labeled with an intraperitoneal injection of either 500  $\mu$ Ci of uridine-<sup>3</sup>H (2 Ci/mmole) or 1 mCi of L-methyl-methionine-<sup>3</sup>H (100 mCi/mmole) (New England Nuclear Corp., Boston).

### *Buffers*

RSB: 0.01 M Tris, pH 7.4 at 20°C, 0.01 M NaCl, 0.0015 M MgCl<sub>2</sub>. HSB: 0.01 M Tris, pH 7.4 at 20°C,

<sup>1</sup> Abbreviations: rRNA, ribosomal RNA; r-pre-RNA, precursors of rRNA; RNase, ribonuclease; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetate; PVS, polyvinylsulfate; DOC, deoxycholate; SDS, sodium dodecylsulfate; sa, specific activity; RSB, reticulocyte standard buffer; HSB, high-salt buffer; UTP, uridine triphosphate.

0.5 M NaCl, 0.05 M MgCl<sub>2</sub>. Resuspension buffer: 0.01 M Tris, pH 7.4 at 20°C, 0.1 M NaCl, 0.001 M EDTA.

### *Isolation of Nuclei*

Two kidneys were decapsulated and homogenized in 20 ml of RSB containing 20 µg/ml of polyvinyl-sulfate (PVS) (Eastman Organic Chemicals, Rochester, N.Y.) with 10 strokes of the loose pestle and 10 of the tight pestle in a glass Dounce homogenizer. All procedures were carried out in the cold. The homogenate was expressed through a stainless steel screen (170 mesh, diameter 2.5 cm, Newark Wire Cloth Co., Newark, N.J.) covering the bottom of the barrel of a 50 ml plastic syringe and then through four Swinny filter holders (0.8 cm<sup>2</sup> filter area; Millipore Co., Bedford, Mass.) containing more screens and attached in series to the end of another syringe. The Swinny filter holders contained a total of 44 layers of tightly packed screens (13 mm in diameter): eight screens in the adapter nearest the syringe and 12 in each of the other three.

From the semi-pure suspension of nuclei extruded by this process, nuclei were spun down at 1100 *g* for 3 min. Resuspended pellets were washed three times by centrifugation. The washed nuclei were resuspended in 6 ml of RSB containing PVS, and 0.15 vol of a 2:1 mixture of 10% Tween-40 (Sigma Chemical Co., St. Louis, Mo.): 10% Na-deoxycholate (DOC) was added (20). After being agitated for 8 sec, the suspension was diluted with 20 ml of RSB containing PVS and centrifuged immediately at 2500 *g* for 5 min to deposit the detergent-cleansed nuclei.

### *Extraction of RNA*

The purified nuclear pellets were digested with electrophoretically purified DNase (200 µg, Worthington Biochemical Co., Freehold, N.J.) in HSB (20) containing 100 µg/ml PVS for 1.5 min at 37°C. The mixture was cooled and layered onto two 10.5 ml 15–30% (w/w) linear sucrose gradients in HSB for centrifugation for 15 min at 22,000 rpm (Spinco SW-41 rotor). The supernatant was discarded, and the pellets of nucleoli were immediately extracted with phenol.

The nucleolar pellets were suspended in 4 ml of resuspension buffer. SDS was added to 0.5% and stirred continuously at 30°C for 1 min. An equal volume of phenol (88%, Mallinckrodt Chemical Co., St. Louis) was immediately added, and extraction was performed as described by Penman (20), except that the temperature was 22°C. Before precipitation of the RNA from the aqueous phase with 2 vol of ethanol at –20°C, 0.5 vol of a 5-fold concentrate of HSB was added to chelate the EDTA.

Phenol-extracted RNA was dissolved in 2 ml of a 10-fold concentrate of RSB and again digested with

DNase (100 µg) for 1.5 min at 37°C. After the addition of SDS to 0.5% and EDTA to 0.01 M, an equal volume of phenol was added and another extraction was carried out, after which the purified RNA was precipitated with ethanol.

### *Display of RNA*

All phenol-extracted RNA recovered was layered on a 7 cm gel of 2.65% (w/v) polyacrylamide and 0.25% (v/v) ethylene diacrylate (13). Electrophoresis was for 5 hr at 5 ma/gel. The gels were then scanned at 260 nm and sliced, and the slices were dissolved in 0.4 ml of concentrated NH<sub>4</sub>OH. After the ammonia had evaporated, 10 ml of Aquafuor scintillator (New England Nuclear Corp., Boston) was added in the experiments with methyl-methionine-<sup>3</sup>H (efficiency 44%), and a toluene-methoxyethanol mixture (3:2) containing 4 g/l of Omnifluor (New England Nuclear Corp.) was used in the experiments with uridine-<sup>3</sup>H (efficiency 25%).

## RESULTS

### *Preparation of the Nuclear Fraction*

RNA extracted from nucleoli isolated according to a procedure, developed earlier in this laboratory (17, 21), which involved a separation of nuclei in 2.2 M sucrose (22), showed a substantial amount of 32S RNA, but did not reveal appreciable quantities of the larger precursors of ribosomal RNA, such as 45S RNA. Since it was possible that the lengthy isolation procedure allowed endogenous nucleolytic activity to destroy the precursors, other methods were sought to shorten the time necessary for isolation and purification of nuclei. The steel screens of 170 mesh were used to remove the unbroken cells from the homogenate. Although the cross-sectional size of the pores (120 µm) in one such 170-mesh screen is large enough to let the tubular fragments (cylinders of 70 µm diameter and several hundred of µm length) through, when the screens are tightly packed as described, almost all the tubular fragments are retained, while 75% of the nuclei go through, as estimated from recovery of DNA.

For removal of cytoplasmic contamination from these nuclei, resuspension in the hypotonic buffer, RSB, proved superior to resuspension in other media, presumably because the perinuclear ribosomes stripped more readily from swollen nuclei (20). After the final cleansing with the mixed detergents Tween-40: DOC, the nuclei were digested in HSB with DNase and centrifuged through a

sucrose gradient to remove nucleoplasm. Nuclei before treatment with DNase had an RNA to DNA ratio of 0.2.

Nonetheless, RNA extracted from the presumptive nucleolar pellet did not show any peaks migrating more slowly than 32S following gel electrophoresis until after all the RSB and HSB used were supplemented with PVS. With this ribonuclease inhibitor included, the absorbancy pattern of the nuclear RNA showed at least two distinct peaks with electrophoretic mobility less than that of 32S RNA. These two peaks were rapidly labeled after the mice were injected intraperitoneally with uridine-<sup>3</sup>H.

The omission of PVS during any step in the washings and during the mixed-detergent treatment led to a considerable reduction in these high-molecular weight peaks. Although the PVS concentration employed was low enough to prevent extensive lysis of the nuclei, some lysis probably accounted for the decrease in yield of DNA from 75% after extrusion through the screens to 57% after the final purification step. Inhibition of the action of DNase by PVS during the digestion of nuclei in HSB was probably also the reason that some nucleoplasm co-sedimented with the nucleoli.

#### *Extraction of Nuclear RNA*

RNA was extracted from the nucleolar pellet that had been recovered from the sucrose gradient by resuspending the pellet in resuspension buffer with 0.5% SDS for 1 min at 30°C, followed by phenol extraction at room temperature. Extraction with phenol-SDS at 55°C (20) resulted in lower 28S and 18S peaks with the concomitant appearance of additional peaks, of which one in the 24S region became most evident.

#### *Nomenclature of the RNA Species*

The large and the small ribosomal RNA species were identified as coinciding with the two major peaks in cytoplasmic RNA. Therefore, they were called 28S and 18S, respectively. Identification of the other peaks depended upon the logarithmic relationship between molecular weight and electrophoretic mobility (23, 24), using as standards the 28S and 18S RNA, for which molecular weights of  $1.68 \times 10^6$  daltons (25) and  $0.67 \times 10^6$  daltons (26) have been reported. By this method of assigning nominal sedimentation values, the peaks migrating more slowly than 28S had molecular

weights of  $2.1 \pm 0.05$  (32S),  $2.65 \pm 0.15$  (36S), and  $3.9 \pm 0.5$  (45S)  $\times 10^6$  daltons (15). Identification of 32S RNA was further confirmed in other experiments by coelectrophoresis with labeled authentic HeLa nuclear RNA provided by Prof. S. Penman.

#### *Kinetics of Uridine Incorporation*

5 min after an intraperitoneal injection of uridine-<sup>3</sup>H, the electrophoretic pattern (Fig. 1) showed one radioactivity peak, coinciding with the optically detectable 45S RNA and superimposed on a background of heterodisperse label. At 10 min, the radioactivity of the 45S peak was considerably increased. By that time, the labeled 32S RNA first became apparent. After 20 min, when the 45S peak was saturated, label still continued to accumulate into the 32S RNA and did so for another 20 min. These kinetics are consistent with a precursor-product relationship between 45S and 32S RNA under conditions of continuous incorporation of radioactive label. The presumptive relation becomes even more evident when the specific radioactivities of both species are plotted against time (Fig. 2). The ultimate plateau of the 45S and 32S RNA at the same level of specific activity is expected when the specific activity of the nucleotide pool of the immediate precursor, in this instance UTP, is constant for a long period. Studies to be published on the labeling of the UTP pool<sup>2</sup> will, in fact, show that after the specific activity of the UTP reaches its maximum within 10 min of labeling, it decreases at a rate of only 15% per hr. Presence of labeled 45S RNA 24 hr after injection is probably a result of the uridine-<sup>3</sup>H precursor's entering a large, poorly exchanging pool as in HeLa cells (27).

Not until after 40 min did label appear in 18S and in 28S RNA. In experiments designed to show the amount of these species representing contamination of the nuclei with cytoplasm, specific activities of these species in the nucleus were compared with the respective specific activities in the cytoplasm after 2 hr of labeling so as to enable sufficient counts to be incorporated (Table I). Identical specific activities of 18S RNA in the two fractions at this time, when cytoplasmic 18S RNA was still far from saturation, imply that all of the 18S RNA in the ostensible nuclear preparation resulted from cytoplasmic contamination. Although

<sup>2</sup> G. AB, M. L. Pruyne, and R. A. Malt. Nucleotide pools in mouse kidney. Unpublished data.

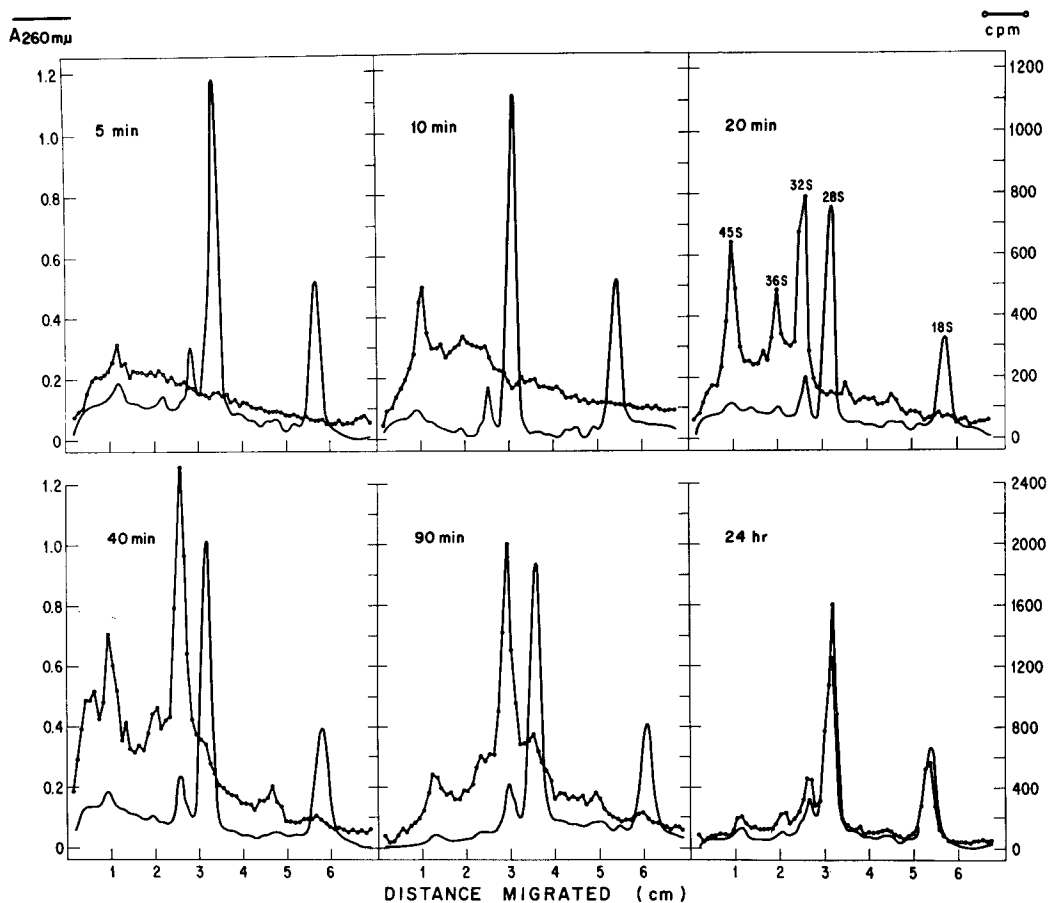


FIGURE 1 Labeling patterns of nuclear RNA. Mice were injected with  $500 \mu\text{Ci}$  of uridine- $^3\text{H}$  and killed after 5, 10, 20, 40, 90 min, and 24 hr. Nuclear RNA from two kidneys was analyzed by gel electrophoresis as described in the text. The nominal sedimentation values of the peaks are indicated in the curve showing incorporation for 20 min.

the equimolar amounts of nuclear 28S RNA and nuclear 18S RNA suggest that the same situation might also be true for 28S RNA, the higher specific activity of nuclear 28S RNA indicates 28S RNA molecules of true nuclear origin. From the fact that, under conditions of continuous labeling, nuclear 28S RNA should reach the same specific activity as 45S and 32S RNA (15 dpm/ng, Fig. 2), and from the data in Table I, we calculate that the ostensible nuclear 28S RNA actually consists of 97% cytoplasmic RNA of specific activity 0.36 dpm/ng and 3% nuclear RNA of specific activity 15 dpm/ng. Admixture of the nuclear preparations with cytoplasmic 28S and 18S RNA does not, of course, influence observations on the kinetics of r-pre-RNA.

#### Kinetics of Methylation

Fig. 1 shows that all of the radioactive peaks after injection of uridine- $^3\text{H}$  are superimposed on a background of heterogeneous, rapidly labeled RNA. In order to see whether the heterogeneous label originated from degraded ribosomal RNA precursors, or more likely from the rapidly turning-over heterogeneous nuclear RNA (21, 28, 29) that sedimented with the nuclei because PVS inhibited digestion with DNase, the mice were injected with methyl-methionine- $^3\text{H}$  instead of uridine- $^3\text{H}$ . Only the ribosomal RNA and its precursors should have been labeled by the radioactive methyl group. Fig. 3 shows the electrophoretograms of nuclear RNA after different times of labeling in vivo with methyl-methionine- $^3\text{H}$ .

After 10 min, radioactivity was detectable only in the 45-32S region, with a major peak coinciding with 45S RNA. The rise in the specific activity of 45S and 32S RNA (Fig. 4), was similar to that after uridine-<sup>3</sup>H injection. The results are consistent with the interpretation that methylation in kidney RNA, as in HeLa cells and liver (7-9), also occurs at the level of 45S RNA. The experiments do not allow distinction between the possibilities that 45S RNA becomes methylated during synthesis or only after completion. Some methylation in later stages of the processing also cannot be ruled out, but it does not seem to be quantitatively important.

Although the kinetics of labeling of 45S and 32S RNA soon after injection of methyl-methionine-<sup>3</sup>H

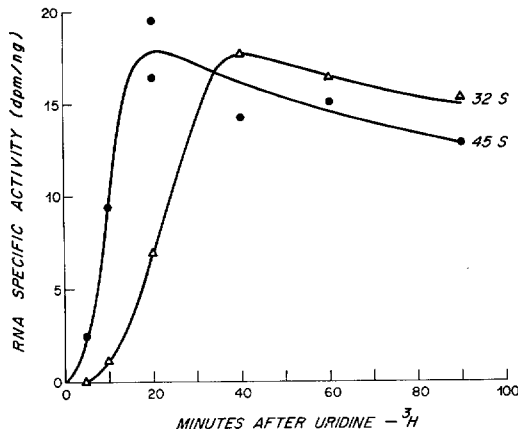


FIGURE 2 Kinetics of labeling of 45S and 32S RNA. The specific activities were derived from nuclear RNA labeled and analyzed as in Fig. 1. The amounts of 45S and 32S RNA were calculated from the area under the peak absorbancy after subtracting the baseline absorbancy, found by connecting the lowest points in the curve by a smooth line. The radioactivity of the peaks was obtained by integrating the counts in the slices after subtracting the heterodisperse label, found by connecting the lowest points between the radioactive peaks. Time needed to synthesize whole 32S

$$\left[ \text{pool} = \frac{\text{sa } 32\text{S}_{\text{max}}}{\text{sa } 32\text{S}_{\text{max}} - \text{sa } 32\text{S}_{20 \text{ min}}} \times \frac{120}{100} \right] \times 15 \pm 5 \text{ min}$$

Turnover time of 45S pool =

$$\frac{\text{synthesis time of 32S pool}}{\text{molar ratio 32S:45S}}$$

TABLE I

Comparison of the Specific Activities of Species of rRNA in the Nuclear and the Cytoplasmic Fractions 2 Hr after Injection of Uridine-<sup>3</sup>H

Subcellular fraction	Specific activity	
	28S RNA	18S RNA
	(dpm/ng)	
Nuclei	0.8 ± 0.06	0.37
Cytoplasm	0.36	0.37

Nuclear RNA was isolated and analyzed as described in the text. Cytoplasmic RNA was extracted from the supernatant remaining after the first nuclear centrifugation. An amount equivalent to 5% of one kidney was extracted with phenol in the presence of SDS (0.5%) and EDTA 0.01 M. The procedure was the same as that for nuclear RNA, except that the treatment with DNase was omitted.

were similar to the kinetics of labeling with uridine, the curves became quite different later. After 20 min the methyl-labeled 45S RNA and after 40 min the methyl-labeled 32S RNA decreased rapidly in specific radioactivity. This finding means that methyl-methionine-<sup>3</sup>H is available only during a short period after injection. After 24 hr, the 45S RNA was no longer labeled, in contrast to the finding after injection of uridine-<sup>3</sup>H.

## DISCUSSION

The method of screening of nuclei reported here reduces the time to recovery of the semi-pure nuclear pellet from mouse kidney to 30 min, in comparison with separation of the nuclear pellet by centrifugation through 2.2 M sucrose (17, 21, 22), which takes 1.5 hr. Moreover, with this method the major source of impurities, the whole cells, is removed during the first step. The sieved nuclei can be resuspended and swollen in a hypotonic buffer in the presence of PVS to inhibit RNase for further cleansing with mixed detergents with far less risk of degrading labile RNA.

Although the final nuclei are clean by the criterion of RNA/DNA of 0.2, some cytoplasmic contamination remains. In the absence of electron micrographs, the source of the cytoplasmic contamination cannot be identified. We think that it is more likely a result of unbroken whole cells escaping the sieving process rather than of inadequate cleansing of the nuclei. Furthermore, residual cytoplasmic contamination is far

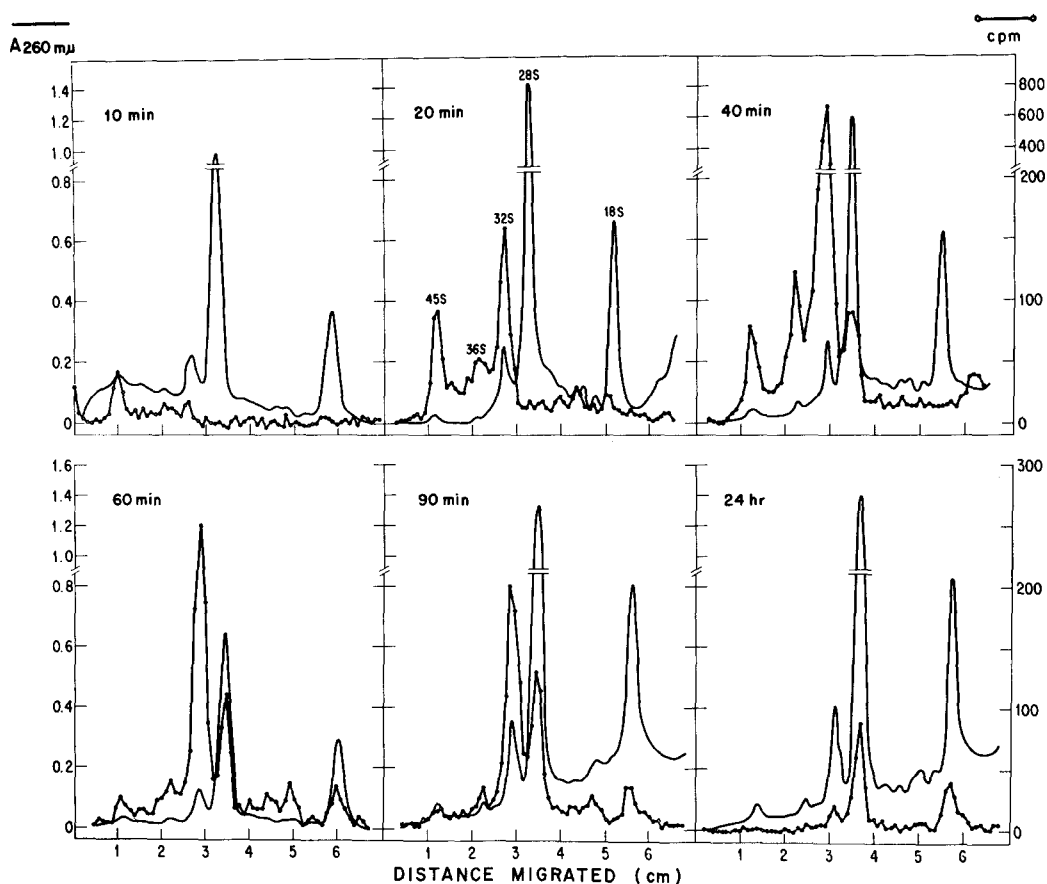


FIGURE 3 Labeling patterns of nuclear RNA after injection of methyl-methionine- $^3\text{H}$ . Mice were injected with 1 mCi of methyl-methionine- $^3\text{H}$  and killed after 10, 20, 40, 60, 90 min, and 24 hr. Nuclear RNA from two kidneys was analyzed as in Fig. 1 and in the text. The nominal sedimentation values of the peaks are indicated.

more obvious in kidney cells than in HeLa cells because kidney cells contain far less nuclear precursor RNA, as will be discussed below.

The kinetics of labeling of RNA in nuclei from mouse kidney are in agreement with the apparently universal mammalian scheme in which 28S RNA derives from a long-lived 32S immediate precursor, which, itself, derives from a short-lived 45S precursor. In kidney, in contrast to HeLa cells (13), 36S RNA is the major species in the transformation of 45S RNA to 32S RNA, and there is almost no indication of 41S RNA. In this respect, mouse kidney closely resembles mouse L cells (16). On the other hand, the radioactive species migrating between 28S and 18S (estimated molecular weight  $1 \times 10^6$  daltons) in the electrophoretic pattern of RNA from mouse kidney may

be analogous to the final precursor for 18S RNA identified in HeLa cells (15), or it may be an artifact.

Rapid incorporation of both uridine- $^3\text{H}$  and methyl-methionine- $^3\text{H}$  into 32S RNA of mouse kidney indicates a much faster rate of processing of its 45S precursor than is the case in HeLa cells (30). After labeling with uridine- $^3\text{H}$  for 20 min, a time when both the specific activities of UTP and 45S RNA have leveled off, it takes another  $15 \pm 5$  min for the 32S RNA pool to become saturated. From this figure, we estimate that it would take  $21 \pm 7$  min to synthesize the whole 32S RNA pool, (legend, Fig. 2), taking into account that, at the time saturation is reached, 20% of the 32S RNA will already have been processed into 28S RNA. From the absorbancy curves of Figs. 1 and 3, we

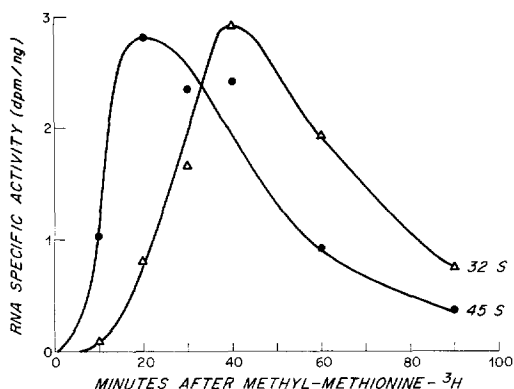


FIGURE 4 Kinetics of labeling of 45S and 32S RNA after injection of methyl-methionine-<sup>3</sup>H. The specific activities were derived from the electrophoretic patterns shown in Fig. 3. The amounts of 45S and 32S RNA were calculated as indicated in Fig. 2. The radioactivity of the peaks was obtained by integrating the total number of counts in the slices, without correction.

estimate that there are 6–14 times more 32S RNA molecules than 45S RNA molecules (calculated from a mass ratio 3–7 and a molecular weight ratio of about 0.5). Therefore, the whole 45S RNA pool should turn over one time every  $3 \pm 2$  min (legend, Fig. 2). The mass ratio of 3–7 may be too high because of preferential extraction of 32S RNA at room temperature and because of breakdown of 45S RNA. However, even with the molar ratio of 4 reported for HeLa cells (31), we still calculate the turnover time of 3.5–7 min for the whole 45S RNA pool.

The half-life of 4.1 days for mouse kidney ribosomes (32) and the estimate of 6 pg DNA/aver-

age diploid nucleus (33) permit calculation of the rate of rRNA synthesis as 0.06 pg/nucleus per hr, the same as in normal rat liver (19), compared with 0.8 pg/nucleus per hr in regenerating liver (19) and in HeLa cells during logarithmic growth. From these data and from the turnover times of the rRNA precursors, we estimate that nuclei of mouse kidney cells, like those liver cells (19), contain about 20 times less 45S RNA than nuclei of HeLa cells. The validity of this estimate is strengthened by the figure of 200–600 molecules of 45S RNA/kidney nucleus, calculated from the area of absorbance of 45S RNA in the electrophoretic patterns of kidney RNA and from the empirical observation that, under the conditions of these experiments, 1 cm<sup>2</sup> on the recording paper is equivalent to 0.33 μg RNA. The average number of 45S RNA molecules in the nucleus of a HeLa cell is 10,000 (31). Preliminary observations suggest that the kidney undergoing compensatory hypertrophy, perhaps as has been inferred for hyperplastic liver (19), increases its store of ribosomes by processing more intermediaries at one time rather than by accelerating the rate of synthesis of mature rRNA.

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