MEMBRANE-BOUND RIBOSOMES IN KIDNEY

Methods of Estimation and Effect of Compensatory Renal Growth

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ABSTRACT

Membrane-bound ribosomes are thought to secrete protein for export and free ribosomes to secrete protein for intracellular use. The proportion of the total ribosomes that is bound to membranes in normal mouse kidneys has been estimated by three different methods, and the results have been compared with those obtained by a fourth method used by us previously. The most valid estimates appear to be those obtained (*a*) by comparison of radioactivity in peaks representing free and membrane-bound ribosomes on linear sucrose gradients after labeling for 24 hr with ^{14}C-orotic acid, and (*b*) by measurements of optical density in free and bound ribosomes that had been separated by centrifugation on discontinuous gradients of 0.5 M/2.0 M sucrose.

Analyses by these methods show that about 20–25% of the ribosomes in a postnuclear supernatant prepared from mouse kidneys, but only 10–15% of the ribosomes in a post-mitochondrial supernatant, are membrane-bound. About 75% of the bound ribosomes sediment as polysomes of many different sizes. The proportion of membrane-bound ribosomes and their aggregation into polysomes were unchanged in kidneys undergoing compensatory hypertrophy after removal of the opposite kidney.

These experiments show that, unlike liver, kidney has a predominance of free ribosomes compared to bound ribosomes; those ribosomes that are membrane-bound do not become free during compensatory renal growth.

INTRODUCTION

In organs like pancreas and liver that secrete much protein, most ribosomes are bound to the endoplasmic reticulum (rough endoplasmic reticulum) (1–4). Caro and Palade (5) have demonstrated that protein synthesized by membrane-bound ribosomes in pancreatic acinar cells is at first within the vesicles of the rough endoplasmic reticulum, then within smooth vesicles, and finally is condensed into discrete packets of protein in the Golgi vesicles before being discharged from the cell. Serum proteins are probably secreted in much the same way by the liver (6–8), in which about 75% of the ribosomes are membrane-bound (3, 4). In organs like kidney that appear to secrete little protein, however, the proportion of ribosomes bound to endoplasmic reticulum and the function of these ribosomes have received less attention.

Several groups (9–11) have shown in electron micrographs of renal tubule cells that most of the ribosomes lie free in aggregates unattached to endoplasmic reticulum, and we have demonstrated (12) in the developing kidney that virtually no rib-

osomes are bound to membranes until 2 days after birth, when the proportion rises sharply to 20% and then more gradually to reach about 30% in the kidneys of the adult mouse. The work reported here was undertaken to compare other methods for estimating the proportion of ribosomes bound to membranes with the method used previously, to determine whether the membrane-bound ribosomes occur as polysomes, and to study the effect of unilateral nephrectomy on the membrane-bound ribosomes of the surviving kidney. Work on the specific function of the membrane-bound ribosomes is in progress.

MATERIALS AND METHODS

Homogenates

Kidneys were taken from male Charles River mice 45–55 days old, killed by overstretching the neck. Subsequent manipulations were carried out at 4°C. Usually, four kidneys in 3.5 ml of homogenization medium were disrupted by three strokes of the loose pestle (0.004 inch clearance) and one stroke of the tight pestle (0.001 inch) in a Dounce glass homogenizer. This method breaks about 60% of the cells, and further homogenization breaks few more. Histologic examination of the broken cells, consideration of the clearance of the Dounce pestle, and correlation of cyclic mitotic activity in proximal tubules with changes in ribonucleic acid (RNA) metabolism in homogenates thus prepared (13), all indicate that homogenization in this manner breaks chiefly proximal tubule cells. Postnuclear supernatants were prepared by centrifuging homogenates at 850 *g* for 5 min; postmitochondrial supernatants were prepared by centrifuging at 20,000 *g* for 5 min.

Buffer Solutions

Homogenization medium: 0.25 M sucrose containing 0.1 M Tris, 0.025 M KCl, 0.0015 M $MgCl_2$, pH 7.6 at 4°C.

RSB: 0.01 M Tris, 0.01 M KCl, 0.0015 M $MgCl_2$, pH 7.6 at 4°C.

TKM: 0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$, pH 7.5 at 20°C.

SDS: 0.5% SDS (sodium dodecyl sulfate) in 0.05 M Tris, 0.1 M NaCl, pH 7.6 at 23°C.

Chemicals

Ribonuclease (RNase) was obtained from Worthington Biochemical Co., Freehold, N.J.; ^{14}C-protein hydrolysate (640 μc/mg) and ^{14}C-orotic acid (256 μc/mg) were obtained from Nuclear-Chicago Corporation, Des Plaines, Ill.

Sucrose Gradients

The optical density (OD) of centrifuged sucrose gradients was recorded automatically, on a Gilford Model 2000 spectrophotometer (Gilford Instrument Co., Oberlin, Ohio), in effluent collected from the bottom of the centrifuge tube. Radioactivity in fractions collected from the effluent was assessed by coprecipitation of the RNA with carrier protein in cold trichloracetic acid; preparations to be studied for synthesis of nascent protein were first briefly incubated in 1 N NaOH. Precipitates were retained and washed on Millipore filters and counted in a Nuclear-Chicago gas-flow counter (background 2 cpm).

Estimation of Membrane-Bound Ribosomes

METHOD 1: (*A*) This method was similar to that of Talal and Exum (14). Postnuclear or postmitochondrial supernatants were layered over a 24 ml linear gradient of 15–40% sucrose (w/w) in RSB prepared over a 2 ml "cushion" of 68% sucrose in RSB. Gradients were centrifuged for 90 min at 24,000 rpm (Spinco SW 25.1 rotor, Spinco Div., Beckman Instruments, Palo Alto, Calif.). The areas under the fast-sedimenting and slow-sedimenting peaks of OD on the recorder charts (Fig. 1), representing the bound and free ribosomes, respectively, (2, 14) were measured planimetrically after the charts had been redrawn to compensate for the slower flow of the concentrated sucrose in the first few milliliters of effluent from the gradients. The area of the rapidly-sedimenting material was expressed as a percentage of the total OD, excluding that due to protein at the top of the gradient.

(*B*) Similar estimations were carried out with kidneys from mice that had received 20 μc of ^{14}C-orotic acid intraperitoneally 24 hr previously. Acid-precipitable radioactivity associated with the fast-sedimenting peak of OD was expressed as a percentage of the total OD, excluding that due to ribosomal subunits.

METHOD 2: Free ribosomes and total ribosomes were separately deposited through discontinuous gradients (15, 16). Postnuclear supernatants (2 ml) in 0.25 M sucrose-TKM were layered over a discontinuous gradient of 4 ml of 0.5 M sucrose-TKM and 3 ml of 2.0 M sucrose-TKM, each made 10% with a high-speed supernatant fraction (60 min at 270,000 g_{av}; Type 65 rotor) from mouse liver to inhibit RNase (16, 17), and centrifuged for 24 hr at 40,000 rpm (Type 65 rotor) to deposit the free ribosomes. A replicate supernatant in 10% liver supernatant was made 1% with sodium deoxycholate (DOC) to release all ribosomes from membranes and was treated similarly. All supernatant was removed from the transparent pellets, which were dissolved in 0.5%

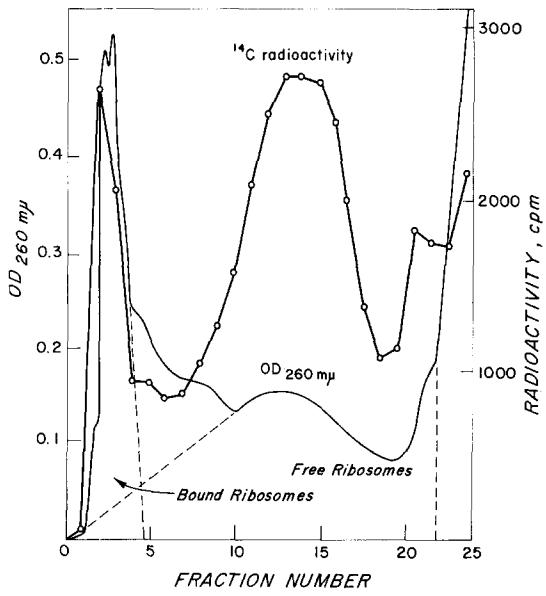

FIGURE 1 Sedimentation profile of a postnuclear supernatant from mouse kidney. Mice were injected with 20 μc ^{14}C-orotic acid 24 hr previously. 15–40% linear gradients of sucrose in RSB were prepared over a 2 ml "cushion" of 68% sucrose; gradients were centrifuged for 90 min at 24,000 rpm in an SW 25.1 rotor. Areas between dotted lines were measured planimetrically and designated "bound" and "free" ribosomes as indicated. Radioactivity in fractions 1–6 was taken to be associated with the bound ribosomes and was expressed as a percentage of the total radioactivity in fractions 1–22. In this gradient the bound ribosomes account for 31% of the total ribosome OD and 18% of the total ribosome radioactivity.

SDS buffer for determination of OD at 232, 260, and 280 mμ.

METHOD 3: Polyribosomes in purified free and membrane-bound ribosomes were displayed as follows. Native postnuclear and postmitochondrial supernatants were centrifuged on discontinuous gradients as in Method 2. The membranous material from the interface was made 1.0% with DOC. The pellets of free ribosomes were resuspended in 0.25 M sucrose-TKM with 10% liver supernatant and made 1% with DOC. Each preparation was layered over 2 ml of 2.0 M sucrose-TKM containing 10% liver supernatant and centrifuged for 3 hr at 65,000 rpm (Type 65 rotor). The pellets of purified ribosomes from this centrifugation were resuspended in homogenization buffer containing 10% liver supernatant and layered over 16 ml of 15–30% linear gradients of sucrose in RSB for centrifugation at 24,000 rpm for 210 min (SW 25.3 rotor) to display polysomes. This experiment was also carried out with kidneys that had been decapsulated and incubated for 15 min at 37°C in Hanks' salt solution containing ^{14}C-amino acids to label the nascent protein attached to polyribosomes (12).

Proportions of bound ribosomes were estimated by measuring planimetrically the total area of OD (excluding that due to ribosomal subunits and supernatant protein) on the recorder charts and expressing the OD due to bound ribosomes as a percentage of the total OD due to all the ribosomes in each cytoplasmic preparation.

Effect of Unilateral Nephrectomy

Postnuclear supernatants from the right kidneys of mice that had undergone left nephrectomy 1, 2, 4, and 7 days previously were examined by Methods 1 *A* and 2. Kidneys from sham-operated and normal control mice were analyzed concurrently. Sedimentation analysis of purified free and bound ribosomes to display polyribosomes (Method 3, above) was also carried out on the right kidneys of mice 1 and 3 days after left nephrectomy.

RESULTS

Estimation of Proportion of Membrane-Bound Ribosomes

METHOD 1: Postnuclear supernatants from the kidneys of normal mice gave the sedimentation profile shown in Fig. 1. The peak of OD representing free ribosomes was usually more clearly defined, and the peak representing bound ribosomes was sometimes less clearly defined than in this example. Measurements of OD tracings (Table I: Method 1 *A*) suggested that 33% of the ribosomes in postnuclear supernatants were bound compared with only 21% indicated by radioactivity measurements (Method 1 *B*).

Method 1 has the advantage that the OD and radioactivity under the two peaks are compared directly within each sucrose gradient. (Free and bound ribosomes in kidney show substantially equal labeling after a 24-hr pulse of ^{14}C-orotic acid

TABLE I

Proportions of Free and Membrane-Bound Ribosomes in Postnuclear and Postmitochondrial Supernatant Preparations from Mouse Kidneys

Source of data	Per cent ribosomes bound			
	PNS	*N*	PMS	*N*
Method 1 Centrifugation on continuous gradients				
1 *A* Comparison of OD	33 ± 1*	12	15 ± 2	13
1 *B* Comparison of ^{14}C	21 ± 1	6		
Method 2 Centrifugation on discontinuous gradients	16 ± 3	6		
Method 3 Comparison of OD in profiles of purified polysomes	24 ± 2	2	12 ± 1	7
Revised values from reference 12			21 ± 1	8

PNS, postnuclear supernatant; PMS, postmitochondrial supernatant; *N*, number of determinations.

* Standard error.

(18).) Disadvantages are the imperfect definition of the fast-sedimenting peak in some postnuclear supernatants, the fact that light-scattering also contributes to the OD comprising the fast-sedimenting peak, and the possibility that some RNA is present in the membranes themselves (19). That these factors exaggerate the true proportion of bound ribosomes is confirmed by the lower proportion indicated when radioactivity in the two classes of ribosomes is compared instead of OD (Table I). Incomplete sedimentation of single free ribosomes and of small polysomes may also cause small errors in this method.

METHOD 2: Table I shows an estimate of only 16% bound ribosomes by this method. No difference was observed when the preliminary (nuclear) centrifugation was for 3 min rather than 5 min. As the 232/260 mμ ratio was uniformly about 0.81 and the 260/280 mμ ratio was uniformly 1.76, OD at 260 mμ was accepted as a basis for comparison of RNA in the pellets.

Errors in this method are the loss of ribosomes along the nonsectoral sides of the tube and the possible activation of nucleases in spite of the presence of mouse-liver supernatant as an RNase inhibitor. The effect of these potential variables is unknown, but is likely to be small.

METHOD 3: When purified free and bound ribosomes from postmitochondrial supernatants were analyzed, about 75–80% of both the free and the bound ribosomes sedimented as polysomes (Fig. 2). The free and bound polysomes had a similar wide range of sizes, but polysomes of intermediate size (5–10 ribosomes) appeared to be slightly less numerous among the bound ribosomes. Treatment of the resuspended ribosomes with 2 μg of RNase for 10 min at 4°C before centrifugation caused almost all the ribosomes to sediment as monomers. Gradients prepared from kidneys labeled with ^{14}C-amino acids contained acid-precipitable radioactivity associated with the whole range of membrane-bound polysomes (but little associated with the single ribosomes), demonstrating that the membrane-bound polysomes had been active in protein synthesis. Although no signs of degradation of polysomes to smaller polysomes and single ribosomes were evident in gradients prepared from postmitochondrial supernatants, gradients prepared from postnuclear supernatants showed exaggerated peaks due to trimers, dimers, and single ribosomes, and an accompanying reduction in the OD representing larger polysomes. These findings suggested that some degradation had occurred despite the use of RNase inhibitor.

The advantage of this method is that purified preparations of both classes of ribosomes are compared directly and that any RNA present in the membranes themselves (19) is not taken into account. One possible source of error might be

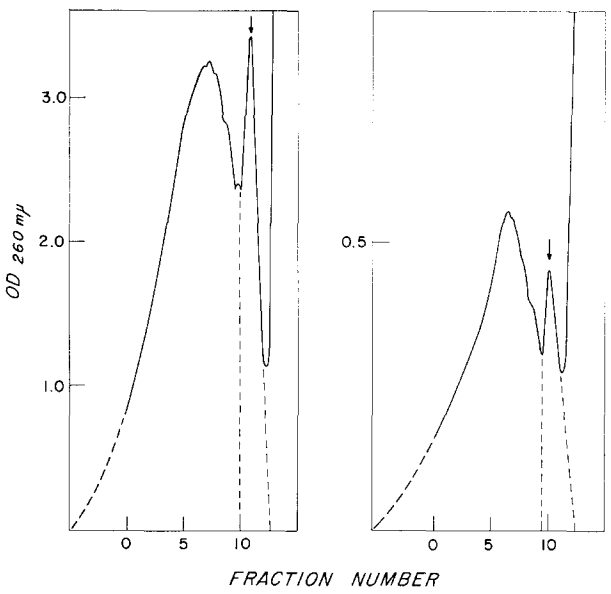

FIGURE 2 Sedimentation profile of purified free and bound ribosomes, from mouse kidneys, on 15–30% sucrose gradients. Free and bound ribosomes were separated from postmitochondrial supernatants by centrifugation on discontinuous gradients of 0.5/2.0 M sucrose, treated with DOC in the presence of liver supernatant, again centrifuged through 2 M sucrose, and resuspended in homogenization buffer. The final gradients were centrifuged for 210 min at 24,000 rpm in an SW 25.3 rotor. The position of the single ribosomes in each gradient is marked by an arrow. Proportions of ribosomes sedimenting singly or as polysomes were determined by planimetry of the areas under the curves as indicated. The area under the extrapolated part of the curve on the left of each chart, representing polysomes that sedimented into the pellet, was included with the polysomes.

loss of membranous material along the sides of the tubes in the discontinuous gradients, but use of 1.3 M sucrose instead of 0.5 M sucrose to counter this effect (16) did not change the results. Two other possible errors are the loss of ribosomes during resuspension of the pellets and the degradation of bound ribosomes with enzymes activated by DOC. In fact, the proportionality of free and bound ribosomes was independent of up to three resuspensions, and postmitochondrial preparations of purified bound ribosomes showed no sign of degradation; postnuclear preparations, although somewhat degraded, could still be used to estimate proportions of bound ribosomes by comparison of optical densities (Table I). The value for postnuclear supernatants obtained in this way (24%) is lower than those values indicated by Method 1 *A* (33%), but close to the values obtained by Methods 1 *B* (21%) and 2 (16%).

Estimates of free and membrane-bound ribosomes made directly from comparisons of chemically-determined RNA (20, 21) in the crude membranous fraction at the interface of the 0.5 M and 2.0 M sucrose layers and in the pellet were unsatisfactory even if the RNA was ethanol-precipitated from solution in 0.5% SDS buffer. A series of replicate aliquots of postnuclear supernatants analyzed by such a method compared with Method 1 *A* consistently showed 49% ribosomes bound by this method compared with 35% by Method 1 *A*. The large estimate cannot be correct since it is at wide variance with electron micrographs (9–11), which show that few kidney ribosomes are bound to membranes. The discrepant estimation may, in part, have resulted from contamination with nonribosomal RNA.

Revision of Estimates from Previous Work

Table I gives revised values for the proportion ribosomes in adult kidneys obtained in previous work (12). The method used then was to centrifuge postimitochondrial supernatants on 15–30% sucrose gradients and to compare the RNA content of the pellet at the bottom of the gradient with the RNA content of free polysomes and single ribosomes still in the gradient. The percentage of the total RNA found in the pellet was operationally defined as the proportion of bound ribosomes. The mean value from eight determinations on adult mice of various ages was 30%. Table I shows this value corrected for contamination of the pellet by the heaviest free polysomes. The correction was derived by extrapolating the OD tracing on the original recorder charts through the ordinate until it met the baseline (16). The triangular area so defined was expressed as a percentage of the whole area of ribosomal OD on each chart, and the percentages from the eight determinations were

combined into a mean (9%) representing the contamination by free polysomes. Subtraction of this value from the original mean value of 30% gave the amended value for bound ribosomes (21%) shown in Table I. The correction used is supported by data from experiments in which postmitochondrial supernatants were centrifuged for intermediate times (60 min in the SW 25.1 rotor) on 15–40% sucrose gradients. The heaviest free polysomes were then still seen in the gradients, and the OD tracings resembled the extrapolated outline used to calculate our correction factor. A small error due to sedimentation of polysomes into the microsomal pellet after deflection by the wall of the nonsectorial tube (22) is still present.

Effect of Unilateral Nephrectomy

In the postnuclear supernatants examined by Method 1 *A*, the mean values for percentage of membrane-bound ribosomes in nephrectomized, sham-nephrectomized, and control mice were all between 31% and 38%. In supernatants examined by Method 2, the values were all between 18% and 23%. No trend was detectable.

Polysome profiles of membrane-bound ribosomes in postmitochondrial supernatants 1 and 3 days after nephrectomy were identical with those from control mice.

DISCUSSION

Because cells known to secrete much protein, like those of the liver and pancreas, have many bound ribosomes, and cells that do not secrete much protein, like erythrocytes and epidermal cells, have mostly free ribosomes, Birbeck and Mercer (23) and others have suggested that membrane-bound ribosomes synthesize the protein destined to be secreted (exported), while free ribosomes synthesize the protein for intracellular use. If this is true for kidney, then the proportion of ribosomes that are attached to membranes might give a rough guide to the proportion of the total protein synthesized that is exported.

An ideal procedure for estimating the bound ribosomes in a homogeneous organ would release the contents of all the cells and permit an estimation to be performed directly on the whole cytoplasm. Homogenization of kidney to preserve polysomes as employed in the experiments reported here has the advantage of rupturing chiefly proximal tubules and leaving other cortical and medullary cells intact, to be removed together with nuclei by preliminary centrifugation. Ribosomes attached to large fragments of endoplasmic reticulum may be lost at this stage, and if the cytoplasm is further fractionated to remove mitochondria and lysosomes, the loss of membrane-bound ribosomes will be still greater. The loss is not necessarily reproducible because manual homogenization is difficult to standardize, and effective *g* forces vary between different volumes of homogenate centrifuged at the same speed. We tried to minimize these sources of variation by doing many individual assays on both postnuclear and postmitochondrial supernatants; and, because variations in cytoplasmic preparations may be compounded by errors inherent in the method of assay, we used several methods. Each gave consistent results with regular use, but each had certain disadvantages, which are discussed above.

The results (Table I) obtained by comparison of ^{14}C-labeled RNA in free and bound ribosomes (Method 1 *B*), by comparison of free ribosomes with total ribosomes released with DOC (Method 2), and by comparison of total OD in sedimentation profiles of purified free and bound ribosomes (Method 3), are consistent, both internally and with electron micrographs (9–11). The data agree in showing that about 20% of the ribosomes are membrane-bound in postnuclear supernatants; the results from Method 1 *A* are also compatible with this value when the known errors are taken into consideration. Bcause of possible loss of ribosomes in the preliminary (850 *g*) centrifugation, our results may underestimate the true proportion of bound ribosomes, but perhaps less so than in similar work with liver (15), since perinuclear ribosomes are a less conspicuous feature of kidney cells (9–11).

We suggest that about a quarter of the ribosomes must be attached to membranes in the normal kidney. Using this proportion and the information that about 75% of both free and bound ribosomes exist as polysomes (Fig. 2), we calculate that the disposition of ribosomes in mouse kidney is: 18% membrane-bound polysomes, 7% membrane-bound single ribosomes, 56% free polysomes, and 19% free single ribosomes. This calculation does not allow for the presence of ribosomal subunits, which in liver account for only 1% of the total ribosomes (16).

The demonstrated preponderance of free kidney polysomes is in keeping with other work from this

laboratory in which the rate of labeling of all subcellular fractions with ^{14}C-orotic acid was studied. If newly-synthesized 28S RNA enters polysomes directly (24, 25), then the major fraction of polysomes, either free or membrane-bound, should be labeled first. In fact, free kidney ribosomes were always labeled before the membrane-bound ribosomes (18). The work described here further confirms that our earlier estimates of the proportion of membrane-bound ribosomes in kidneys from adult mice (12) were substantially correct and thus reinforces our conclusion that the proportion of ribosomes attached to membranes changes suddenly after birth.

It is important to question whether polysomes liberated from membranes with detergent and displayed on sucrose gradients exist in the same form in the native kidney, or whether they are artifacts produced by degradation of larger polysomes or by nonspecific aggregation of smaller polysomes. Postnuclear preparations excepted, there were none of the characteristic signs of degradation in polysome profiles. Some aggregation might arise by incomplete resuspension of ribosomal pellets before the final centrifugation, but profiles of freshly prepared and of resuspended ribosomes were identical when analyzed concurrently.

The membrane-bound ribosomes in mouse kidney exist as polysomes with a wide range of sizes. This finding suggests that many different proteins or protein subunits are made by the bound ribosomes, apparently more than just the proteins exported from the kidney if these proteins were limited to the renal hormones renin and erythropoietin. Many sizes of membrane-bound polysomes have also been demonstrated in rat liver (16), rat spleen (14), and in cultured mammalian fibroblasts (26).

That the proportion of the total ribosomes bound to membranes was not changed in the response to unilateral nephrectomy supports ultrastructural illustrations of an increase in smooth, but not rough membranes, after nephrectomy (9, 11). Presumably the increased synthesis of ribosomal RNA (rRNA) after nephrectomy (18), by which RNA per cell is increased by about 20% in 2 days (20), leads to a proportionate increase in both free and bound ribosomes, and attachment of a predetermined proportion of nascent ribosomes to endoplasmic reticulum occurs quickly, at least before 1 day. The apparent stability of membrane-binding of ribosomes during compensatory renal growth confirms the findings of Webb et al. (27), and is at issue with the findings of Cammarano et al. (28) with respect to ribosome binding in hyperplasia of the liver after partial hepatectomy.

With the addition of this new information to data previously accumulated (12, 13, 18), the mouse kidney begins to represent a well-characterized system in terms of its nucleic acid metabolism during normal and compensatory growth. As a readily accessible mammalian tissue with a predominance of free polysomes approaching that seen in HeLa cells, the mouse kidney may be more suitable for some investigations of protein and nucleic acid metabolism than organs like the liver that require the addition of detergent to liberate their ribosomes from membranes.

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