SEQUENTIAL CHANGES IN CLASSES OF RNA DURING COMPENSATORY GROWTH OF THE KIDNEY*

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The amount of RNA in the average renal cell begins to increase soon after contralateral nephrectomy and approaches its maximum 2–4 days later (1–3).[1] During this time there is a two- to fourfold increase in the rate at which ribosomes are labeled from radioactive precursors (4), and more polyribosomes, particularly more large polyribosomes, are recoverable from equivalent masses of renal tissue (5).

Here we report experiments undertaken to show alterations in the classes of RNA that contribute to these events. Ribosomal RNA production was estimated from the amount of nuclear precursor rRNA[2] and messenger RNA from specific activity of rapidly labeled nonribosomal RNA in polyribosomes. The initial increase in RNA in the renoprival state appears to be caused by enhanced synthesis of the nuclear precursors of rRNA. When the wave of *rnuc*RNA synthesis subsides coincident with a fall in the mitotic activity of the proximal tubule cells, mRNA synthesis rises.

Materials and Methods

Animals.—Young adult male mice of the Charles River strain (42–50 days old, 30 g) were subjected to nephrectomy through the left flank; the adrenal was spared. Operations were performed under light ether anesthesia in less than 2 min between 9:00 and 10:00 a.m. The mice were permitted to have food and water up to the time of surgery and immediately thereafter. Nephrectomized and sham-operated animals were decapitated at intervals from 12 hr

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[2] The following abbreviations are used in this paper: mRNA, messenger RNA; rRNA, ribosomal RNA; *rnuc*RNA, nuclear precursor to cytoplasmic rRNA; *rcyto*RNA, cytoplasmic rRNA; tRNA, transfer RNA; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetracetic acid; BSA, bovine serum albumin; TCA, trichloracetic acid; S.A., specific activity (cpm/OD unit RNA); OD, optical density; hpf, high-power field.

to 46 days. Right kidneys, quickly removed and cooled to 4°C in the buffer for homogenization, were used in all experiments.

Radioisotope.—Uridine-5-^{3}H (20 c/mmole, Schwarz BioResearch, Orangeburg, N.Y.) was given by intraperitoneal injection of 0.5 ml aqueous solution 45 min before termination of an experiment. For intervals after nephrectomy of 8 days or less, 125 μc were given to each of six mice; for longer intervals 250 μc were given to each of three mice.

Extraction.—RNA was prepared by phenol extraction of nuclei and cytoplasm in hypertonic buffer by the methods previously described (6, 7) with a few modifications: crude nuclei were washed twice instead of once by centrifugation through 0.34 M sucrose (0.003 M $MgCl_2$). Extractions were carried out at 57°C to avoid aggregation of RNA. The nuclear extract was washed an additional time with chloroform-isoamyl-alcohol to assure recovery of mRNA from the interface, and the aqueous phase of the cytoplasmic extract was similarly washed immediately before precipitation with ethanol to remove final traces of protein. Polyribosomes were prepared as before (5, 7) except that the homogenization medium contained 0.25 M sucrose.

Analysis.—Details of preparative ultracentrifugation, measurement of OD, and counting of radioactivity have been published (7). The efficiency of counting in the Nuclear-Chicago Mark I Liquid Scintillation Spectrometer (Nuclear-Chicago Corp., Chicago, Ill.) was about 20%. Quenching was determined by external standard channels ratio.

RESULTS

Ribosomal RNA.—Sedimentation coefficients of OD peaks were calculated (8) with reference to a value of 18S for the slower sedimenting fraction of cytoplasmic rRNA. The sedimentation value of the larger r*cyto*RNA in all animals studied thus calculated was 28.41S (±0.03 SE). To eliminate the effects of systematic error in the gradients the position of the faster sedimenting r*nuc*RNA (nominal 30S (6)) was calculated from the following formula:

$$Srnuc_{30} = \frac{18(Drnuc_{30})(Lcyto)}{(Drcyto_{28})(Lnuc)}$$

where D = the distance of the nuclear or cytoplasmic rRNA peak from the top of the gradient and L = the total length of the gradient as translated on the recording spectrophometer chart. The peaks were clearly shown on the actual recordings (taken at 0.5 OD full-scale deflection) compared with the blunted appearance of some of them when transposed to a common scale for purposes of publication. The validity of the method of calculation was proved by several experiments in which purified 18S rRNA labeled for 24 hr with uridine-^{3}H was added to the nuclear extracts before layering on the gradients. Sedimentation values of the heavier r*nuc*RNA compared to the reference peak of 18S acid-precipitated radioactivity were almost identical to sedimentation values calculated from the formula.

The difference in the sedimentation value of the heavier r*nuc*RNA compared to the cytoplasmic 28S rRNA was taken as a measure of the amount of nuclear ribosomal precursor present (7); that is, the more precursor >32S the faster would the heavier r*nuc*RNA sediment. Control experiments showed that injection of an aqueous solution of isotope 45 min before removal of the kidneys

had no effect on sedimentation values. For reasons that are not clear, the calculated Sr*nuc*$_{30}$ was 3.2S greater than Sr*cyto*$_{28}$ in kidneys removed 3 min after injection, only 0.5S greater at 10 min, but as usual 1.5S greater thereafter.

Differences between the sedimentation values of r*nuc*RNA and r*cyto*RNA from two typical experiments are given in Table I and are shown in Figs. 1 and 2. There was an increase in the S value of r*nuc*RNA within the 1st day after nephrectomy and a maximal difference of about 2.59S on the 2nd day. Thereafter the difference dropped quickly so that by the 4th day the sedimentation values were almost the same. There was a slower secondary rise to the 8th day, then another gradual fall-off to the 21st day. Sham-operated animals showed no such systematic changes.

TABLE I

Postnephrectomy	Sr*cyto*$_{28}$		Sr*nuc*$_{30}$		ΔS	
	1	2	1	2	1	2
days						
0.5	28.52	28.08	29.86	29.70	1.34	1.62
1	28.48	28.80	30.35	31.16	1.87	2.36
2	29.45	28.88	32.04	30.95	2.59	2.07
4	28.42	28.08	29.43	28.80	1.01	0.72
8	27.58	28.16	29.97	30.12	2.39	1.96
14	28.20	27.22	29.81	30.61	1.61	1.39
21	28.08	28.37	29.16	29.59	1.08	1.22
33		28.45		30.80		2.35
46	27.73		29.69		1.96	

Relative sedimentation values of the faster sedimenting rRNA in nuclei and in cytoplasm at different times after unilateral nephrectomy in two experiments.

Messenger RNA.—mRNA was operationally defined as the heterogenous rapidly labeled nonribosomal RNA in polyribosomes (9–11). Such RNA liberated with SDS from polyribosomes labeled with uridine-^{3}H for 30 min and displayed on sucrose-SDS gradients sedimented between 4S and 40S with the principal concentration between 8S and 18S (Fig. 3). Although labeled tRNA undoubtedly contributed some of the material sedimenting between 2S and 6S, mRNA similarly prepared from polyribosomes from which tRNA was removed by solubilization in 2 M LiCl also showed radioactivity in the 2S-6S regions. Recovery of the material from the small pellet at the bottom of these DOC-treated polysome gradients from which the SDS gradients were made revealed in the pellet only a little RNA with an inconsequential amount of radioactivity (DOC appears to precipitate rapidly-labeled 45S particles under such circumstances (10)).

The sedimentation profile of the putative mRNA was almost identical to

that of the rapidly labeled RNA from whole kidneys extracted with phenol in hypertonic buffer (7). Furthermore, other experiments show that preparations containing this mRNA have the ability to stimulate amino acid incorporation in in vitro systems.

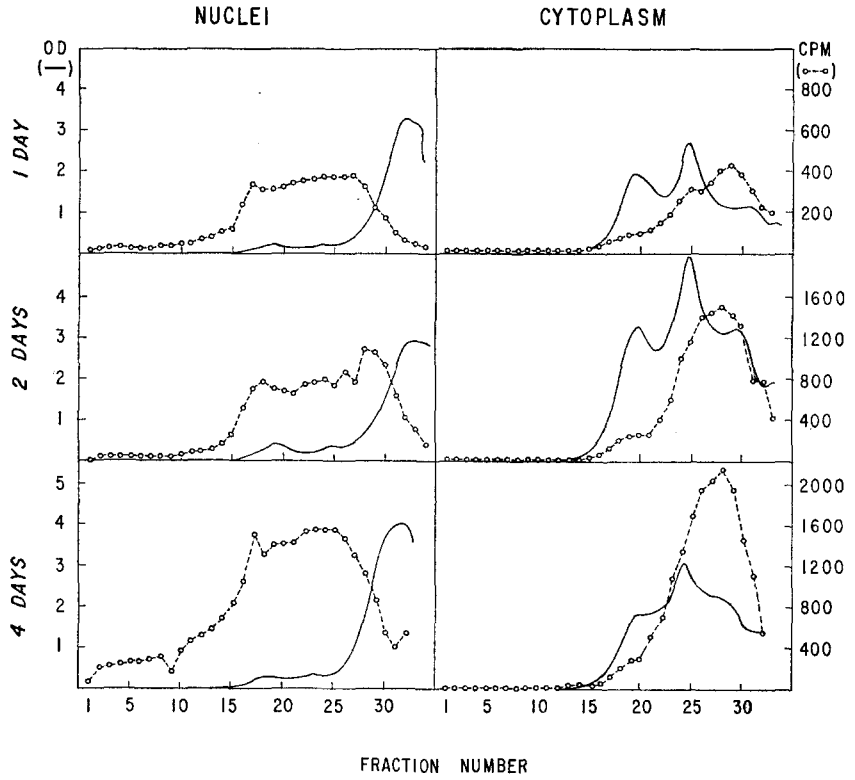

FIG. 1. Patterns of labeling of renal nuclear and cytoplasmic RNA in kidneys 1, 2, and 4 days after contralateral nephrectomy. Details of labeling and of extraction are given in the text. Centrifugation on 26 ml 15–30% sucrose-SDS gradients was for 16 hr at 17,000 rpm (Spinco SW 25.1) at 25°C.————————, OD; -o-o-o-o-o, radioactivity.

Even though the mRNA labeled after 30 min exposure to uridine-^{3}H is apparently uncontaminated with rapidly labeled rRNA and the mRNA labeled at 40 min has some detectable rRNA superimposed on it, we chose a 45 min period of incorporation for the experiments reported in this paper because it offers a good compromise between 30 min, best for showing mRNA, and 60 min, best for showing sedimentation differences between r*nuc*RNA and r*cyto*RNA (7), without introducing appreciable artifact. To reduce the possibility of overlap from labeled rRNA or tRNA influencing results, S.A. (cpm/OD) at 12S was chosen as the arbitrary measure of mRNA. The S.A. at 12S

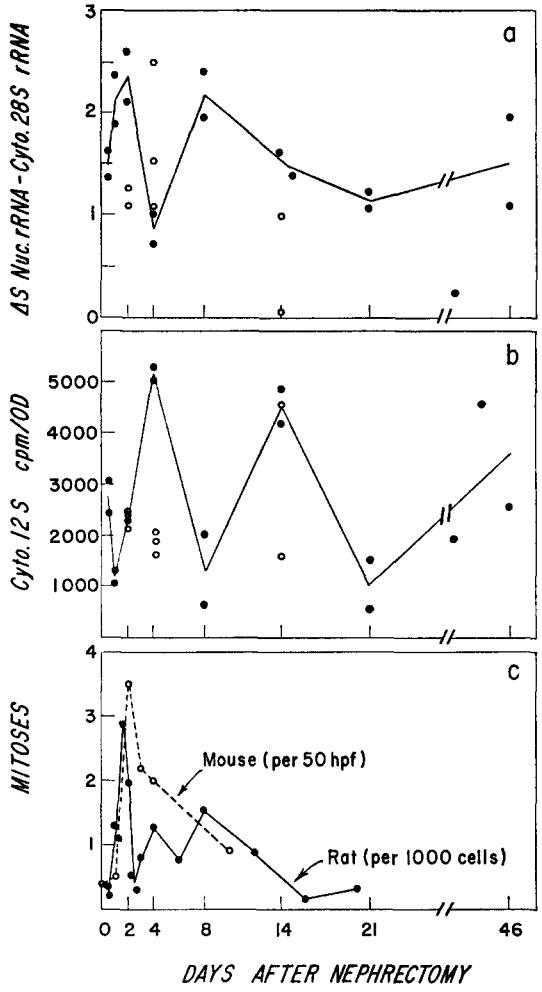

FIG. 2. Changes in the remaining kidney after unilateral nephrectomy. (*a*) Differences between the S value of the nuclear precursor of rRNA and of 28S cytoplasmic rRNA. •, nephrectomized mice; ○, sham-operated mice. (*b*) S.A. of 12S cytoplasmic rRNA, an assay of mRNA. •, nephrectomized mice; ○, sham-operated mice. (*c*) Mitotic index of proximal tubule cells of rats and mice derived from data of Williams (16) and Reiter (18).

following unilateral nephrectomy is shown in Fig. 2. Ratios of S.A. at 12S to S.A. at 18S and at 28S are constants for every time studied, showing that changes in S.A. at 12S cannot be attributed to spreading from occasional alterations in labeling of 18S and 28S rRNA.

The 1st day after nephrectomy there was a fall in the amount of mRNA present. Some recovery was present at 2 days, but the peak value, approxi-

mately twice normal, was not found until 4 days after nephrectomy. Thereafter there was a decline to 8 days after nephrectomy, followed by a rise to a second maximum at 14 days, and another decline to 21 days. Sham-operated animals showed no such changes except for one group at 14 days.

Acid-Soluble Pool.—Although other experiments (unpublished) had shown that the pool of acid-soluble material 3–40 hr after injection of uridine-^{14}C were

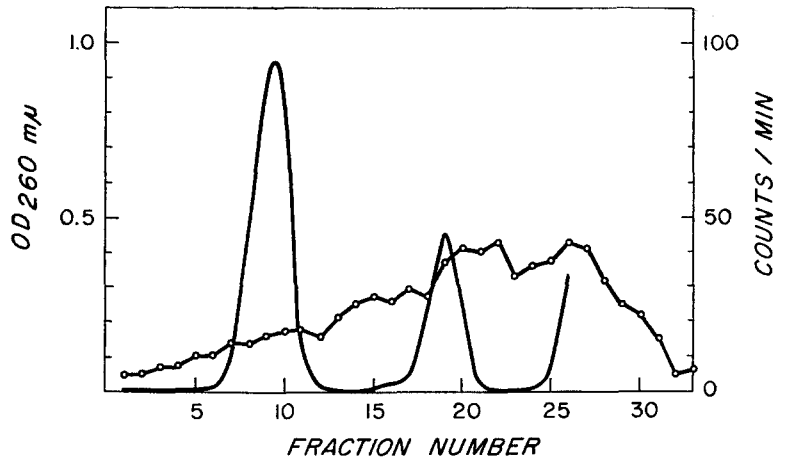

FIG. 3. Rapidly labeled RNA from renal polyribosomes. The right kidney was removed from four mice 30 min after intraperitoneal injection of 250 μc uridine-^{3}H and polyribosomes prepared as described in the text from a postmitochondrial supernatant made 0.5% with DOC. 1 ml supernatant was layered on each of two 15–30% sucrose gradients and centrifuged for 2.5 hr at 24,000 rpm at 4°C (Spinco SW 25.1). The polysome portions and the small amount of sediment at the bottoms of the tubes were separately pooled. RNA was released with 0.5% SDS buffer, precipitated with 2 volumes ethanol at −20°C, and redissolved in 0.5 ml 0.5% SDS buffer for display on 13 ml 15–30% sucrose-SDS gradients (SW 25.3 rotor, 24,000 rpm × 20 hr, 23°C). Fractions were collected and analyzed for OD at 260 mμ and for acid-precipitable radioactivity as described (7).————, OD, -o-o-o-o-, radioactivity. From the sediments at the bottoms of the tubes the most 28S and 18S rRNA extracted was 0.05 OD units; little radioactivity was recovered, and the spectrum of precipitated radioactivity was identical with that in the polysomes.

not materially different in normal kidneys and in kidneys undergoing compensatory hypertrophy, the possibility arose that the high specific activity at 12S in kidneys 4 days after nephrectomy and 45 min after injection of uridine-^{3}H might have been a consequence of an increased radioactive precursor pool at that stage. The size of the total radioactive acid-soluble pool was therefore determined in another experiment. Individual nucleotide and endogenous pools were not measured.

Each mouse of two pairs was injected with 250 μc uridine-^{3}H in the normal state or at 2, 4, and 7 days after left nephrectomy or sham operation. 45 min

after injection, the right kidneys were rapidly removed and homogenized in pairs in 6 ml 10% TCA. Acid-soluble and acid-precipitable radioactivity were counted and total protein was estimated (Fig. 4). The acid-soluble counts/kidney remained constant at least for 4 days after nephrectomy. Since the wet mass of the kidney was increasing 10–23% during the same time,[1] the average number of counts delivered/unit mass of kidney must have actually been less.

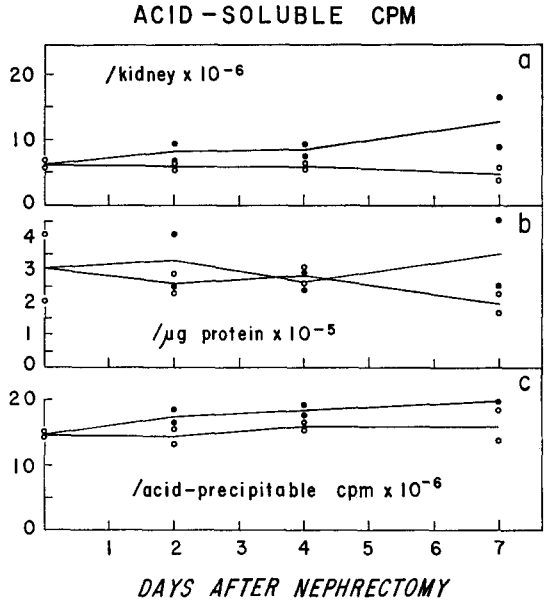

FIG. 4. Acid-soluble radioactive pools in kidneys after contralateral nephrectomy. Pairs of kidneys were labeled and processed as described in the text. TCA precipitates were washed 3 times with 5% TCA. Radioactivity of the pooled supernatants was determined by liquid scintillation counting (7), and the precipitates were hydrolyzed by a modification[1] of the method described by Wennemacher et al. (12). Protein was estimated by the biuret method from the KOH hydrolysate (BSA standard). An aliquot of the same hydrolysate was also counted as the acid-precipitable radioactivity. •, renoprival kidneys; ◦, sham-operated kidneys. (*a*) Acid-soluble counts/kidney; (*b*) acid-soluble counts/mg kidney protein; and (*c*) acid-soluble counts/acid-precipitable counts.

The constancy of radioactive acid–soluble counts/mg protein confirms the impression that an increased amount of precursor delivered on the 4th day was not responsible for the increased S.A. at 12S in uninephrectomized mice.

Heterogeneous Nuclear RNA.—Neither in this work nor in the earlier investigation were we concerned with nuclear species other than those that served as precursors to cytoplasmic rRNA. With attention lately drawn to the possibility that the heterogeneous nonribosomal rapidly labeled nuclear RNA might represent an unstable precursor to mRNA (13–15), we looked for a way to describe

changes in this material during the course of renal compensatory growth. OD was not available as a reference because the top parts of the nuclear gradients contain DNase-digested DNA as well as RNA. The ratio of radioactivity at 12S to radioactivity at the 30S peak (dpm 12S/dpm 30S) was chosen as the reference. Fig. 5 shows that no consistent change was detected in this ratio for the first 2–4 days after nephrectomy, but there was a sharp increase to the 8th day, followed by a decline to the 14th day, and a subsequent secondary rise to the 21st day. From the 4th day, therefore, this curve paralleled that of rRNA described above except for the 21st day. This data will not be further discussed as its significance is for the present unclear.

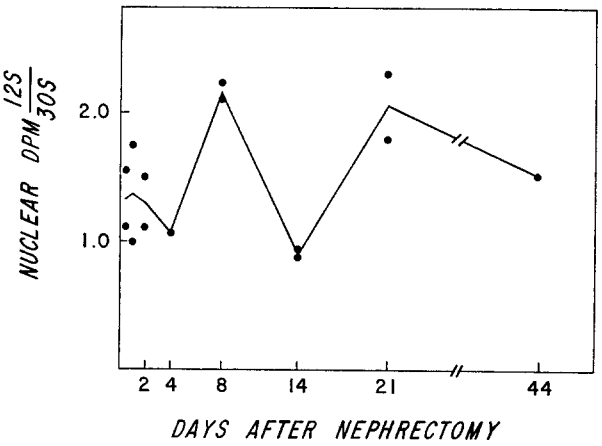

FIG. 5. Heterogeneous renal nuclear RNA after contralateral nephrectomy. The graph shows the ratio of radioactivity (dpm) in the 12S material compared to radioactivity in 30S material—an arbitrary reference. These animals are the same as those in Fig. 2.

DISCUSSION

Perhaps one of the reasons there is no conclusive experiment revealing the cause of renal compensatory growth is that the phenomenon itself was not adequately described until the last decade. The effect of diurnal rhythms and of differences caused by withholding food and water, for example, were not appreciated. By rigidly controlling such variables, Williams (16) (see also reference 17) confirmed unequivocally that renal cell division as well as hypertrophy is a consequence of uninephrectomy in the rat. The normal low level of mitoses in proximal tubule cells increases about seven times on the 2nd day following nephrectomy, returns to normal, and then rises again on the 8th day. The presence of at least the first mitotic burst has been demonstrated in the mouse as well (18).

Early histochemical and biochemical work on compensatory growth were

naturally hampered by unawareness of environmental variables as well as by the technical difficulties of the time. However, the experiments of Vegni (1) found confirmation in the exacting work of Halliburton and Thomson (3), which described an increase of about 29% RNA/cell on the 4th day after uninephrectomy in rats. In mice, RNA/cell increases about 22% by the same time.[1] Descriptions of labeling of specific RNA fractions after nephrectomy (19, 20) have heretofore been concerned chiefly with the inhibitory effect of diphosphopyridine nucleotide on labeling.

The experiments reported in this paper were done to answer the question of how much of the increased number of polyribosomes in the renoprival kidney is due to increased ribosome synthesis and how much is due to increased mRNA synthesis, since these are the two major intracellular components thought to be responsible for the structure of functioning mammalian polyribosomes. The experiments show that the first definitely identifiable change in RNA metabolism provoked by nephrectomy is an increased synthesis in the nuclear precursors of rRNA. This activity is responsible for the increases of RNA/average renal cell in this strain of mice following uninephrectomy. The steadily greater amount of *rnuc*RNA until the 2nd day, its fall, and its rise again to the 8th day coincide nicely with the waves of mitotic activity in mice and in rats (16–18, 21). Not only are the peaks of mitosis and *rnuc*RNA synthesis themselves coincident, but the proportion of change between different days and the general shapes of the curves agree.

The changes in mRNA are quite the reverse. mRNA is present in low amounts on the 2nd day after nephrectomy, when *rnuc*RNA is high, and in high amounts on the 4th day, when *rnuc*RNA is low. It should be emphasized that these measurements were actually determinations of labeling or specific activity rather than of synthesis of the molecules per se. Changes in the total free precursor pools during renal growth could conceivably have influenced these results. It was demonstrated that the total radioactive pool sizes were stable. Changes in specific nucleotide pools rather than total pool size could also conceivably have been important as in the first few hours after partial hepatectomy (22), but even in the liver the changes did not persist beyond the 1st day, and they were not major in animals fed up to the time of surgery (23). Finally, variations in absorption of the radioactive precursor could have been responsible for the results, but the random variations in the sham-operated mice and the constancy of radioactive acid-soluble pools in the renoprival kidneys in other experiments reported here render this possibility unlikely.

These data on rRNA and mRNA production obtained from whole kidneys in vivo parallel in many ways data obtained by other workers from rabbit kidney fragments stimulated to grow by explantation to tissue culture (24, 25). Although one might now dispute equating mRNA with rapidly labeled metabolically unstable nuclear RNA as was done in the in vitro experiments with

kidney fragments reported in 1963, one is nevertheless struck by the three stages of RNA synthesis identified in the period of 32 hr before DNA synthesis began in vitro. For the first 12 hr total RNA synthesis remained stable, but rRNA synthesis increased sharply and immediately. The phase of rRNA synthesis was followed by a 10-hr period during which nuclear RNA synthesis inhibitable by actinomycin increased two- to threefold. In the last 10-hr period, RNA synthesis stabilized at a new, high rate. In regenerating liver as well as in explanted kidney endogenous *rnuc*RNA synthesis is an outstanding feature in the 1st day after hepatectomy (26, 27).

Likewise, there is considerable area of agreement between our results and those reported by Mach and Vassalli (28), who studied RNA synthesis in rat spleen and lymph nodes stimulated to produce antibody by immunization. In these cells taken at the peak of RNA synthesis there was a major shift towards the synthesis of the precursors of rapidly labeled rRNA compared to non-immunized cells as evidenced by an increase in the labeled material sedimenting >30S. Nonimmunized cells, by contrast, under the same conditions of rapid labeling produced chiefly slow sedimenting RNA consistent with mRNA.

Predominant synthesis of rRNA in the exponential phase of randomly growing tissue culture cells has been recorded from the first reports of Darnell's group (29, 10). More recently Kubinski and Koch (30), using synchronized amnion cells, have found that although rRNA production characterizes the dividing cell, mRNA accumulates in the nondividing cell. Since the curve of rRNA synthesis from our experiments almost exactly parallels the plot of mitotic activity in renoprival mouse and rat kidney, it is difficult to avoid the similarity of our data with those of Kubinski and Koch. Furthermore, the simple analogy would be teleologically satisfying. One would suppose that since the production of mRNA is rapid, the production of ribosomes slow, and the pool of ribosomes unattached to mRNA probably small (31, 32), the cell would require new ribosomes during the course of growth before it could use any mRNA that could later be produced quickly. Several lines of evidence support the contention that ribosomes are necessary to permit further transcription of RNA (33–36), and if this is the case, the presence of new ribosomes would seem to be a preliminary to new mRNA production. An alternative explanation might also be considered: that uninephrectomy inhibits production of new mRNA and that depletion of mRNA provokes the formation of new ribosomes (Fig. 2).

These hypotheses suggest that the stimulus or stimuli to compensatory growth of the kidney may operate to stimulate rRNA synthesis either directly or by depletion of mRNA and that the production of mRNA could be triggered by the early increase in rRNA or by the precipitous decrement in rRNA after the 2nd day. If so, then it is possible that the stimulus to compensatory hypertrophy needs to operate only for a short time to set in motion a complex series of events. These hypothetical events would be further evidence (21) against a

"functional load" theory of compensatory growth and perhaps more in keeping with the idea that simple events that stimulate disproportionate growth of the cytoplasm are responsible for cellular hypertrophy and hyperplasia (37).

If the data we report are derived mainly from the proximal tubule cells as we believe they are, the simple explanation may actually be correct. However, until the nature of the cells of origin for our preparations is proved, the reservation must be held that one class of renal cells responds to the renoprival state by early synthesis of rRNA and another responds later with mRNA. It is even possible that there could be separate stimuli for these two classes of cells, for it has been shown that sensitized lymphocytes stimulated to grow by addition of antibody will produce mainly rRNA, but if stimulated by phytohemagglutinin, the product is nonribosomal, presumably mRNA (38).

SUMMARY

The synthesis of nuclear precursors of rRNA and of cytoplasmic mRNA has been estimated in mouse kidneys following contralateral nephrectomy.

1. The production of nuclear rRNA parallels mitotic activity in the proximal tubule cells. Its peak is 2 days after nephrectomy; there may be a second peak at 8 days.

2. The production of mRNA is almost the reciprocal of rRNA production. It is least at 2 days and most at 4 days after nephrectomy. It may also decrease immediately after nephrectomy.

3. Growing kidney cells may build a store of ribosomes before they elaborate mRNA. The sequential relation between rRNA and mRNA synthesis raises the possibility that production of rRNA could regulate the production of mRNA and that the stimulus to compensatory hypertrophy might need to act only long enough to set into motion the machinery for making rRNA, either directly or as a consequence of exhausting mRNA.

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