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AMINO ACID INCORPORATING ACTIVITY AND SYNTHESIS OF FREE AND MEMBRANE BOUND POLYSOMES IN THE RAT KIDNEY AFTER FOLIC ACID

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Abstract—A single injection of 1·13 mM folic acid/kg body wt. causes an increase in the protein content of the rat kidney accompanied by changes in the protein synthesis rate.

The *in vitro* [^{14}C]phenylalanine incorporating activity per kidney ribosome is decreased during the first 6 hr after folic acid injection and increased from the 12th to the 24th hr to twice the control activity, this level being maintained till the 96th hr. The decreased and increased amino acid incorporating activity occurs in the presence of endogenous or exogenous mRNA (polyuridylic acid).

Since the RNA content in the kidney homogenate increases by two and a half times that of controls while the protein/RNA mass ratio in the DOC treated kidney microsomes remains constant during the first 96 hr after folic acid there may be an increase in the number of ribosomes due to increased synthesis of ribosomes. The incorporation of 5-T-orotic acid into the cytoplasmic rRNA was three times that of controls 20 hr after folic acid.

Dissociation of the kindey ribosomes in free and membrane bound ribosomes by sucrose gradient centrifugation shows that the free kidney ribosomes from folic acid treated rats were labelled very strongly and earlier than the membrane bound ribosomes using 5-T-orotic acid as RNA precursor. The results suggest that after folic acid first a stimulated synthesis of free kidney ribosomes occurs and later some of them associate with endoplasmic membranous structures. The association of kidney ribosomes to endoplasmic membranes seems to be of shorter duration than the half life of newly synthesized ribosomes.

AS SHOWN in earlier studies[1–3] the application of certain pteridines causes hypertrophy in rat kindey. This kidney proliferation results in an increase in the nucleic acid and protein content. The total protein content per kidney is increased by 35 per cent 4 days after the injection of 1·13 mM folic acid/kg body wt.[2]

The early increase in protein synthesis seems to play a special role in the initiation of proliferation.

On kidney cell cultures Kishimoto and Lieberman[4] showed by experiments with actinomycin D and puromycin that mitosis is dependent on protein synthesis in the late interphase which itself depends on some RNA synthesis. Recent results[5] show that this early stimulation of RNA synthesis is mainly a synthesis of rRNA precursors or rRNA. Before the stimulation of rRNA synthesis a 4 to 5-fold increase in the amino acid incorporation occurs, accompanied by an increase of the activities of glucose-6-phosphate-dehydrogenase, lactate dehydrogenase, and hexokinase.[6,7]

The inductive increases of activities of the glucose-6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase[8] in the rat kidney after folic acid possibly influence the synthesis of precursors of RNA and DNA by a regulation of the flow through the oxidative pentosephosphate pathway. Threlfall and Taylor[1,9] who dealt with the interrelationships between changes in the rates of protein and RNA synthesis and the rise in DNA synthesis on the folic acid induced kidney proliferation came to the conclusion that a continuous need for protein synthesis exists both before and during the period of increased DNA synthesis.

In order to characterize the protein synthesis in the folic acid-induced kidney proliferation itself we have studied the *in vitro* amino acid incorporating activity of kidney microsomes and the synthesis of kidney ribo- and polysomes and their distribution in membrane bound and free ribo- and polysomes.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 140–160 g were fed on Altromin R and tap water *ad lib.* until 15 hr before removal of the kidneys. The kidneys were removed at the same time in all experiments, at 9 a.m.

Chemicals

Folic acid (E. Merck, Darmstadt) was dissolved in 0·3 M $NaHCO_3$ solution and injected in a dose of 1·13 mM/kg body wt. i.v.

5-T-orotic acid (Radiochemical Centre Amersham), sp. act. 1c/m-mole was used for labelling cytoplasmic rRNA.

[^{14}C]Phenylalanine (u), (Radiochemical Centre Amersham), sp. act. 369 mc/m-mole was used in the amino acid incorporating system.

Preparation of kidney microsomes

All work was done at 4°. After decapitation of rats the kidneys were decapsulated, sliced and thrown into ice cold medium A (0·25 M sucrose, 0·01 m $MgCl_2$, 0·08 M KCl, 0·05 M tris–HCl, pH 7·8 at 4°)[10] and homogenized in a Potter–Elvehjem homogenizer. The nuclei and mitochondria were sedimented by centrifugation of the homogenate at 13,000 *g* for 15 min. The postmitochondrial supernatant was centrifuged at 105,000 *g* in a fixed angle Spinco-rotor type 50.1, for 2 hr. The microsomal pellet was resuspended in medium A and frozen by liquid nitrogen and stored at −35° until further use.

Deoxycholate (DOC) was added to the microsomal suspension in medium A to give a final concentration of 1 per cent, thoroughly mixed, layered over medium B (1·0 M sucrose, 0·01 M $MgCl_2$, 0·08 M KCl, 0·05 M tris–HCl, pH 7·8 at 4°), centrifuged at 100,000 *g* for 2 hr. After thorough removal of the DOC solution the pellet was resuspended in medium A, frozen by liquid nitrogen and stored at −35° until further use.

Extraction and determination of the total RNA from kidney homogenates and from the microsomal fraction was done by a modified method of Ogur and Rosen.[11] We repeated the extraction with cold 1·0 M PCA (perchloric acid) over 20 hr twice because a single extraction yields only 60–70 per cent of the total RNA content.

RNA was determined by the method of Mejbaum[12] with soluble yeast RNA as standard.

After the extraction from the microsomal pellet by the phenol method[13] in the presence of 0·5% SDS the ribosomal RNA species were differentiated by centrifugation over a 8–20 per cent sucrose gradient. As a measure of the RNA content of each gradient fraction the extinction at 260 nm was used; 60 μg RNA/ml give at 260 nm an $E_{1\,cm} = 1{\cdot}1$. This relation was directly proportional from 5 to 60 μg RNA/ml. For the radioactivity determination 0·5 resp. 1·0 ml of the neutralized RNA extract was mixed with 10 ml scintillation fluid (4 g PPO, 0·2 g POPOP, 20 ml ethylene glycol, 10 ml ethanol, 60 g naphthalene and 1000 ml dioxane) and counted in a Packard Liquid Scintillation Spectrometer type 3375. The counts were corrected for background and quenching.

Extraction and determination of protein

The total microsomal protein was extracted by a modified method of Schmidt and Tannhauser.[14] To the microsomal pellet 1·0 M PCA was added in the cold and the PCA insoluble fraction was washed twice with the same PCA. After extraction of the nucleic acids with 1·0 M PCA over 15 min at 75° the pellet was washed once with ethanol, twice with ethanol–ether–chloroform (2:1:1, by vol.) once with ether, then dried and dissolved in 0·1 N NaOH by incubation over 2 hr at 60°. From this solution aliquots were taken for the determination of protein by the method of Lowry[15] with bovine serum albumin as standard.

Amino acid incorporating system

This was based on the method of Wool and Cavichi.[16] In preliminary experiments we optimized the system for incubation time, Mg^{2+}- and K^{+}-concentration. The incubation assay contains in a final volume of 1·1 ml 55 μmoles tris–HCl, pH 7·8 at 37°, 88 μmoles KCl, 11 μmoles $MgCl_2$, 2·75 μmoles ATP, 0·88 μmoles GTP, 11·0 μmoles phosphoenolpyruvate, 0·25 mg tRNA (as soluble yeast RNA from Boehringer, Mannheim), 0·1 mg pyruvate kinase, 0·35 μl mercaptoethanol, 300 μg RNA microsomal suspension, 1·0 mg protein 105,000 *g* supernatant derived from the brain of untreated rats, 0·5 μc [^{14}C]phenylalanine(u). The microsomal suspension served as a starter. After 30 min incubation at 37° 100-μl aliquots were dropped on Whatman 3 MM paper discs (2·5 cm dia.). The paper discs were thrown into ice cold 10% TCA (trichlor acetic acid) with 5 mM unlabelled carrier phenylalanine, washed three times with the same TCA, three times with ether–ethanol–chloroform (2:2:1, by vol.), once with ether, dried, given in scintillation fluid (4 g PPO, 50 mg POPOP and 1000 ml toluene) and counted in the above mentioned scintillation counter.

Dissociation of membrane bound and free ribo- and polysomes

This was based on the methods of Henshaw[17] and Talal.[18] The microsomal pellet prepared as described above was layered on a 24-ml linear gradient of 15–40 per cent sucrose in 0·01 M tris–HCl, pH 7·6 at 4°, 0·01 M KCl, 0·015 M $MgCl_2$, prepared over a 2-ml cushion of 68 per cent sucrose in the same solution. The gradients were centrifuged for 90 min at 24,000 rev/min in a SW 25.1 Spinco-rotor, fractionated in 35 fractions and their extinctions at 260 nm measured in a PMQ-Zeiss spectrophotometer after appropriate dilution. The first peak of the E_{260}-curve refers to the fast

sedimenting membrane bound ribo- and polysomes and shows an average protein/RNA rate of 4·8 for the controls. The second peak of the slow sedimenting ribo- and polysomes shows an E_{232}/E_{260} of 0·9 and an E_{260}/E_{280} of 1·68.

The radioactivity recovered in the bound or free ribo- and polysomes was assayed as follows: After injection of 200 μc 5-T-orotic acid/kg body wt. at different times and preparation of the above described sucrose gradients an aliquot of 100 μl of each gradient fraction was pipetted on Whatman 3 MM paper discs (2·5 cm dia.), which were dropped into ice cold 5% PCA with 5 mM unlabelled orotic acid, stored for 10 min in the cold, washed three times with the same PCA, then with ethanol–ether (1:1, v/v) and ether. After being dried the discs were transferred into bottles with scintillation fluid and the radioactivity determined as described above.

RESULTS AND DISCUSSION

The folic acid induced changes in the amino acid incorporation by kidney microsomes (Fig. 1) were studied by a system in which only the microsomes were derived from the folic acid treated rats. The other components of the system are exogenous and of the same quantity and quality. Therefore it can be deduced from the disintegrations per minutes per milligram of RNA that the amino acid incorporating activity per ribosome will be first diminished and then stimulated after folic acid application. During the first 6 hr the incorporation of [^{14}C]phenylalanine is inhibited, followed by a gradual, later constant increase which is 92 per cent above the control after 96 hr. The decrease and increase in the amino acid incorporating activity occurred in the presence of endogenous mRNA (Fig. 1) and synthetic exogenous mRNA, polyuridylic acid.[19] Therefore the increased inherent capability for amino acid incorporation of the kidney microsomes from folic acid treated rats may be due to changes in the association of mRNA, to changes in the aggregation of ribosomes, their altered affinity to tRNA, possibly as observed by Nicholls[20] in the aminonucleosid-nephrotic

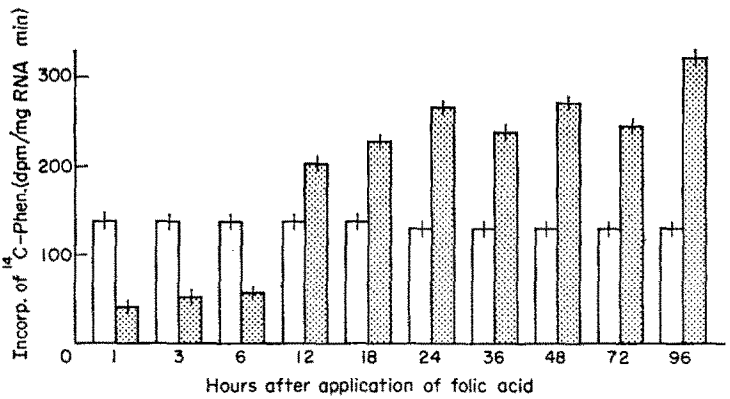

FIG. 1. *In vitro* incorporation of [^{14}C]phenylalanine by kidney microsomes at different times after 1·13 mM folic acid/kg body wt. Incubation assay as given in Methods. Each column is representative for 12 kidneys which were pooled and served for eight incubation assays. Vertical lines are the standard errors of the incubation assays.
□ microsomes of control rats.
▒ microsomes of rats treated with folic acid.

rat kidney or to changes of the ribosomal proteins themselves some of which are essential for function, assembly or fidelity of translation.[21]

In preliminary experiments (Vetter, Brade, 1970, unpublished) the amino acid incorporation by kidney microsomes from control and folic acid treated rats was examined in the presence of the 105,000 *g* kidney supernatant from control and folic acid treated rats. The experiments indicate that the 105,000 *g* kidney supernatant derived from rat kidneys 24 hr after 1·13 mM folic acid/kg body wt. has an additional stimulating effect on the increased amino acid incorporation after folic acid. In this connection further experiments on the binding of aminoacyl-tRNA to ribosomes and determinations of the ribonuclease activity in the kidney hyaloplasm of folic acid treated rats are required.

The diminished amino acid incorporation in the first hours after folic acid resembles the decreased synthesis by liver of excreted proteins which occur 3 hr after CCl_4 administration when rough endoplasmic reticulum dilatation is established.[22] Since both control and treated rats were without food during the decreased amino acid incorporation, fasting cannot account for the diminished amino acid incorporation as claimed by Surks[25] for the decreased amino acid incorporation in the liver of fasting rats. Vetter and Brade[19] were able to demonstrate that the inhibition of the [^{14}C]-phenylalanine incorporation by kidney microsomes 3 and 6 hr after a combined application of 400 μg actinomycin D and 500 mg folic acid per kg body wt. equals that found after a single injection of actinomycin D. This nonadditive inhibition suggests a similar inhibitory mechanism for actinomycin D and folic acid. Under the assumption that the only effect of actinomycin D consists of an inhibition of the DNA dependent RNA synthesis the inhibition of the incorporation of amino acids by microsomes in the first 6 hr after folic acid might be mainly due to an inhibition of rRNA and mRNA synthesis. Experiments about direct inhibiting effects of actinomycin D on the protein synthesis[23–25] restrict this conclusion.

Increase in the number of ribosomes

The RNA content in the kidney homogenate increases from 3324 to 8488 μg RNA/kidney[2] 4 days after 1·13 mM folic acid/kg body wt. Since 85 per cent of the increase of the total RNA refer to the rRNA[26] an increase of the number of ribosomes is assumed. This is supported by the constancy of the protein/RNA ratio in the DOC treated microsomal fraction (Table 1) which is independent of the time of folic acid injection. The microsomal preparation from which the data in Table 1 were derived includes membrane bound and free ribo- and polysomes. The decrease of the protein/RNA ratio of kidney microsomes after folic acid (Table 1) might therefore mainly be due to an increase of the number of free ribo- and polysomes or to the slower increase of membrane protein of the endoplasmic reticulum or to both. An augmentation of free kidney polysomes lying in groups, chains or arcs after folic acid treatment was demonstrated in our earlier electron microscopic studies.[3] The participation of bound and free ribosomes in the increased synthesis of kidney ribosomes after folic acid will be discussed later.

An increase of the ribosome number connected with an increased aggregation to polysomes is characteristic for many proliferative systems and present after temporary renal ischemia,[27] after unilateral nephrectomy,[28] in the nephrotic kidney,[20] and after partial hepatectomy.[29,30] The reason for the augmentation of ribosomes and their

TABLE 1. COMPARISON OF THE PROTEIN/RNA RATIO OF KIDNEY MICROSOMES FROM RATS TREATED WITH FOLIC ACID BEFORE AND AFTER DOC TREATMENT

Time after application of 1·13 mM folic acid per kg body wt. (hr)	Protein/RNA	
	Kidney microsomes	DOC-treated kidney microsomes
0	4·680	1·085
12	3·535	0·901
24	2·608	1·090
36	2·373	0·990
48	2·640	1·010
72	3·003	0·974
96	2·634	—

Each figure is the mean of two undependent experiments. Each experiment includes a pool of eight to ten kidneys.

aggregation to polysomes is still hypothetical. Changes in the intracellular amino acid pool,[31] in the energy supply or in certain dissociation or initiation factors[32] may be involved.

Increased synthesis of ribosomes

The increase of the RNA content in the rat kidney after folic acid is connected with an increased labelling rate of the cytoplasmic rRNA. Table 2 shows that the specific activities in the whole RNA and in the 28-S and 18-S-RNA species from kidney microsomes 20 hr after injection of folic acid and the radioactive precursor are two to three and a half times higher than that of control rats. Though, at present, changes in the endogenous precursor pools of cytoplasmic rRNA could not be taken into account, a stimulated formation of cytoplasmic rRNA after folic acid seems very likely. Thereby it remains unknown whether the increase of store of ribosomes is effected either by processing more intermediaries at one time or by accelerating the rate of synthesis of mature rRNA. Table 2 shows that the labelling of the 28-S- and 18-S-RNA extracted from the microsomal fraction of kidneys from folic acid treated rats is equivalent. The same effect, a parallel and equivalent incorporation of protein

TABLE 2. SPECIFIC ACTIVITIES (dis./min/μg RNA) IN THE RNA OF KIDNEY MICROSOMES FROM RATS TREATED WITH FOLIC ACID

	RNA extracted by the method of Ogur and Rosen[11] (dis./min/μg RNA)	RNA extracted by the phenol method[13] (dis./min/μg RNA)	
		28-S-RNA	18-S-RNA
1·13 mM folic acid per kg body wt. 20 hr before	1082	1675	1640
10 ml 0·3 M $NaHCO_3$ per body wt. 20 hr before	362	490	472

Pulse labelling with 1 mc 5-T-orotic acid/kg body wt. over 20 hr. RNA extraction as given in methods. The data are expressed as the arithmetic mean of two undependent experiments.

and RNA precursors into both the 30-S and 50-S subunit was observed by Hanoune and Feigelson[33] under comparable conditions of a stimulated synthesis of liver ribosomes after cortisone application. Because of our knowledge about dissociation and reassociation of animal ribosomal subunits between each round of translation of mRNA[34,35] it seems probable that the newly built ribosomal particles are involved in the stimulated protein synthesis after folic acid.

Distribution of newly synthesized membrane bound and free ribosomes

The decrease of the protein/RNA ratio in the kidney microsomes from folic acid treated rats leads to the assumption that an increase of the number of ribosomes is connected with changes in the proportion of free to bound ribosomes. In order to study this question we dissociated the membrane bound and free kidney ribosomes by centrifugation of the microsomal pellet over a sucrose gradient as given in Methods. The radioactivity recovered in the PCA insoluble, lipid free gradient fraction after a pulse labelling for at least 24 hr with 5-T-orotic acid serves as a measure for the synthesis of ribosomes. In Fig. 2 the fast sedimenting peaks of the extinction curves at 260 nm (first fractions) represent the membrane bound ribosomes. The slow sedimenting peaks represent the free ribo- and polysomes.

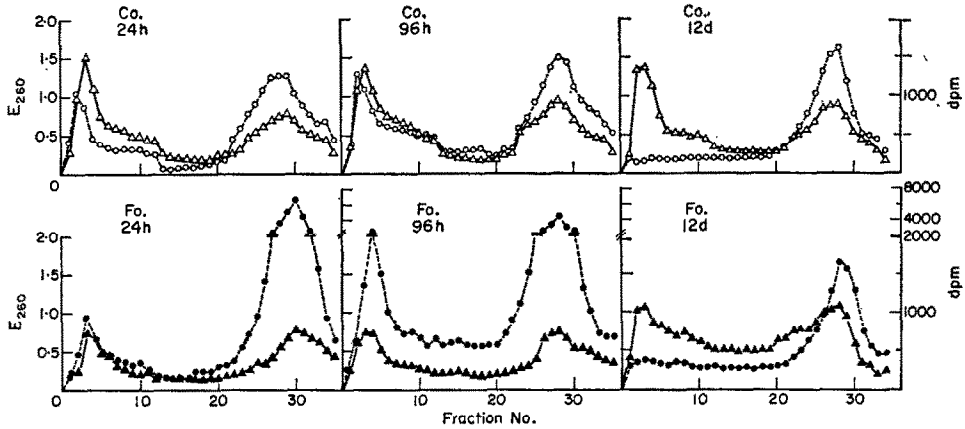

FIG. 2. Sedimentation profiles of the 105,000 *g* pellet from rat kidneys over a 15–40 per cent linear gradient of sucrose centrifuged for 90 min at 24,000 rev/min in a SW 25.1 Spinco-rotor. The left peak refers to the bound the right peak to the free ribo-, polysomes. PCA insoluble, lipid free radioactivity (dis./min/ml diluted gradient fraction) recovered in kidney ribo-, polysomes from rats treated with folic acid ●——● and control rats ○——○ after different labelling times with 5-T-orotic acid. Injection of 200 μc 5-T-orotic acid/kg body wt. and 1·13 mM folic acid/kg body wt. at the same time 24 hr, 96 hr, 12 days before removal of the kidneys. Control rats received 0·3 M $NaHCO_3$-solution and 5-T-orotic acid at equal amounts and times. Extinction at 260 nm (E_{260}) of ribo-, polysomes from folic acid treated rats ▲——▲ and control rats △——△. Extinction refers to the same dilution of gradient fraction as radioactivity.

Twenty-four and 48 hr (not shown) after folic acid the protein/RNA ratio in the fast sedimenting bound ribo- and polysomes is decreased from 7·8 (controls) to 4·6. This confirms the assumption made from Table 3 that the augmentation rate of ribosomes in the bound ribosome fraction is greater than the proliferation rate of

membranous structures and that membranous structures were possibly partly dissolved at that time. Similar findings after toxic agents as CCl_4[36] and after partial hepatectomy[37,38] consisting of fragmentation and dilatation of rough surfaced cisternae with fewer attached ribosomes were observed. On the other hand experiments on the formation of liver ribosomes and endoplasmic membranes during hormone-induced tadpole metamorphosis[39] showed that the aggregation of polysomes is connected with a stimulated synthesis of phospholipids in the smooth and rough membranes of the endoplasmic reticulum. In this connection the exact half life of endoplasmic membranes and ribosomes has to be studied in order to decide whether the turnover of certain membrane components and ribosomes in the folic acid induced kidney proliferation shows the same rate as stated for the normal and proliferation liver.[40,41]

Figures 2 and 3 show that the labelling patterns of free and bound ribosomes 24 and 96 hr after injection of the RNA precursor were nearly the same. This agrees with the findings of Malt *et al.*[42] for mouse kidney ribosomes. Figure 2 further shows that the recovered radioactivity in the slow sedimenting free ribosomes 24 and 96 hr after folic acid is markedly higher than the radioactivity in the control ribosomes. The increased labelling of the free kidney ribosomes 96 hr after folic acid is very probably due to the long lasting half life of the previously synthesized ribosomes because there is no increased incorporation of 5-T-orotic acid with a 24-hr pulse labelling at 96 hr after folic acid, as could be seen in Fig. 3. The stronger labelling of the free kidney ribosomes after folic acid makes a difference to the labelling pattern in the renoprival kidney[42,43] where an equal labelling of the free and bound ribosomes and an unchanged proportion of membrane bound ribosomes and their aggregates were observed. This may be due mainly to the difference between the two experimental systems employed, namely the compensatory hypertrophy after unilateral nephrectomy and the regenerative proliferation after tubular damage by folic acid which is probably accompanied by a changed ribosome–membrane interaction. The role of the ribosome association to endoplasmic membranes still remains unexplained. Stabilizing of mRNA, restriction of free exchange of certain ribosome populations, facilitating the formation of certain proteins by compartmentalization[44] are under discussion.

Figure 2 shows that there is no increased labelling of the fast sedimenting membrane bound kidney ribosomes 24 hr after folic acid, at a time when the free kidney ribosomes are strongly labelled 96 hr after folic acid the bound ribosomes are more strongly labelled than the controls. This could mean that newly synthesized free ribosomes are layered on the endoplasmic membranes, possibly by an exchange mechanism, at a later time or that different precursor pools for bound and free ribosomes exist, of which that for the bound ribosomes supplies the radioactive material at a later time. The assumption that first the synthesis of free kidney ribosomes is stimulated and later some of the free ribosomes associate with the endoplasmic membranes is supported by Fig. 3 which shows that after a 24-hr pulse labelling, no radioactivity was recovered in the membrane bound kidney ribosomes 96 hr after folic acid. This would also be in keeping with the following facts: Free kidney ribosomes were always labelled with [^{14}C]orotic acid earlier than the membrane bound ribosomes.[45] In the developing kidney no ribosomes are bound to membranes until 2 days after birth when the proportion rises to 20 per cent.[46] Similar findings and conclusions for free and bound liver ribosomes were made by Halliman and Munro.[47]

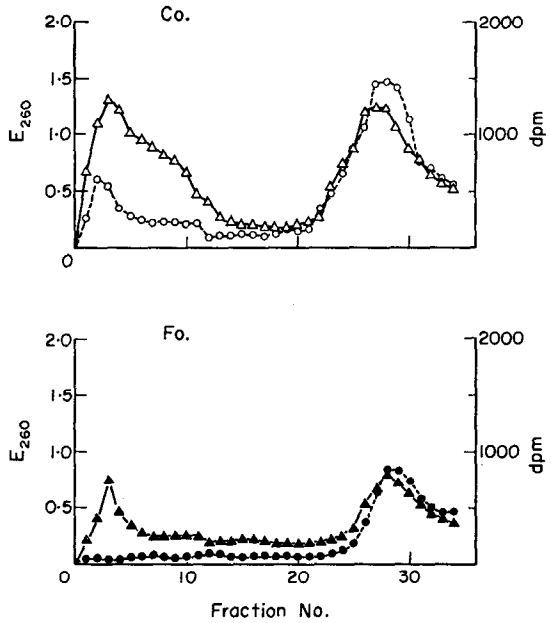

FIG. 3. Sedimentation profile of the 105,000 *g* pellet from rat kidneys. See Fig. 2 for details. Recovered radioactivity in ribo-, polysomes from rats treated with folic acid ●——● and control rats ○——○ after labelling with 5-T-orotic acid. Injection of 1·13 mM folic acid/kg body wt. 96 hr and injection of 200 μc 5-T-orotic acid 24 hr before removal of the kidneys. Extinction at 260 nm (E_{260}) of ribo-, polysomes from folic acid treated rats ▲——▲ and control rats △——△.

The duration of association of newly synthesized ribosomes to membranes seems to be shorter than the half life of newly synthesized ribosomes: 12 days after injection of the RNA precursor, the slow sedimenting free kidney ribosomes from control and treated rats are still labelled while the fast sedimenting bound ribosomes are not, as shown in Fig. 2. This statement is restricted by a possible different reiterated use of radioactivity by bound and free ribosomes.

REFERENCES

1. G. THRELFALL and D. M. TAYLOR, *Eur. J. Biochem.* **8,** 591 (1969).
2. W. BRADE, H. HERKEN and H.-J. MERKER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **266,** 95 (1970).
3. W. BRADE, H. HERKEN and H.-J. MERKER, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **262,** 228 (1969).
4. S. KISHIMOTO and I. R. LIEBERMAN, *J. cell. Biol.* **25,** 103 (1965).
5. M. J. LEE, M. H. VAUGHAN and R. ABRAMS, *J. biol. Chem.* **245,** 4525 (1970).
6. I. R. LIEBERMAN and P. OVE, *J. biol. Chem.* **235,** 1765 (1960).
7. I. R. LIEBERMAN, R. ABRAMS, N. HUNT and P. OVE, *J. biol. Chem.* **238,** 3955 (1963).
8. H. HERKEN, P. VOSS, W. BRADE and H. KOLBE, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **267,** 297 (1970).
9. G. THRELFALL, D. M. TAYLOR and A. T. BUCK, *Lab. Invest.* **15,** 1477 (1966).
10. J. R. FLORINI and CH. B. BREUER, *Biochemistry* **5,** 1870 (1966).
11. M. OGUR and G. ROSEN, *Archs. Biochem.* **25,** 262 (1950).
12. W. MEJBAUM, *Hoppe-Seylers Z. physiol. Chem.* **258,** 117 (1939).
13. H. L. SÄNGER and C. A. KNIGHT, *Biochem. biophys. Res. Commun.* **13,** 455 (1963).
14. G. SCHMIDT and S. J. THANNHAUSER, *J. biol. Chem.* **161,** 83 (1945).

15. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
16. J. G. WOOL and P. CAVICCHI, *Biochemistry* **6,** 1231 (1967).
17. E. C. HENSHAW, T. B. BOJARSKI and H. H. HIATT, *J. molec. Biol.* **7,** 122 (1963).
18. N. TALAL and E. D. EXUM, *Proc. natn. Acad. Sci. U.S.A.* **55,** 1288 (1966).
19. J. VETTER and W. BRADE, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **269,** 462 (1971).
20. D. M. NICHOLLS, M. P. RYAN, S. H. MIALL, C. G. WESTALL and I. D. CAPPON, *Can. J. Biochem.* **48,** 308 (1970).
21. M. NOMURA, S. MIZUSHIMA, M. OSAKI, P. TRAUB and C. W. LOWRY, in *Cold Spring Harbor Symposia on Quantitative Biology* Vol. XXXIV. Cold Spring Harbor, L.I. New York (1969).
22. E. A. SMUCKLER, O. A. ISERI and E. P. BENDITT, *J. exp. Med.* **116,** 55 (1962).
23. G. HONIG and M. R. RABINOVITZ, *Science* **149,** 1504 (1965).
24. R. SOEIRO and H. AMOS, *Biochim. biophys. Acta* **129,** 406 (1966).
25. M. I. SURKS, *Am. J. Physiol.* **218,** 842 (1970).
26. J. R. WARNER and R. SOIERO, in *Exploitable Molecular Mechanisms and Neoplasia*, p. 57. Williams & Wilkins, Baltimore (1969).
27. W. THOENES, *Mikromorphologie des Nephrons nach temporärer Ischämie.* Thieme, Stuttgart (1964).
28. R. A. MALT, *J. Surg. Res.* **6,** 152 (1966).
29. K. TSUKADA and I. R. LIEBERMAN, *Biochem. biophys. Res. Commun.* **19,** 702 (1965).
30. R. J. STENGER and D. B. CONFER, *Exp. molec. Path.* **5,** 455 (1966).
31. B. S. BALIGA, A. W. PRONCZUK and H. N. MUNRO, *J. mol. Biol.* **34,** 199 (1968).
32. A. R. SUBRAMANIAN, B. D. DAVIS and R. J. BELLER, in *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. XXXIV. Cold Spring Harbor, L.I., New York (1969).
33. J. HANOUNE and F. FEIGELSON, *Biochim. biophys. Acta* **199,** 214 (1970).
34. B. L. HOGAN and A. KORNER, *Biochim. biophys. Acta* **169,** 71 (1968).
35. M. H. VAUGHAN, J. R. WARNER and J. E. DARNELL, *J. molec. Biol.* **25,** 235 (1967).
36. N. KRISHNAN and R. J. STENGER, *Am. J. Path* **49,** 239 (1967).
37. G. DELHUMEA DE ONGAY, Y. MOULE and C. FRAYSSIMEL, *Expl. Cell Res.* **38,** 187 (1965).
38. I. BARTOK and S. Z. VIRAGH, *Z. Zellforsch.* **68,** 741 (1965).
39. J. R. TATA, *Biochem. J.* **105,** 783 (1967).
40. G. DALLNER, P. SIEKEVITZ and G. E. PALADE, *J. cell Biol.* **30,** 73 (1966).
41. J. N. LOEB, R. R. HOWELL and G. N. TOMKINS, *Science.* **149,** 1093 (1965).
42. R. A. MALT and S. K. STODDARD, *Biochim. biophys. Acta* **119,** 207 (1966).
43. G. C. PRIESTLEY and R. A. MALT, *J. cell Biol.* **41,** 886 (1969).
44. A. R. WILLIAMSON, in *Essays in Biochemistry* **5,** 139 (1969).
45. R. A. MALT and D. A. LEMAITRE, *Biochim. biophys. Acta* **145,** 190 (1967).
46. G. C. PRIESTLEY and R. A. MALT, *J. cell. Biol.* **37,** 703 (1968).
47. T. HALLIMAN and H. N. MUNRO, *Biochim. biophys. Acta* **108,** 285 (1965).