

RIBOSOMAL RNA GENES IN SALIVARY GLAND AND OVARY OF *RHYNCHOSCIARA ANGELAE*

A. G. GAMBARINI and R. MENEGHINI. From the Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 20780, São Paulo, Brasil

INTRODUCTION

Two facts make it important to determine whether the proportion of ribosomal RNA (rRNA)¹ genes in salivary glands of *Rhynchosciara angelae* varies throughout the end of the last (fourth) instar of larval development, namely: (a) In this instar, during the appearance of "DNA puffs," it is possible to observe many micronucleoli around the chromosomes (1). Perhaps the micronucleoli may be ascribed to an extra synthesis of ribosomal DNA (rDNA), in analogy to what has been proposed in *Hyboscara*, to explain similar microbodies (2). (b) *In situ* hybridization experiments showed that the rRNA genes are located mainly at the heterochromatic ends of chromosomes X and C (3). As heterochromatic regions are less active in replication in polytene chromosomes (4-6), one would suspect an under-replication of the rRNA genes during polytenization, similar to what has been observed in *Drosophila hydei* (7). However, this was not observed for *Rhynchosciara* (18).

To test both these hypotheses, we hybridized rRNA with salivary gland DNA from larvae of different ages at the end of larval life. An alteration in the hybridization level during the polytenization would indicate either an under-replication or amplification of the rRNA genes.

In this paper, we verified that the proportion of rRNA genes does not change during a period of time corresponding to almost two cycles of DNA replication at the end of larval life. It was also observed that the number of rRNA genes in ovary

¹ Abbreviations used in this paper: rDNA, the DNA which codes for rRNA; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; SSC, 0.015 M sodium citrate, 0.15 M sodium chloride.

tissue of adult *Rhynchosciara* is higher than in salivary glands. The possible implications of these facts are discussed.

METHODS

Animals

All experiments were performed with fourth instar female larvae or with adult females, raised in the laboratory as previously described (8). The fourth instar corresponds to nearly 65% of the total larval life and, for convenience, was divided into six periods, characterized by morphological and physiological events, as detailed elsewhere (9). Salivary gland DNA from three different larval ages was used in the hybridization experiments. The larvae used were: (a) 44-46 days old, corresponding to period III of the fourth instar; (b) 52-54 days old, when puffs are at maximum size and corresponding to period IV; (c) 55-56 days when most of the DNA puffs have contracted, corresponding to period V. The DNA from these three periods will be called "pre-puff DNA," "puff DNA," and "after-puff DNA," respectively.

Ribosomal RNA Preparation

About 500 newly born larvae were placed on agar, in Petri dishes, with 1 g of food (8), containing 2 mCi of uridine-5-³H (28 Ci/mmol), and 1 mCi of orotic acid-5-³H (10 Ci/mmol). The food-isotope mixture was changed every 3 days, until the 10th, when the larvae were starved for 1 day, and then used for RNA extraction. Larvae were homogenized in a Potter-Elvehjem homogenizer with Tris buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 10 mM Mg acetate) containing 1% sodium dodecyl sulfate (SDS) at 2-3°C. The homogenate was shaken with an equal volume of phenol saturated with Tris buffer, and the suspension was centrifuged. The aqueous phase was again mixed with phenol, shaken, and centrifuged.

This step was repeated until a clear interphase was observed. The NaCl concentration of the final aqueous phase was brought to 0.15 M, and 2 vol of ethanol were added. After standing overnight at -20°C , RNA was collected by centrifugation and dissolved in an appropriate volume of a buffer containing 10 mM Tris-HCl (pH 7.4) and 50 mM NaCl. About 100 μg of RNA was applied to each 4.5 ml sucrose gradient (10–30% w/v), in 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 100 mM NaCl. Centrifugation was carried out at 4°C at 45,000 rpm for $3\frac{1}{2}$ hours in the SW 50 L rotor (Spinco) (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). Fractions were collected by puncturing the bottom of the tube and diluted to 0.5 ml for absorbance readings. 0.05 ml-samples of each fraction were used for radioactivity determination in order to show the coincidence of absorbance and radioactivity profiles. A typical result is shown in Fig. 1. Fractions corresponding to peaks 28S and 18S were pooled, the RNA was precipitated by addition of 2 vol of ethanol, and dissolved in 10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl. The specific activity of this RNA was 12,000 cpm/ μg and was determined in a Tri-Carb scintillation spectrometer. The ratios A_{260}/A_{230} and A_{260}/A_{280} for the RNA solution were 2.40 and 2.07, respectively.

DNA Preparation

The same procedure was applied to about 600 salivary glands or 300 ovaries. Homogenization was

done in a Dounce homogenizer with 10 ml of saline-ethylenediaminetetraacetate (EDTA) (10 mM EDTA, 100 mM NaCl, pH 8.3) containing 0.5% Nonidet P-40 (Shell do Brasil S. A.). Chromatin was collected by centrifuging at 3000 g and washed once by centrifuging at 10,000 rpm with saline-EDTA. Chromatin was then resuspended in 5 ml of saline-EDTA containing 100 $\mu\text{g}/\text{ml}$ RNase (Worthington Biochemical Corp., Freehold, N. J., previously freed of DNase by heating at 80°C for 5 min) and 100 $\mu\text{g}/\text{ml}$ α -amylase (Worthington). After incubation for 15 min at 37°C , SDS and pronase (predigested at 60°C for 5 min) were added to final concentrations of 1% (w/v) and 500 $\mu\text{g}/\text{ml}$, respectively. Incubation continued at 60°C for 15 min and then for 30 min at 37°C . The suspension was then cooled, NaCl was added to a final concentration 1 M, and proteins were extracted by two cycles of shaking with equal volume of chloroform-isoamyl alcohol (25:1 v/v), followed by centrifugation. DNA was precipitated from the aqueous solution by addition of 2 vol of ethanol, collected by centrifugation, and resuspended in 0.01 M Tris-HCl (pH 7.4). Before using, DNA was further purified by banding in CsCl gradient, as indicated below.

Centrifugation in CsCl Density Gradient

The technique described by Flamm et al. (10) was used. To 3.36 ml of a DNA solution containing Tris 0.01 M, pH 7.4, 4.271 g of CsCl were added. This yields 4.5 ml of a solution with a density of 1.700

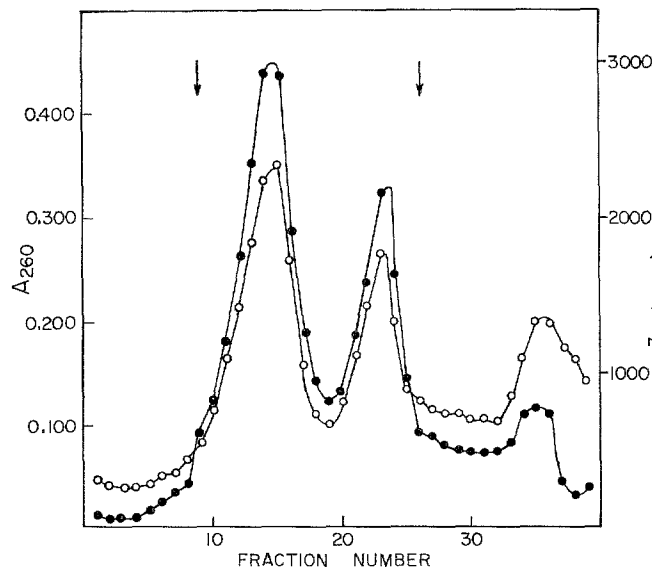


FIGURE 1 Sedimentation in sucrose gradient of larval RNA- ^3H RNA was extracted and centrifuged (100 μg) as described in Methods. 40 fractions were collected and diluted to 0.5 ml for absorbance readings (O—O). 0.05-ml samples of each fraction were applied to pieces of Whatman No. 1 paper, for radioactivity determination in a Beckman Beta spectrometer (●—●). Samples included in the interval indicated by the arrows were pooled and processed as indicated in Methods.

g/cm³. After centrifugation at 42,000 rpm for 36 hr, fractions were collected by puncturing the bottom of the tube and diluted to 0.6 ml with 10 mM Tris-HCl (pH 7.4) for absorbance readings. To perform hybridization experiments with separate fractions from the CsCl gradient, the DNA was sheared by sonication before centrifugation. Unsheared DNA was used in saturation experiments. In this case, the fractions corresponding to the DNA band were pooled and treated with 2 vol of ethanol, the precipitated DNA was collected by centrifugation and dissolved in 10 mM Tris-HCl (pH 7.4).

Hybridization Experiments

Essentially the technique described by Gillespie and Spiegelman (11) was followed. DNA was denatured by adding an equal volume of 1 M NaOH and maintained for at least 10 min at room temperature before neutralizing with 4 vol of a solution containing 0.25 M Tris-HCl (pH 8.0), 0.25 M HCl, and 1 M NaCl. 10 μg of denatured DNA were applied to each nitrocellulose membrane filter (Millipore, GS 13 mm diameter, Millipore Corp., Bedford, Mass.), dried overnight in a desiccator, and then for 2 hr in

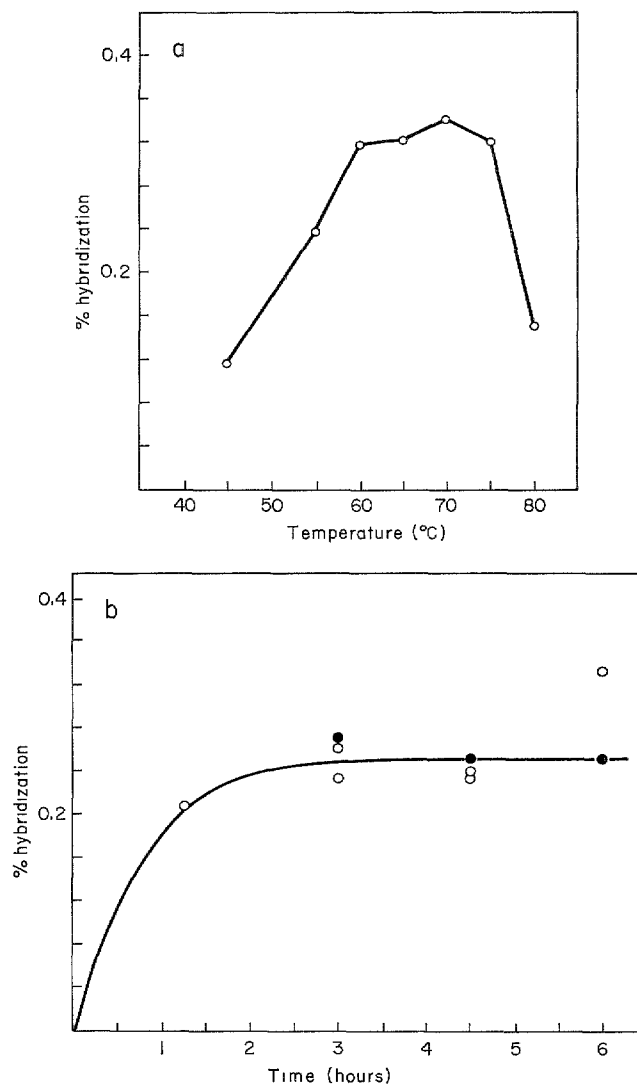


FIGURE 2 Influence of temperature and time in the annealing reaction. Hybridization was performed with 3.0 μg/ml of rRNA-³H (12,000 cpm/μg) as described in Methods, except that temperature (a) or time (b) was varied as indicated. In both cases the DNA used is a mixture of DNA from several tissues of *Rhynchosciara*. In Fig. 2 b, more than one determination was made for some points, and in this case full circles correspond to sonicated DNA and empty circles to nonsonicated DNA.

an oven at 80°C. The annealing reaction was carried out in 2 ml of 6X SSC (0.015 M sodium citrate, 0.15 M sodium chloride), at 68°C. Above this temperature, an accentuated decrease in hybridization efficiency is observed (Fig. 2 a). Time of annealing was chosen as 4 hr, beyond which no increase in hybrid formation is observed (Fig. 2 b). At the end of the reaction, the filters were washed by swirling in a beaker with 6X SSC and then with 2X SSC. RNase treatment was carried out with 20 µg/ml RNase (Worthington) in 2X SSC, at room temperature, for 30 min. This was followed by washing with 2X SSC. After determining the radioactivity of each filter, they were washed with chloroform, dried, and incubated in HCl, at 98°C for 20 min. DNA retention averaged 90–95% of the input DNA as determined by reading the absorbance of the acid solution, at 268 mµ (27.8 units of absorbance corresponding to 1 mg/ml DNA).

Before the annealing reaction, rRNA was heated in a boiling water bath for 3 min and immediately chilled in an ice bath. This procedure increases the formation of hybrid by about 70%, presumably due to loss of secondary structure of the rRNA, which has a melting temperature of 66°C in 6X SSC.

RESULTS

Hybridization of rRNA to DNA

Fractionated by CsCl Centrifugation

Previous results have shown that by hybridizing *Xenopus* rRNA-³H to *Rhynchosciara* DNA fractions obtained by CsCl centrifugation, the peak of the hybrid appears in a higher density position than the main band DNA (3, 12, and A. G. Gambarini, M. Birnstiel, and F. J. S. Lara, unpublished results). As shown in Fig. 3, the same pattern of hybridization is obtained by using the homologous RNA. This result is a good indication that *Rhynchosciara* rRNA is a satisfactorily pure preparation for the experiments which are described below, since nonribosomal RNA hybridizes in the region of the main peak of DNA (12).

Hybridization of rRNA to Salivary Gland and Ovary DNA

In the experiment shown in Fig. 4, salivary gland DNA from three different stages of development and ovary DNA were hybridized with increasing concentration of rRNA-³H. Just one saturation curve was traced by the set of points of salivary gland DNA hybridization, since a slight dispersion of 4% of the results at the saturation level does not allow one to discriminate between

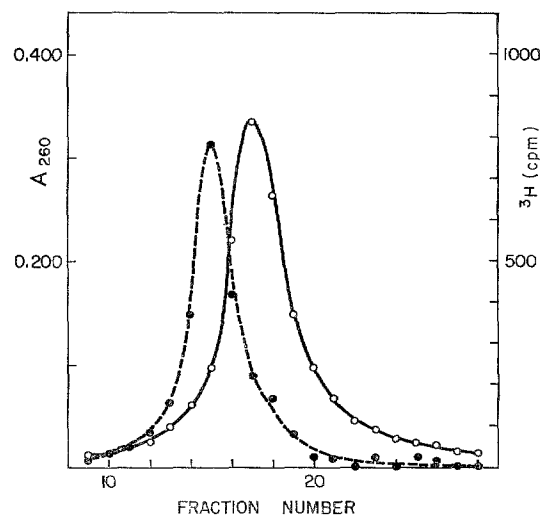


FIGURE 3 Hybridization of rRNA-³H with CsCl fractionated DNA from *Rhynchosciara*. 70 µg of ovary DNA was sonicated and centrifuged for 36 hr at 42,000 rpm in the Beckman No. 50 Ti rotor (Spinco) at 20°C. 0.1 ml-fractions were collected, the absorbance at 260 mµ was determined, and the DNA was hybridized with 2.0 µg/ml rRNA-³H (12,000 cpm/µg), under the conditions described in Methods. (O) absorbance at 260 mµ; (●) cpm hybridized.

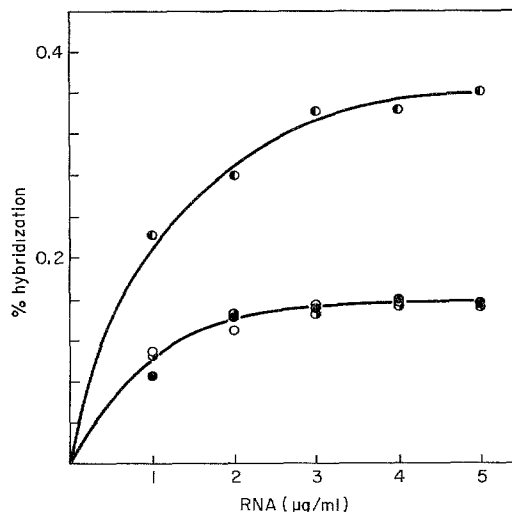


FIGURE 4 Hybridization of rRNA-³H with salivary gland and ovary DNA. Salivary gland DNA from three different larval ages and ovary DNA were immobilized in nitrocellulose filters and hybridized with increasing concentrations of rRNA-³H (12,000 cpm/µg) as described in Methods. Pre-puff DNA (O—O); puff DNA (●—●); after-puff DNA (●—●); ovary DNA (●—●).

possible small differences. However, a clear distinction between saturation curves for salivary gland DNA and ovary DNA can be observed. Ovary DNA hybridizes about twice as much as salivary gland DNA.

Rhynchosciara salivary gland cells have a haploid genome of 1.3×10^{11} daltons as determined by DNA renaturation kinetics (13). Taking 0.160% as the value for rRNA hybridization in this tissue (Fig. 4) and multiplying by two, in order to correct for duplex DNA, one can calculate the figure of 4.2×10^8 daltons of rDNA per haploid genome. The molecular weights of major and minor rRNA in *Rhynchosciara* are 1.4×10^6 and 0.73×10^6 , respectively (F. J. S. Lara and W. Loening, unpublished results). This gives the value of 4.2×10^6 daltons for the cistron of these two RNAs. The number of cistrons is, according to this, about 100 per haploid genome. The haploid genome should be understood here as the simplest genome of a cell in a specific tissue, at a specific time of development, since chromosomal constitution in somatic and germinative cells is different (14). Moreover, there are indications that in salivary glands certain DNA species may be amplified (12, 15–17). Ovary cells also have a haploid genome of 1.3×10^{11} daltons (J. Balsamo, J. Hierro, and F. J. S. Lara, unpublished results). Fig. 4 shows that 0.36% of ovary DNA hybridizes with rRNA. Therefore, the number of cistrons, calculated as above, is about 220. However, in this case, we are probably dealing with a mean value since the three different types of ovary cells have different chromosome constitutions (14).

DISCUSSION

The results presented here show clearly that rRNA genes are reiterated in *Rhynchosciara* genome. In salivary gland cells the number of these genes is about 100, and this number seems to be maintained virtually constant throughout the end of the fourth instar (Fig. 4). This result indicates that rRNA genes are not amplified during the final part of the fourth instar. Therefore, the micronucleoli which appear at this time and which contain DNA complementary to rRNA (3) should not be involved in rDNA amplification, as proposed in the case of *Hybosciara* (2). In this respect, our data are in accord with those previously reported by Gerbi (18).

It has been observed that genes for rDNA in *Rhynchosciara* are located in heterochromatin of

chromosomes X and C (3). In other diptera, the heterochromatin of polytene chromosomes is less active in replication (4–6, 19). *Rhynchosciara* seems to agree with other diptera in this respect, since it has been shown that a highly repetitive DNA fraction, presumably belonging to heterochromatin, is not replicated in the polytenic chromosomes of salivary glands (13). Apparently, rDNA is not under the same control, despite its location in heterochromatin, since its proportion remains unchanged during a period of 15 days, when the DNA content in salivary gland cells increases almost four times (R. Meneghini and M. Cordeiro, unpublished results). Therefore, genes for rRNA appear to be replicating at the rate of euchromatic DNA, perhaps due to some independent control as proposed by Gall et al. (19).

rDNA amplification has been observed in the ovaries of some insects (20–22). It is possible that the same is occurring in the case of *Rhynchosciara*. However, the cytology of *Rhynchosciara* does not suggest that such an amplification has taken place (14). In fact, the nurse cells of the ovary produce large amounts of RNA, part of this RNA being transferred to the oocyte, which has little activity in nucleic acid metabolism (14). This would be a typical case where amplification of rRNA genes in the oocyte would not be expected (20). One possible alternative is that in the process leading to the enormous polyploidy in nurse cells (14), the chromosomes which contain genes for rRNA are preferentially replicated. Another possibility is that the saturation value found with ovary DNA represents that of a normal haploid genome. In salivary glands this value would be lower due to an under-replication of rDNA during polytenization. This under-replication would not be necessarily observed in a short space of time (Fig. 4) either because it is very slow or because it occurs at an earlier time of the development. This possibility is speculative and is just mentioned because of some preliminary results showing that fatty body cells of *Rhynchosciara*, whose chromosomes are much less polytenic than those from salivary glands, present a higher proportion of rRNA genes than the latter tissue (A. G. Gambarini, M. Birnstiel, and F. J. S. Lara, unpublished results).

SUMMARY

The number of rRNA genes per haploid genome in salivary glands and ovary of *Rhynchosciara angela*

was determined by RNA-DNA molecular hybridization. In salivary glands, this number is about 100 and does not change throughout a period of 10 days, at the end of the fourth instar of larval development, when amplification of other genes is observed. In the mature ovary tissue, the number of ribosomal genes is about 220. The possible explanation for the difference of ribosomal genes between ovary and salivary gland is discussed.

We wish to thank Professor F. J. S. Lara for helping to translate the manuscript and for the facilities offered at his laboratory. We also thank Dr. H. Chaimovich and Dr. J. Balsamo for critically reviewing the manuscript.

Received for publication 20 December 1971, and in revised form 17 April 1972.

REFERENCES

1. GABRUSEWYCZ-GARCIA, N., and R. G. KLEINFELD. 1966. *J. Cell Biol.* **29**:347.
2. DA CUNHA, A. B., C. PAVAN, J. S. MORGANTE, and M. C. GARRIDO. 1969. *Genetics*. **61** (Suppl.):335.
3. PARDUE, M. L., S. A. GERBI, R. A. ECKHARDT, and J. G. GALL. 1970. *Chromosoma*. **29**:268.
4. RUDKIN, G. T. 1965. *Genetics*. **52**:470.
5. BERENDES, H. D., and H. G. KEYL. 1967. *Genetics*. **57**:1.
6. MULDER, M. P., P. VAN DUYN, and H. J. GLOOR. 1968. *Genetika*. **39**:385.
7. HENNIG, W., and B. MEER. 1971. *Nat. New Biol.* **233**:70.
8. LARA, F. J. S., H. TAMAKI, and C. PAVAN. 1965. *Am. Nat.* **99**:189.
9. ARMELIN, H. A., R. MENEGHINI, and F. J. S. LARA. 1969. *Genetics*. **61**(Suppl.):351.
10. FLAMM, W. G., H. E. BOND, and H. E. BURR. 1966. *Biochim. Biophys. Acta*. **129**:310.
11. GILLESPIE, D., and S. SPIEGELMAN. 1965. *J. Mol. Biol.* **12**:829.
12. MENEGHINI, R., H. A. ARMELIN, J. BALSAMO, and F. J. S. LARA. 1971. *J. Cell Biol.* **49**:913.
13. BALSAMO, J., J. HIERRO, M. BIRNSTIEL, and F. J. S. LARA. 1971. Gene Expression and Its Regulation. International Symposium on Protein Synthesis and Nucleic Acids, La Plata, Argentina, December 1971. Plenum Publishing Corporation, New York.
14. BASILE, R. 1969. *Genetics*. **61**(Suppl.):261.
15. BREUER, M. E., and C. PAVAN. 1965. *Chromosoma*. **7**:371.
16. RUDKIN, G. T., and S. L. CORLETTE. 1957. *Proc. Natl. Acad. Sci. U. S. A.* **43**:964.
17. FICQ, A., and C. PAVAN. 1957. *Nature (Lond.)*. **180**:983.
18. GERBI, S. A. 1971. *J. Mol. Biol.* **58**:499.
19. GALL, J. G., E. H. COHEN, and M. L. POLAN. 1971. *Chromosoma*. **33**:319.
20. GALL, J. G. 1969. *Genetics*. **61**(Suppl.):121.
21. GALL, J. G., H. C. MACGREGOR, and M. E. KIDSTON. 1969. *Chromosoma*. **26**:169.
22. LIMA-DE-FARIA, A., M. BIRNSTIEL, and H. JAWORSKA. 1969. *Genetics*. **61**(Suppl.):145.

A SIMPLE METHOD FOR THE PREPARATION OF RENAL BRUSH BORDERS

ROBERT J. CHERTOK and SUZANNE LAKE. From the Bio-Medical Division, Lawrence Livermore Laboratory, University of California, Livermore, California 94550

INTRODUCTION

The brush-border membranes of intestinal epithelial cells have been studied rather extensively and are known to contain several enzyme activities (1-4). The renal brush border is located exclusively on the luminal surface of the plasma membrane of the proximal convoluted tubule. Since 80% of the glomerular filtrate is absorbed in the proximal tubule, this suggests that the renal brush border is intimately involved with the reabsorptive mechanisms. Much is known about the

relationships between fine structure and function in the kidney, but biochemical studies of the brush-border membrane of the proximal tubule are just beginning.

Several methods of isolating brush borders from kidney homogenates have recently been developed (5, 6, 7). Biochemical studies of such preparations show them to be relatively free of subcellular contamination.

There is still a need, however, for a method of isolating renal brush borders that is neither

tedious nor time-consuming but yields a preparation relatively free of subcellular contamination. The procedure to be described, a modification of the method of Miller and Crane (1), is such a method.

METHODS

Brush Border Preparation

The renal brush borders were isolated from New Zealand white rabbits, weighing approximately 3 kg, by a modification of the procedure of Miller and Crane (1). The animals were killed by a blow to the base of the skull, and the kidneys were removed and placed in cold physiological saline. All subsequent procedures were conducted in the cold. The kidneys were decapsulated and the cortex was removed with a glass slide into cold 5 mM ethylenediaminetetraacetate (EDTA), pH 7.3. The scrapings were homogenized for 30 sec in a Waring Blender with a Variac control set at 90. 50 ml of 5 mM EDTA were used per two rabbits, and a 50-ml volume was homogenized at a time. A small sample of this homogenate was rehomogenized in a Sorvall Omni-Mixer and diluted to be used in the enzyme assays to establish cortex tissue activity.

The remaining homogenate was filtered through glass wool and then through 40- μ mesh nylon cloth. This resulted in removal of the unbroken pieces of cortex, larger subcellular pieces of cortex, and the nuclei. The volume of the filtrate was then increased to 150 ml per two rabbits and centrifuged at 270 *g* for 10 min. The sediment was resuspended in 5 mM EDTA pH 7.3 and centrifuged again. The sediment on the side of the tube and the loosely packed portion of the pellet were aspirated and discarded. The pellet was washed twice in EDTA, then resuspended in 90 mM NaCl-5 mM EDTA and allowed to stand on ice for 20 min. The suspension was filtered through glass wool, centrifuged, and washed in NaCl-EDTA. The resulting pellet was resuspended in distilled water. The enzyme studies were done with this suspension of brush borders.

Protein was determined by the biuret technique (8).

Enzyme Studies

The degree of purification of brush-border material between the original homogenate and the final preparation was assessed by comparative assays for alkaline phosphatase, trehalase, and maltase. The degree of contamination by other subcellular structures was assessed as follows. for lysosomes, by comparative assays for acid phosphatase (9); for microsomes, by assays of glucose-6-phosphatase (9); for mitochondria, by assays of succinic cytochrome *c* reductase (10);

and for nuclei, by assays for adenosine triphosphate (ATP):nicotinamide mononucleotide (NMN) adenyl transferase (11).

For the alkaline phosphatase assay, each reaction mixture contained 3 μ moles of *p*-nitrophenyl phosphate, with the addition of (3) 0.2 μ moles Zn acetate, 0.5 μ moles CoCl₂, and 0.02 μ moles MgCl₂, in a total volume of 3.2 ml. The reaction mixture contained a minimum of 0.03 mg/ml of protein. The buffer was 0.05 M glycine pH 10.5. Acid phosphatase was determined by a similar method. The enzyme was incubated with 3 μ moles *p*-nitrophenyl phosphate in acetate buffer pH 5.4. Acid phosphatase is inhibited by 2 mM KF and alkaline phosphatase is inhibited by 4 mM EDTA (12). Potassium fluoride was added to the appropriate reaction mixtures; EDTA was added to all. The mixtures were incubated for 10 min at room temperature. One drop (0.05 ml) of 2 M NaOH was added to each to stop the reaction, and to increase the color of the *p*-nitrophenol. The optical density was determined at 410 μ . Acid phosphatase activity was taken to be the fluoride-sensitive activity. Since the optical density of the two different cuvettes with and without KF was compared to the blank, the turbidity of each reaction mixture was determined before the addition of substrate.

Glucose-6-phosphatase, a marker enzyme for microsomes, was assayed according to the method of Hübscher and West (12), with slight modifications. The assay volume was increased to 2 ml, and inorganic phosphate was determined by the method of Fiske-SubbaRow (13).

Succinic cytochrome *c* reductase, the marker for mitochondria, was assayed by the method of Slater and Planterose (14).

Trehalase and maltase, markers for brush border, were determined by the indirect method of Sacktor (15), with modifications. Homogenate or brush-border preparation was incubated with 5 μ moles of substrate (trehalose or maltose) with a minimum of 0.16 mg/ml protein for 30 min at 37°C. This reaction was linear up to 30 min under the conditions used. The reaction mixture was centrifuged at 48,000 *g* for 5 min, and 0.3 ml of the supernatant was added to a reaction mixture containing 1 μ mole nicotinamide adenine dinucleotide phosphate (NADP), 1 μ mole ATP, 1.5 μ mole MgCl₂, 2.5 U hexokinase, 2.5 U glucose-6-phosphate dehydrogenase, and potassium phosphate buffer, pH 6.3 in a total volume of 2.5 ml.

ATP:NMN adenyl transferase, the marker for nuclei, was determined by the method of Kornberg (16). Paired reactions were run using nicotinamide adenine dinucleotide (NAD) in place of NMN to determine if any NAD formed during the incubation was degraded by the homogenate or the brush-border preparation.

RESULTS

The average yield was 7.5 mg of protein compared to the starting material of about 5 g of protein. In a typical experiment, approximately 70% of the protein was lost in the initial filtration and 25% was lost after the first centrifugation.

The results of the enzyme assays (Table I) were used to determine the efficiency of the isolation procedure for purifying renal brush borders. The enzyme values show that much of the contaminating subcellular material is removed in the washing procedure. The microsomes seemed to be the most difficult to remove. The amount of mitochondrial contamination varied with the preparation over a range of about 6–22% and, like the marker activities, seemed to be unrelated to the quantity of starting material or to the degree of protein loss.

The absence of nuclear material in the brush border was determined indirectly, as no values for ATP:NMN adenylyl transferase could be obtained for the homogenate. Therefore, this enzyme was not included in Table I.

Alkaline phosphatase is closely associated with the brush-border membrane of intestinal epithelium (4) and kidney (6), but is less specific than trehalase and maltase, both of which have been shown to be associated with the renal brush borders and lacking in other parts of the kidney (5). Changes in the specific activities of the three enzymes, taken together, should then be informative as to the degree of purification of the brush-border material.

Alkaline phosphatase averaged a 5-fold increase in specific activity (Table I). Both trehalase and maltase averaged 9- to 10-fold increases. Maltase activities were consistently less than those of trehalase, but the purification factors were quite similar from one experiment to the next.

DISCUSSION

The success of any isolation and purification procedure is based on the ability of the resulting preparation to fulfill the requirements set forth by investigators with a specific problem to be studied. Here it was our objective to prepare renal brush borders relatively free of subcellular contamination with a minimum of time and effort, for the purpose of studying transport and enzyme characteristics. The procedure described in this paper seems to fulfill these requirements. The time required from the decapsulation of the kidneys to the final step is under 2 hr. The final preparation appears to be less free of subcellular particles than preparations obtained by other, more tedious and time-consuming methods, but it is adequate for use on the day of preparation. Fig. 1 is an electron micrograph of a preparation.

Microsomes seem to be the primary source of contaminants that are found in our first preparation; this is evident in the high (0.50) purification factor of glucose-6-phosphatase (Table I). When brush-border membranes are released from the tissue, they tend to curl back on themselves, trapping varying amounts of cytoplasm within the confines of the membrane. Even though the

TABLE I
Specific Activities of Representative Enzymes of Subcellular Constituents of the Homogenate and Brush-Border Preparation

	N	Homogenate	Brush borders	Relative specific activities*	Recovery
					%
Alkaline phosphatase‡	5	0.048 ± 0.002	0.239 ± 0.014	4.9	0.75
Acid phosphatase‡	4	0.086 ± 0.008	0.023 ± 0.006	0.24	0.36
Glucose-6-phosphatase‡	5	0.028 ± 0.004	0.014 ± 0.006	0.50	0.08
Trehalase§	5	0.014 ± 0.001	0.133 ± 0.023	9.2	1.54
Maltase§	4	0.010 ± 0.001	0.108 ± 0.026	9.9	1.26
Succinic cytochrome <i>c</i> reductase	5	0.0164 ± 0.0014	0.0024 ± 0.0008	0.14	0.02

* Relative specific activities are the averaged purification factors for each experiment.

‡ Alkaline phosphatase, acid phosphatase, and glucose-6-phosphatase are given as IU per mg protein.

§ Trehalase and maltase are given as μ moles glucose formed per minute per mg protein.

|| Succinic cytochrome *c* reductase is given as ΔOD_{510} per minute per mg protein.

Each value is the mean \pm standard error.

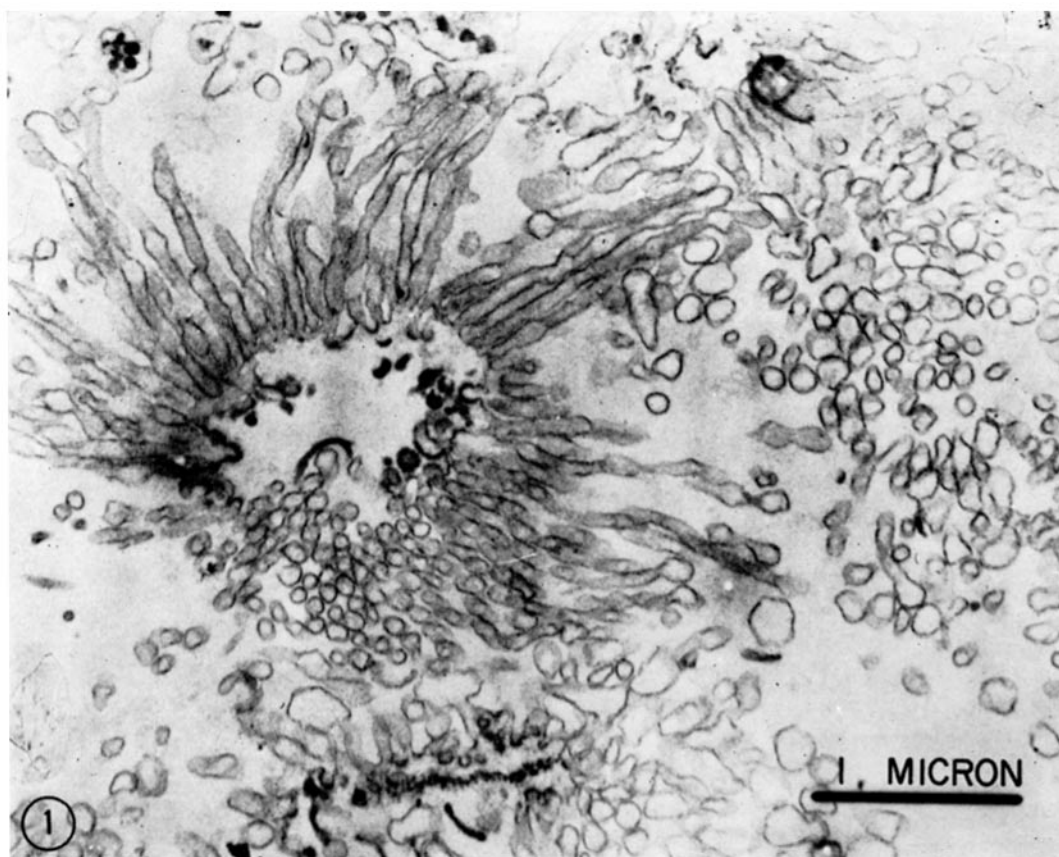


FIGURE 1 Electron micrograph of brush-border preparation. $\times 28,000$

washing procedure reduces the amount of trapped material, it is probable that some of the contaminating subcellular material comes from the trapped cytoplasm.

The variable contamination by mitochondria was mentioned previously as being unrelated either to the quantity of starting material or to the degree of protein loss during the procedure. However, mitochondria, being heavier than the other organelles in question, could have been caught up to varying degrees in the formation of the pellet during centrifugation if not successfully removed by the filtration procedure.

Difficulties were encountered in the assay of ATP:NMN adenylyl transferase. No activity could be demonstrated in the original homogenate, even though niacin had been added to inhibit nucleosidases (16). When NMN was replaced by NAD in the initial incubation mixture, it could not be detected when the incubation mixture was assayed with alcohol dehydrogenase (17). The

alcohol dehydrogenase assay system was tested directly by the addition of NAD and found to be functioning properly. It was then assumed that the NAD was either (a) still degraded in the presence of the homogenate and niacin, or (b) rendered incapable of participating in the alcohol dehydrogenase reaction. When brush-border preparations were tested in the same manner with NMN and with NAD, the problem was not encountered. Since alcohol dehydrogenase activity was found with the brush-border preparations containing added NAD but not with added NMN, it was concluded that there was no detectable nuclear contamination in the brush borders.

Alkaline phosphatase has been demonstrated to be associated with the brush-border membrane in the kidney (6), and therefore has been considered a good candidate as a marker enzyme for the brush border even though it is not highly specific for that structure. Its activity was concentrated 5-fold by our procedure. Trehalase and

maltase have also been found associated with the brush-border membrane (5), and also have been considered as marker enzymes for the brush borders. These enzymes were concentrated 9- or 10-fold. However, trehalase would seem to be the best choice as a marker enzyme even though the assay is less simple than that for alkaline phosphatase. Trehalase is found nowhere else in the kidney (5); alkaline phosphatase has a more general distribution (5). Trehalase activity, then, would be more indicative of the amount of brush border in the starting material. This is true of maltase as well, but its activity is not so great as that of trehalase.

SUMMARY

A simple method has been developed for the isolation of renal brush borders from rabbit kidney. The procedure requires less than 2 hr and yields a preparation that is relatively free of subcellular contamination.

The authors thank Dr. Enoch Houser, Dr. Robert Grabske, William Tocher, and Darryl President for their assistance. The authors also thank Dr. Arthur Nunn for his many helpful suggestions. This work was performed under the auspices of the United States Atomic Energy Commission. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the United States Atomic Energy Commission to the exclusion of others that may be suitable.

Received for publication 18 February 1972, and in revised form 25 April 1972.

REFERENCES

- MILLER, D., and R. K. CRANE. 1961. The digestive function of the epithelium of the small intestine. II. Localization of disaccharide hydrolysis in the isolated brush border portion of intestinal epithelial cells. *Biochim. Biophys. Acta.* **52**:293.
- FAUST, R. G., M. G. LEADBETTER, R. K. PLENGE, and A. J. McCASLIN. 1968. The effects of sugars, amino acids, hexosamines, sulfhydryl-reacting compounds, and cations on the preferential binding of D-glucose to Tris-disrupted brush borders. *J. Gen. Physiol.* **52**:482.
- EICHLIZ, A., and R. K. CRANE. 1965. Studies on the organization of the brush border in intestinal epithelial cells. I. Tris disruption of isolated hamster brush borders and density gradient separation of fractions. *J. Cell Biol.* **26**:687.
- HOLT, J. H., and D. MILLER. 1962. The localization of phosphomonoesterase and aminopeptidase in brush borders isolated from intestinal epithelial cells. *Biochim. Biophys. Acta.* **58**:239.
- BERGER, S., and B. SACKTOR. 1970. Isolation and biochemical characterization of brush borders from rabbit kidney. *J. Cell Biol.* **47**:637.
- KINNE, R., and E. KINNE-SAFFRAN. 1969. Isolierung und enzymatische Charakterisierung einer Bürstensaumfraktion der Rattenniere. *Pfluegers Arch. Eur. J. Physiol.* **308**:1.
- WILFONG, R. F., and D. M. NEVILLE, JR. 1970. The isolation of a brush border membrane fraction from rat kidney. *J. Biol. Chem.* **245**:6106.
- LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In *Methods in Enzymology*. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**:450.
- WATTIAUX-DECONNINCK, S., M. J. RUTGEERTS, and R. WATTIAUX. 1965. Lysosomes in rat-kidney tissue. *Biochim. Biophys. Acta.* **105**:446.
- JOHNSON, D., and H. LARDY. 1967. Isolation of liver or kidney mitochondria. In *Methods of Enzymology*. R. W. Estabrook and M. E. Pullman, editors. Academic Press Inc., New York. **10**:94.
- MAHLER, H. R., and E. H. CORDES. 1966. Intracellular organization and particles. In *Biological Chemistry*. Harper & Row, Publishers, New York. **378**.
- HÜBSCHER, G., and G. R. WEST. 1965. Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. *Nature (Lond.)*. **205**:799.
- LELOIR, L. F., and C. E. CARDINI. 1957. Characterization of phosphorus compounds by acid lability. In *Methods in Enzymology*. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**:843.
- SLATER, T. F., and D. N. PLANTEROSE. 1960. An assay procedure for a succinate-neotetrazolium-reductase system. *Biochem. J.* **74**:591.
- SACKTOR, B. 1968. Trehalase and the transport of glucose in the mammalian kidney and intestine. *Proc. Natl. Acad. Sci. U. S. A.* **60**:1007.
- KORNBERG, A. 1950. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic phosphate. *J. Biol. Chem.* **182**:779.
- HORECKER, B. L., and A. KORNBERG. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. *J. Biol. Chem.* **175**:385.