*EVOLVING PATTERNS OF RNA METABOLISM DURING TRANSITION FROM RESTING STATE TO ACTIVE GROWTH IN LYMPHOCYTES STIMULATED BY PHYTOHEMAGGLUTININ**

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Phytohemagglutinin (PHA), an extract of the kidney bean (*Phaseolus vulgaris*), has been extensively employed to produce mitotic activity in cultures of human peripheral blood white cells.[1, 2] Much evidence suggests that in such cultures the lymphocyte is the principal cell responding to this mitogenic stimulus.[3–5] After treatment with PHA, small lymphocytes have been shown to undergo progressive enlargement, DNA synthesis, and mitosis during a period of about 60–72 hr.[3, 4] Ordinarily, untreated human peripheral blood lymphocytes will not grow *in vitro* and therefore stimulation by PHA provides a unique opportunity to study a human cell population during its conversion from the resting to the growing state.

A previous report described the initial changes in RNA metabolism following PHA stimulation in a population of >97 per cent pure small lymphocytes from human peripheral blood.[6] RNA production was found to increase abruptly following treatment with PHA and to progress exponentially for at least 24 hr thereafter.

The present communication will describe density gradient sedimentation studies which revealed progressive changes in the pattern of synthesis of different classes of lymphocyte RNA during PHA-stimulated growth. It will be shown that the prompt increase in RNA synthesis observed immediately after PHA treatment occurs primarily in nonribosomal components. Increased ribosomal RNA (rRNA) production, which would be anticipated to occur in growing cells, is minimal for several hours after the onset of increased RNA synthesis. It will also be shown that upon prolonged incubation with PHA an increasingly greater proportion of RNA newly synthesized by lymphocytes appears to be unstable.

Methods.—Lymphocyte cultures: The technique for obtaining purified lymphocyte suspensions from human peripheral blood, and details of culture preparation have been described.[6] In some of the present experiments, commercially obtained nylon fiber columns were used for leukocyte separation (Fenwall leukopak^R) instead of cotton columns previously employed.[6]

Phytohemagglutinin was obtained from Difco Laboratories (PHA-P, lots 456637 and 471373).

Tritiated uridine was obtained from New England Nuclear Corp. (3.4–3.8 c/mmole) or Nuclear-Chicago Corp. (5 c/mmole) and used in concentrations of 10–20 μc/ml for pulse experiments and 2 μc/ml for experiments involving prolonged exposure to precursor.

In pulse-chase experiments, paired cultures were exposed to uridine-H^3 for a 30-min pulse. One member of the pair (pulse) was then harvested while actinomycin D (10 μg/ml) and unlabeled uridine (to a concentration of 0.1 mM) were added to the other member (chase). After an additional 1 hr of incubation, the chase cultures were harvested. In these chase experiments, actinomycin D and unlabeled precursor were introduced together to prevent further incorporation of radioactive precursor. This followed the suggestion of Harris[7] that actinomycin D alone does not arrest further incorporation as quickly as when an effort is made to dilute the labeled precursor pool. A precaution such as this has particular application in the lymphocyte, which appears to maintain a large intracellular pool of nucleotides.[8] Cultures pretreated with actinomycin D for 20–30 min incorporated no label into RNA.

After each appropriate incubation period, cultures were rapidly chilled by pouring into an equal volume of a frozen slurry of Earle's salt solution.

Extraction of RNA: The technique for extraction of RNA from lymphocyte suspensions with phenol and sodium dodecyl sulfate (SDS) at room temperature has been described.[6, 9] In some experiments employing smaller numbers of cells, 750 μg of freshly prepared, unlabeled rat liver RNA[10] was added as carrier during the extraction procedure.

The technique employing extractions with phenol and SDS at room temperature[6] was selected as the best available method of obtaining lymphocyte RNA in maximal yield with the least possible degradation. Studies comparing total RNA yields from lymphocytes by this method with total acid-insoluble cellular RNA determined by the orcinol method[11] showed 70–80% of the total RNA to be extracted by the phenol-SDS technique as employed. This was in good agreement with the yields reported by Salzman *et al.*[9] working with HeLa cells. Elevation of temperature during the phenol shake did not appreciably increase total extraction. Pulse-incorporated radioactivity extracted by the phenol-SDS technique was similarly compared with the total incorporation into acid-insoluble RNA, as determined by direct acid precipitation from intact cells.[12] It was found that 60–80% of the pulse-labeling material was extracted by the phenol-SDS method as described. The remainder of the pulse-labeled RNA could be found in the phenol-aqueous interphase layer. Similar findings have been reported by others.[13, 14] More recent studies in our laboratory, to be reported elsewhere, have shown that this material is consistently extracted at elevated temperatures and that it is apparently an additional portion of the pulse-labeled nonribosomal RNA to be described in the present communication.

The concentration of SDS in the extractions at room temperature could not be raised above 0.34% without producing gross DNA contamination, and even at the concentration employed, a small amount of DNA was present in the RNA preparations. The presence of this amount of DNA, however, had no effect on the experimental results. When RNA preparations were treated with DNAse[15] and most of the resulting polydeoxynucleotides removed by two precipitations in 2 *M* potassium acetate and 25% ethanol,[10] sedimentation patterns of radioactivity were obtained that were identical to those from preparations not treated with DNAse.

Sedimentation of RNA: RNA obtained from phenol-SDS extractions was dissolved in 1 ml of buffer without bentonite and layered on a 29-ml 5–20% sucrose gradient[16] prepared in buffer without $MgCl_2$. The preparation was then spun for 12 hr at 25,000 rpm in the SW25 rotor of the Spinco model L ultracentrifuge. Fractions were collected by means of a peristaltic pump through a puncture in the bottom of the centrifuge tube. RNA content of each fraction was measured by its ultraviolet absorption at 260 mμ (OD_{260}) in the Beckman model DU spectrophotometer. Aliquots of each sample were taken for determination of radioactivity in a Packard Tricarb liquid scintillation counter.[17]

Between three and five repetitions were performed for each of the experiments described below.

Results.—RNA synthesis in resting lymphocytes: As shown in a previous report, resting lymphocytes obtained by the methods used in this study incorporate uridine-H^3 into acid-insoluble RNA at a rate which remains constant for the first 4–6 hr *in vitro* and then falls off sharply, effectively halting new incorporation.[6] To determine the types of RNA synthesized during this linear phase of incorporation, freshly isolated lymphocytes were incubated with uridine-H^3 (2 μc/ml) for 6 hr, and the RNA was extracted and sedimented without carrier in a sucrose density gradient. Optical density peaks sedimenting at 28*S*, 18*S*, and 4*S*, characteristic of mammalian cellular RNA, were evident (Fig. 1).[18] During the prolonged exposure to labeled precursor, the largest proportion of incorporated radioactivity was found in the 4*S* region, with relatively small amounts of incorporation in the 28*S* and 18*S* (rRNA) peaks. It is apparent that during the linear phase of activity, the resting lymphocytes had synthesized only small amounts of ribosomal RNA.

RNA synthesis in PHA-stimulated lymphocytes; stimulated versus unstimulated cells: Augmented RNA synthesis in lymphocyte cultures has been detected within 1 hr after treatment with PHA.[6] To characterize the RNA synthesized during this initial rapid response to PHA treatment, lymphocyte cultures were incubated for 1 hr with PHA and then exposed to a 30-min pulse of uridine-H^3. The sedimenta-

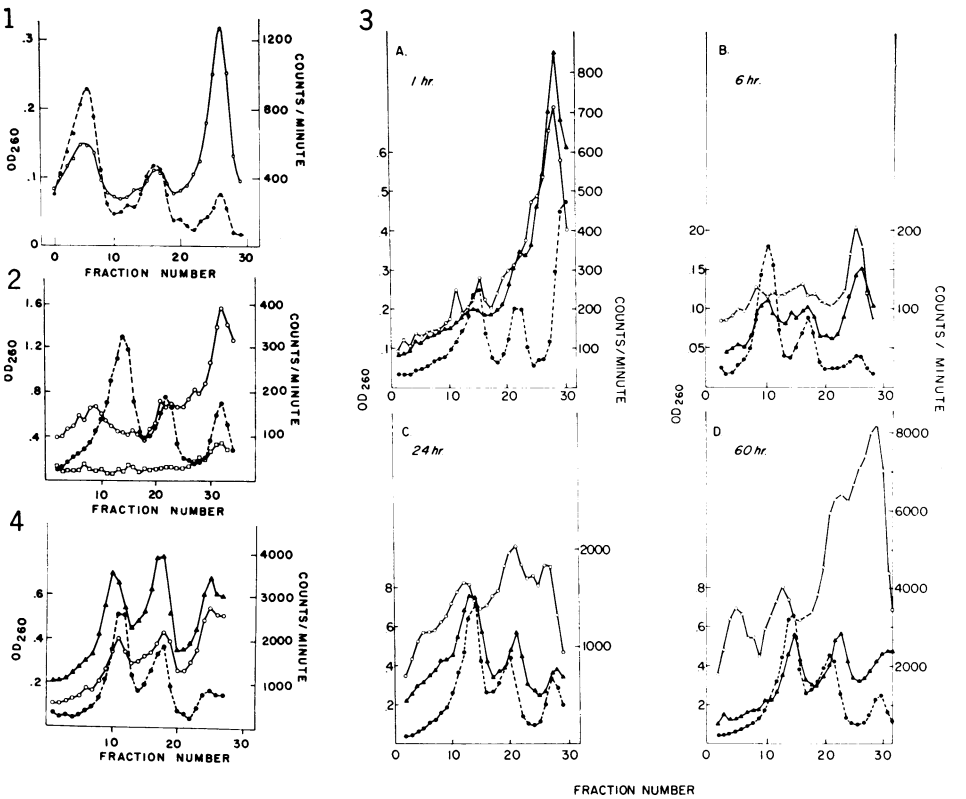

FIG. 1.—Sucrose gradient sedimentation of RNA from resting lymphocytes. A culture containing 1.5 × 10^8 lymphocytes was incubated for 6 hr with uridine-H^3 (2 μc/ml). RNA was extracted and layered on a 5–10% sucrose gradient, which was spun at 25,000 rpm for 12 hr. One-ml fractions were collected. OD_{260}, ●- - - - -●; cpm, O—O.

FIG. 2.—Sucrose gradient sedimentation of pulse-labeled RNA from lymphocytes incubated with and without PHA. A suspension of 2 × 10^8 freshly isolated lymphocytes was divided into two equal cultures, one of which was treated with PHA. Cultures were incubated for 1 hr and then given a 30-min pulse of uridine-H^3 (10 μc/ml). RNA was extracted as in Fig. 1 except that 750 μg of unlabeled rat liver RNA was added as carrier before precipitation. RNA was dissolved and layered on a 5–20% sucrose gradient and spun for 12 hr at 25,000 rpm in an SW25 rotor. One-ml fractions were collected. There was no difference in optical density pattern between PHA-treated and untreated cells in experiments where carrier was omitted. OD_{260}: ●- - ●; cpm, PHA-treated cells: O——O; cpm, untreated cells: □——□.

FIG. 3.—Pulse-chase experiments using lymphocytes incubated with PHA for varying periods of time. Suspensions of freshly isolated lymphocytes (1–4 × 10^8 cells) were each divided equally into two paired cultures, both of which were treated with PHA. Cultures were incubated for varying times and then given a 30-min pulse of uridine-H^3 (10–20 μc/ml). One member of each pair (pulse) was then harvested while the other member (chase) received actinomycin D (10 μg/ml) and was made 0.1 mM to unlabeled uridine. Incubation was continued for 1 hr before harvesting. RNA was prepared and analyzed as in Fig. 2. (*A*) 1-hr incubation with PHA; (*B*) 6-hr incubation; (*C*) 24-hr incubation; (*D*) 60-hr incubation. OD_{260}: ●- - -●; cpm, pulse: O——O; cpm, chase: ▲——▲. There were no differences in optical density profiles between the respective pulses and chases. The large amount of optically dense material at the top of the gradient in (*A*) is due to unlabeled polydeoxynucleotides resulting from treatment of the RNA preparation with DNAse and not completely removed by reprecipitation in 2 *M* $KC_2H_3O_2$ and 25% ethanol.

FIG. 4.—Pulse-chase experiment with RNA extracted under mild conditions. A suspension of 1.4 × 10^8 freshly isolated lymphocytes was divided into two equal cultures, and both were treated with PHA. After 60 hr of incubation, both cultures received uridine-H^3 (10 μc/ml) and were treated as in Fig. 3, except that phenol extractions were performed at 0°C instead of room temperature. OD_{260}: ●- - -●; cpm, pulse: O——O; cpm, chase: ▲——▲.

tion profile of RNA extracted from these cells was compared to that obtained from duplicate cultures treated in identical fashion except for the omission of PHA. RNA production in PHA-stimulated lymphocytes was found to be increased over control levels across the entire gradient, with evident new synthesis of both polydisperse and 4*S* RNA's (Fig. 2). Similar studies performed on paired cultures incubated for longer periods are not included in this report as RNA synthesis in non-stimulated cells after 4–6 hr of incubation was nil, and comparison to corresponding PHA-stimulated cells yielded no additional information.[19] The sedimentation pattern of newly synthesized RNA in PHA-stimulated cells at various times during prolonged incubation was studied as part of the following series of experiments.

Pulse-chase studies: The nature of pulse-labeled RNA in PHA-stimulated lymphocytes was investigated in a series of pulse-chase experiments where, in the absence of further incorporation, the fate of the radioactivity incorporated during specific 30-min intervals could be followed. As has been shown, pulse-labeled RNA extracted by the phenol-SDS method from lymphocytes incubated with PHA for 1 hr was polydisperse, with the largest accumulation sedimenting at 4*S*. After an additional 1-hr chase, there was essentially no alteration in the size distribution of the labeled material, and there was no apparent reduction in its quantity (Fig. 3*A*). When radioactivity incorporated into whole-cell acid-insoluble RNA was determined more quantitatively by direct acid precipitation from intact cells after incubation with PHA for 1 hr, it was found that 30 per cent of the pulse-incorporated radioactivity was lost after a 1-hr chase. Thus, a fraction of RNA was synthesized during the pulse period after 1 hr of incubation which was lost from the macromolecular fraction during the chase. No evidence of this fraction was found in the material from pulsed cultures extracted by phenol-SDS and analyzed by sedimentation, suggesting that this fraction may have remained unextracted by phenol-SDS. It is also possible that a portion of this material could have been extracted from pulsed cultures but was subsequently degraded during manipulation.

The results at this time gave minimal indication of increased rRNA production, since only very slight alteration of the sedimentation pattern suggestive of such production was seen in the chase experiments. Studies of other mammalian systems where rRNA is actively synthesized suggest that it is not produced directly, but is first produced as a high molecular weight precursor and subsequently altered to the more stable 28*S* and 18*S* moieties found in ribosomes.[10, 20–23] It has been shown that a 30-min pulse with uridine-H^3 in such systems results in minimal labeling of the 28*S* and 18*S* peaks, most of the pulse-incorporated radioactivity sedimenting in the region of 30–45*S*.[21–23] A 1-hr chase, however, results in loss of label from the 30*S*–45*S* regions and produces a change in the sedimentation pattern of the remaining labeled RNA causing its profile to approximate that of the optical density curve.[21, 23] If lymphocytes at any time were to produce ribosomal RNA, a similar shift in the size distribution of pulse-labeled RNA, resulting in peaks of radioactivity at 28*S* and 18*S*, should be detectable following a 1-hr chase. It is apparent from Figure 3*A* that, after incubation of lymphocytes with PHA for 1 hr, the observed increase in RNA production included minimal amounts of extractable rRNA, either because of lack of precursor synthesis, or because of failure to convert precursor to its final form.

In pulse-chase studies performed at various times during a 60-hr incubation with

PHA, alteration of the sedimentation pattern of radioactive material suggesting labeling of the ribosomal RNA peaks was first seen in chase experiments after 6 hr of incubation (Fig. 3*B*). However, at that time, the largest proportion of radioactivity which remained after chasing still sedimented at 4*S*. Only at later periods (24 and 60 hr) did the labeled RNA remaining after chasing conform closely to the optical density curve (Figs. 3*C* and *D*). Thus, despite the abrupt increase in the total RNA production after PHA treatment, the onset of augmented rRNA synthesis was delayed for 1–6 hr and became clearly established only thereafter.

From a comparison of pulse and chase curves in each experiment shown in Figure 3, it is evident that in the longer incubations a larger proportion of the pulse-labeling RNA was lost from the sedimentation profile during the chase. By 60 hr about 70 per cent of the extractable pulse-labeling RNA failed to appear on the sedimentation profile after chasing. Assays of whole-cell acid-insoluble radioactivity also showed that 70 per cent of the label incorporated by 60-hr cultures during the pulse was lost after a 1-hr chase. Orcinol assays of bulk RNA from cultures before and 1 hr after treatment with actinomycin D showed no changes. Thus, cell death or indiscriminate loss of pre-existing RNA played no role in this phenomenon.

Inspection of the sedimentation profiles of pulse-labeled RNA over the 60-hr incubation period reveals the progressive emergence of radioactive peaks at 45*S* and 30*S* as well as additional accumulation of radioactivity developing in the 14–18*S* region and at 4–6*S* (Fig. 3*A–D*). While these trends were only suggested after 6 hr of incubation, they were clearly evident after 24 and 60 hr. In accord with the observations made in other mammalian systems, it is likely that the 45*S* and 30*S* peaks of pulse-labeling RNA were precursor to stable rRNA. The time course of their appearance suggests that the delayed onset of increased rRNA production was due to lack of precursor synthesis.

Further evidence for the synthesis, during the pulse period, of a precursor to rRNA is provided by an experiment using a modified RNA extraction procedure in which the temperature of the phenol extractions was reduced to 0°C. When pulse-labeled RNA from lymphocyte cultures incubated with PHA for 60 hr was extracted under these conditions, only about 15 per cent of the expected radioactivity was obtained and the sedimentation profile of this material corresponded with the optical density curve (Fig. 4). Very little of the polydisperse pulse-labeled RNA seen in extractions at room temperature (Fig. 3*D*) was obtained. In particular, peaks of pulse-labeling RNA at 45*S*, 30*S*, and 10–18*S* were absent. It is evident that rRNA and 4*S* RNA are more easily accessible to this less vigorous extraction procedure than are the polydisperse RNA fractions which constitute the largest proportion of pulse-labeled material in 60-hr cultures.

In the corresponding chase determination (Fig. 4), the extracted radioactivity again sedimented with the three major optical density peaks, but the activity in the rRNA peaks had approximately doubled. During the chase with actinomycin D, therefore, more of the pulse-incorporated radioactivity had accumulated in easily extractable rRNA, suggesting conversion from a relatively inaccessible precursor which had been synthesized during the pulse period. If, as seems likely from other work, this relatively inaccessible precursor to rRNA is represented by the 45*S* and 30*S* peaks of pulse-labeling RNA, the remainder of the inaccessible pulse-labeled

material sedimenting with the lighter fractions (4–6*S* and 10–18*S*) would comprise an unstable component.

Pulse-chase experiments, using room temperature extraction after incubation with PHA for 72 hr and for 7 days, yielded findings similar to those observed after 60 hr (Fig. 3*D*). These cultures were in a phase of high mitotic activity and at this time resembled other rapidly growing systems in their active production of rRNA.[21–23] However, although adequate to maintain active cell growth and proliferation in these cultures, rRNA and its precursor still appeared to represent minor proportions of the total pulse-labeling RNA.

Discussion.—Since resting lymphocytes may be stimulated to enlarge and divide, they may be presumed to possess the basic machinery required to support the initial processes leading to cell replication. That these processes are not carried out in the absence of special conditions, such as the addition of specific antigens[24, 25] or PHA, suggests the existence of control mechanisms which are responsive to the stimulating agents. The synthesis of RNA—ribosomal and nonribosomal—appears from our results to be under such control in lymphocytes. Assuming that all types of cellular RNA undergoing synthesis draw upon a common precursor pool, the slow onset of increased rRNA synthesis in the face of the rapid onset of increased synthesis of polydisperse and 4*S* RNA suggests the existence of differing, although possibly interacting, control mechanisms for these types of RNA. The slow acceleration of rRNA synthesis may be related to a requirement for the accomplishment of certain preliminary functions, such as the production of essential new enzymatic or structural protein, or for the elimination of repressor substances. The phenomenon deserves further investigation since it may represent an opening into the area of cell growth regulation.

In other mammalian systems, stable rRNA is the principal product of RNA synthesis.[10, 20–23] Thus, in the HeLa cell, 60–70 per cent of the RNA labeled during a 30-min pulse with uridine-H^3 was conserved and converted into stable rRNA following a chase with actinomycin D. The remaining 30–40 per cent was unstable, being degraded to acid-soluble products during the chase.[21] This degree of conservation was not seen in the present study, since 70 per cent of the pulse-labeled material in lymphocytes producing rRNA at maximal rates was lost during the chase both in phenol-SDS extraction studies and in direct acid-precipitation studies. This finding may be due to a true difference between the RNA metabolism of the HeLa cell and that of the PHA-stimulated lymphocyte, or may reflect a peculiarity of the lymphocyte which renders a large proportion of its newly formed RNA susceptible to degradation when the cells are exposed to actinomycin D. We have established that actinomycin D treatment as employed does not affect the DNA or bulk RNA content of lymphocyte cultures,[8] implying the absence of cell lysis or massive RNA degradation. Further, as shown in Figure 4, conversion from precursor to rRNA does occur in the presence of actinomycin D. If any unstabilizing effect of actinomycin exists, it must, therefore, affect only a fraction of newly synthesized RNA. While the observed instability might then be considered artifactual, it nevertheless would imply a basic difference between the RNA metabolism of the HeLa cell and that of the PHA-stimulated lymphocyte, perhaps related to the rate of production of susceptible material in each cell type.

Scherrer *et al.*[21] suggested that, since 70 per cent of pulse-labeling RNA in HeLa

cells is in 35*S* and 45*S* material, the unstable portion (30%) of the newly synthesized RNA had similar sedimentation values. In our studies, the bulk of pulse-labeling RNA appeared in lighter fractions, and was lost from them after chasing. Whether the unstable RNA in our system is of the same type as that in HeLa cells remains to be determined. It may be that the unstable pulse-labeled RNA in PHA-stimulated lymphocytes is produced originally as high molecular weight material, but is partially degraded during the extraction procedure. Such a possibility exists, since we have found lymphocyte RNA to be highly susceptible to technical breakdown.

An alternative hypothesis, consistent with our data, is that the unstable lymphocyte RNA does, in fact, have a native sedimentation profile as observed, and that it is messenger RNA of short half life, produced in increasing proportions and concerned with successive stages of activity as the lymphocyte progresses from a resting state to one of rapid enlargement and mitosis. Studies currently in progress are designed to test this hypothesis.

Summary and Conclusions.—Stimulation of lymphocytes to enlarge and divide by treatment with PHA provides an opportunity to study the changes in RNA metabolism accompanying the conversion of a human cell population from the resting to the growing state. Sucrose gradient sedimentation studies demonstrated an evolving pattern of RNA synthesis in PHA-stimulated cultures of purified lymphocytes. In the resting state, cells produced small amounts of rRNA. An early transition period occurred after treatment with PHA, which was marked by an abrupt increase in the rate of synthesis of polydisperse and 4*S* RNA. The synthesis of rRNA was increased to a much smaller extent at that time. During a later transition period, beginning 1–6 hr after treatment with PHA, increased rRNA synthesis was more clearly shown. This increase roughly paralleled the emergence of peaks of pulse-labeling RNA at 45*S* and 30*S*. Simultaneously, unstable pulse-labeling RNA began to appear in increasingly greater proportions. Finally, in a phase of active growth (from the 60th hr to the 7th day), there was prominent synthesis of rRNA and its apparent precursors. However, the greatest proportion of incorporated label was found in material which was not conserved following a chase with actinomycin D and unlabeled uridine.

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*ANALYSIS OF HISTONE IN SITU IN DEVELOPMENTALLY INACTIVATED CHROMATIN**

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Since 1950[1] speculations concerning the role of basic proteins associated with DNA have attributed to them, among other functions, the control of development in eukaryotic organisms through differential release of genetic information. Evidence has accumulated that both native and reconstituted associations of DNA with histones are capable of suppression of DNA-directed RNA synthesis and that removal of histone from native nucleohistone results in increased nuclear RNA and protein synthesis.[2, 3]

In addition, X-ray diffraction studies[4, 5] and fractionation of calf-thymus lymphocytes[6] have associated histones with supercoiling of DNA in chromatin and heterochromatin (pycnosis), respectively. Suppressive histone fractions have also been shown, in lampbrush chromosomes, to retract loops active in RNA synthesis or turnover.[7]

In male mealy bugs the paternal (heterochromatic) set of chromosomes, active in the previous generation, has shown genetic inactivity[8] by virtue of discontinued RNA synthesis.[9] The paternal set heterochromatizes during early embryogeny and remains so in most tissues throughout development. During spermatogenesis the heterochromatic and euchromatic elements divide and segregate in an atypical manner which is regular among a wide range of coccids. The end products of meiosis are two nuclei, euchromatic derivatives, which will become viable sperm, and two heterochromatic nuclei which disintegrate during spermiogenesis.[8]

The mealy bug provides a system in which a condensed, inactive mass of chroma-