H^3 URIDINE INCORPORATION IN LAMPBRUSH CHROMOSOMES*

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It is now generally accepted that the nucleus is an active center of ribonucleic acid (RNA) synthesis and perhaps the sole source of cellular RNA.[1] In several cases, uptake of RNA precursors has been demonstrated in the chromosomes themselves. The giant "lampbrush" chromosomes found in vertebrate oocytes are particularly useful for autoradiographic studies, since their large size permits accurate cytological localization of incorporated precursors. The following experiments concern primarily the incorporation of H^3 uridine in RNA of the lateral loops of lampbrush chromosomes. The results of a single experiment with H^3 phenylalanine are also reported.

Materials and Methods.—All experiments were performed on adult females of the crested newt, *Triturus cristatus cristatus* (Laurenti), from an English source. In each experiment, the isotope was given as a single subcutaneous injection of 150 μc. We have used H^3 uridine from Schwarz BioResearch, Mt. Vernon, N.Y. (lot 6001, 1.29 c/mM) and from the Radiochemical Centre, Amersham (lot TRA-27, batch 2, 1.1 c/mM). The sample of H^3 DL-β-phenylalanine came from Amersham (lot TRA-30, batch 1, 0.126 c/mM). Samples of ovary were removed from the living, anesthetized animal at various times after injection. After each sampling, the animal was stitched with gut and returned to an aquarium. The oocytes were transferred to a saline solution (0.1 *M* KCl, 5 parts; 0.1 *M* NaCl, 1 part) in a solid watch glass and their nuclei were isolated free-hand.[2] A nucleus, cleaned of yolk, was pipetted into a drop of saline (the above 5:1 mixture of KCl and NaCl containing 0.5×10^{-4} *M* $CaCl_2$) in a specially designed, flat-bottomed well slide. Each well slide consists of a $3'' \times 1''$ microscope slide perforated by a $1/4''$ hole in the center, sealed by paraffin wax in register with an unperforated slide below. The nuclear membrane was removed free-hand by fine forceps and the well slide at once placed in a sealed chamber containing formaldehyde vapor. The trace of calcium in the saline has no effect on the morphology of the chromosomes but leads to more rapid dispersal of the nuclear sap, thus allowing the chromosomes to spread out and come in contact with the floor of the well slide. Two to 3 hr formaldehyde treatment attaches the chromosomes to this glass surface; thereafter, each well slide can be split into its two component slides, the lower carrying the chromosomes. The fixed preparations were washed with water, treated for 5 min with 5% trichloroacetic acid at 5°C to remove labeled precursor, washed in water again, dehydrated in an ethanol series, washed in xylene to remove paraffin wax, and air-dried from acetone. Kodak AR-10 stripping film was applied and the preparations exposed for from 20 to 100 days at about 17°C. The preparations were developed in Kodak D-19*b* for 10 min at 17°C, washed in water, fixed for 10 min in Kodak acid fixer, hardened for 10 min in 2% formaldehyde, washed in running water, and air-dried. The chromosomes could not be stained satisfactorily through the film, nor did they show up well by phase contrast under the dry film. However, the contrast is good before drying and the preparations have therefore been preserved moist. The film was wetted with distilled water containing a trace of merthiolate as preservative and covered with a glass slip. The edges of the slip were sealed with a resin-lanolin cement. Such preparations have now kept satisfactorily for a year.

Lampbrush Chromosome Structure.—Before presenting the results of the incorporation experiments, we will briefly describe those structural features of lampbrush chromosomes which are relevant to the experiments. Projecting laterally from each homologue of these meiotic bivalents are hundreds of pairs of loops (Fig. 1). Many of the lateral loops have unique morphologies, thus allowing particular chromosome sites to be identified and mapped.[3] All loops contain RNA.[4, 5] The loops arise

from chromomeres lying in the chromosome axis; these chromomeres contain DNA demonstrable by the Feulgen reaction. All loops are asymmetric in the sense that they possess a thinner and a thicker insertion at the chromomere. Each loop possesses a delicate axis, whose diameter is less than 100 Å when freed of its investing ribonucleoprotein matrix.[6] Although the loop matrix is Feulgen-negative, the loop axis must contain DNA, since loops are fragmented by deoxyribonuclease.[7]

The majority of loops, what we have called "normal" loops, have a fine granular or fibrous matrix, and they range in diameter from one to a few microns. Contrasting with these are various "landmark" loops, which can be recognized by their size, unusually high or low optical density, granularity, etc. In order to relate all of our incorporation studies to one particular chromosomal site, we decided to concentrate attention on the "giant granular loops" near the left end of chromosome XII (Fig. 1). These loops are somewhat longer than the average and they possess an exceptionally bulky granular matrix. Our choice turns out to have been lucky, as these giant granular loops differ from most other loops in their pattern of incorporation, and in a particularly revealing way.

Incorporation of H^3 Uridine.—The majority of normal loops, and most of the landmark loops as well, appear uniformly labeled 12 to 18 hr after a single injection of H^3 uridine. By that we mean the grain count per unit of area above a given loop appears to be roughly constant. There are, however, noticeable differences in degree of labeling between different loops. The majority of loops likewise appear uniformly labeled in preparations made at 2, 4, 7, and 14 days after injection. The maximum level of labeling is reached about the fourth day after injection, and there is a gradual falling off from that day on. By 28 days after injection, the chromosomes are only weakly radioactive, and by 56 days they are essentially unlabeled.

The giant granular loops, and a few other loops as yet studied in less detail, display a different pattern of incorporation. Up to one day after uridine injection, when neighboring normal loops are well labeled, the giant granular loops are quite unlabeled except for a short region next to their thin insertion (Fig. 2). Two days after injection, they are labeled about $^1/_5$ of their length from the thin insertions, by four days they are nearly $^1/_2$ labeled (Fig. 3), by seven days only a short region near the thicker insertion remains cold, and at 14 days the whole loop is radioactive (Fig. 4). At 28 days, the giant granular loop is still radioactive throughout its length but less intensely (Fig. 5). This pattern of labeling is found in all giant granular loops examined. Dozens of oocytes from the same animal show precisely the same picture, regardless of oocyte size within the range which we have handled (0.7 to 1.4 mm diameter). Oocytes from six different females have all given comparable results.

Hormone Treatment.—Clever and Karlson[8] have shown that the insect hormone, ecdysone, causes characteristic changes in activity in the polytene chromosomes of *Chironomus*. Macgregor[9] has recently found that injection of gonadotrophic hormones into female newts brings on alterations in the oocyte nuclei, including stiffening of the nuclear sap, reduction in the number of free granules in the sap, and altered morphologies of certain loops. We hoped that gonadotrophic hormone treatment might raise the level of H^3 uridine incorporation in lampbrush chromosomes and thereby give information about the pattern of labeling in normal loops during the first few hours after uridine injection. With this in mind, we gave two

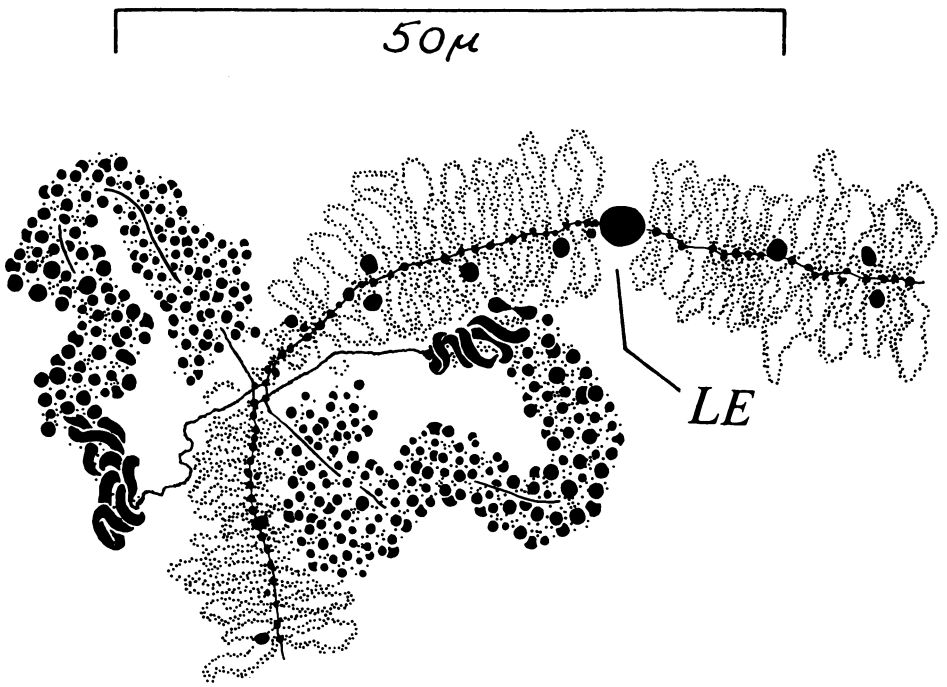
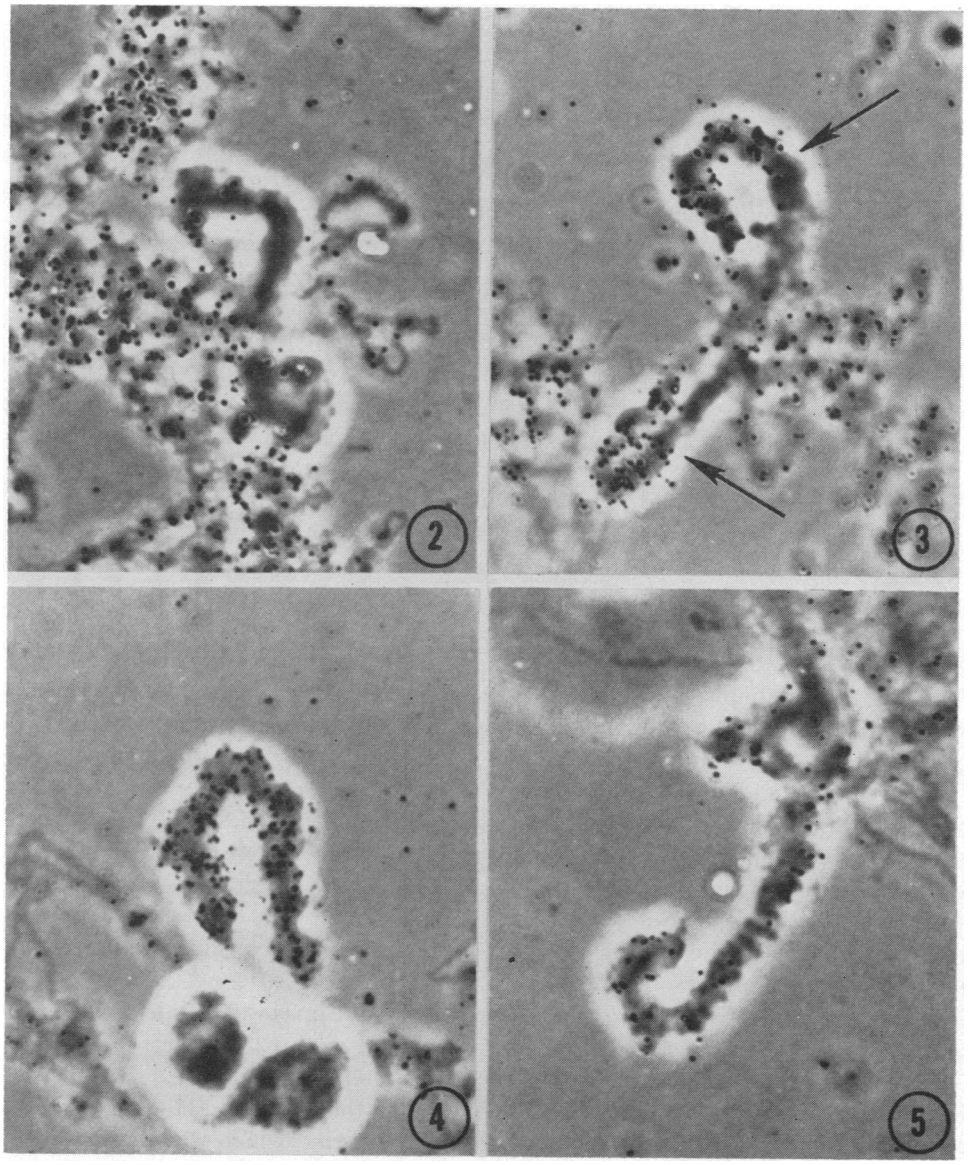


FIG. 1.—The left end of chromosome XII from an oocyte of the newt *Triturus cristatus*. A large number of "normal" loop pairs are shown in addition to the "giant granular loops" characteristic of this chromosome region. All loops are asymmetric in the sense that one end is thinner than the other. In the giant granular loops, the thin insertion consists of a fine thread leading to a dense contorted region. The thick insertion, as well as the bulk of the loop, consists of a coarsely granular matrix surrounding a very delicate axis.

newts subcutaneous injections of 200 I. U. of mixed serum and chorionic gonadotrophins ("Pregnyl" and "Gestyl" from Organon Laboratories, Ltd., London). Three days later, the animals laid eggs. Five days after the first injection, the newts were given another similar hormone injection and one day later they were injected with H^3 uridine. Chromosome preparations were made at intervals thereafter.

One of these newts did show the anticipated increased level of labeling. However, we now think that this was caused by the H^3 uridine injection being made directly into the coelom rather than into the subcutaneous lymph space. The level of labeling in the other newt was not unusually high, but in *both* animals the rate of labeling along the length of the giant granular loops had evidently accelerated by a factor of about two. Thus, one day after H^3 uridine injection, these loops were labeled for about $^1/_5$ of their lengths from the thin insertions, after two days they were nearly $^1/_2$ labeled, after four days less than $^1/_5$ of the length from the thick insertions remained unlabeled, and samples taken seven days after injection were uniformly radioactive.

Dilution and Transfer Experiments.—Although normal loops do not show the sequential pattern of H^3 uridine incorporation characteristic of the giant granular loops, we hoped that a similar pattern might be disclosed by diluting out the uridine some time after the initial injection; that is, we thought it might be possible to cause



FIGS. 2–5.—Autoradiographs of the giant granular loops on chromosome XII of *Triturus cristatus*. A female newt was given a single injection of 150 μc H^3 uridine in the subcutaneous lymph space, and oocyte chromosomes were isolated at various times thereafter. In each photograph, the thin insertion of the loop is to the left. In Figure 3, both members of the loop pair may be seen clearly, but in the others only one member shows to advantage. Exposure 23 days (Fig. 3) or 39 days (Figs. 2, 4, 5). Phase contrast × 1,600.

FIG. 2—One day after injection, the giant granular loops are unlabeled except for a short region near the thin insertion (not well shown here).

FIG. 3.—Four days after injection, the loops are labeled approximately $^1/_2$ their length from the thin insertion. Arrows indicate the sharp boundary between labeled and unlabeled regions.

Fig. 4.—Fourteen days after injection, the giant granular loops are totally labeled.

FIG. 5.—Twenty-eight days after injection, the loops are totally labeled, but the activity is reduced.

an unlabeled segment to appear at the thin insertion and extend around the loop during successive days. To this end, we made two experiments.

In the first (dilution) experiment, we gave the usual injection of H^3 uridine (0.033 mg) to two female newts and followed this three days later by 1,000 times as much cold uridine (33 mg). We chose three days because the maximum level of labeling is reached at about this time and also because the giant granular loops are then labeled a convenient length. In brief, the dilution had no effect on the continued incorporation of labeled uridine by the chromosomes. The normal loops were still labeled throughout their lengths at the end of six days, and without evident diminution of level. The giant granular loops had progressed as usual from about $^1/_3$ labeled at three days, when the cold uridine was given, to about $^2/_3$ labeled at the end of six days. In these loops, too, there was no obvious lowering of the level of labeling.

In the second (transfer) experiment, we gave injections of H^3 uridine to two female newts (animals J and K). At the end of three days, one ovary was removed from each of these animals. The ovary from animal J was placed in the coelom of a non-injected female (animal L), and similarly the ovary from K was placed in a cold female (animal M). Reciprocally, an ovary from L was transferred at the same time to J and an ovary from M to K. Chromosome preparations were made from the hot animals at the time of the operation. Three days later, preparations were made from all of the transferred ovaries, as well as from the hot ovaries remaining in the injected animals.

The transfer of a hot ovary into a cold animal had no effect on the pattern of incorporation. At the end of six days (three in the original hot donor and three in the cold host), normal loops showed uniform labeling. The giant graunular loops had continued to label, so that they showed the picture typical of six days post-injection. The hot controls which were not transferred were similar. The cold ovaries transferred to the coeloms of the hot animals picked up hardly any radioactivity. This last result must be due to lack of circulating isotope in the hot host and not to a disturbance in the transferred ovaries themselves, since isolated ovaries can incorporate RNA precursors.[10]

Incorporation of H^3 Phenylalanine.—Two newts were given single injections of 150 μc of H^3 phenylalanine. At intervals ranging from one to 28 days after injection, samples of ovary were removed and chromosome preparations made. Radioactivity appears as early as one day after injection in all the loops, and labeling is maintained at roughly a constant level throughout the period which we have sampled. On no slide, even after 100 days exposure, is the level of labeling in normal loops sufficiently high for us to determine the spatial pattern of phenylalanine incorporation. We should recall that our sample of H^3 phenylalanine had only about $^1/_{10}$ the specific activity of the H^3 uridine. We were, however, able to assess the labeling of the giant granular loops. In contrast to the sequential labeling of these loops with H^3 uridine, with H^3 phenylalanine they are uniformly radioactive on the first day after injection, and they remain uniformly labeled thereafter.

Discussion.—At least three mechanisms might account for the sequential labeling of the giant granular loops by H^3 uridine: (1) A wave of RNA synthesis may pass over the loop, without a physical movement of materials. The situation would then resemble the wave of DNA synthesis which passes over the macronucleus of

Euplotes.[11, 12] (2) RNA may be synthesized in a restricted region next to the thin insertion. While the loop axis remains stationary, the loop product moves slowly around, to be shed near the thick insertion. (3) The loop axis itself may be paying out the chromomere at the thin insertion and winding back in at the thick insertion (Fig. 6). RNA would be synthesized for a sort time only as each new segment of loop axis comes into action. The RNA product would be carried around the loop still attached to the region of axis on which it was made.

According to either alternative (2) or (3), the asymmetry of the loop is explained by the continuing protein synthesis. The thick insertion of the loop is oldest and the matrix there has been producing protein for a longer time than at the thin insertion.

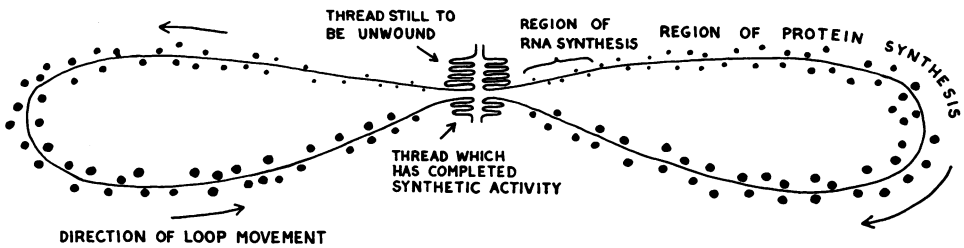


FIG. 6.--Interpretation of labeling experiments in terms of a conveyor belt model of the loops.

We consider the wave explanation extremely improbable. If waves were passing over the giant granular loops, we might expect different oocytes to be in different phases at the time of isotope administration. As already mentioned, however, loops from all oocytes, regardless of age, show the same incorporation pattern. No cases were found, for instance, where incorporation began at a point in the middle of the loop.

The incorporation experiments alone do not permit a decision between alternatives (2) and (3). We prefer (3), that is, moving axis and moving product, on both morphological and theoretical grounds.[13] The granules which make up the bulk of the giant granular loops are attached to the loop axis by fine threads. This fact can be ascertained when the loop matrix is dissolved by dilute saline or RNase.[14] Such threads, with their attached granules, could perhaps move along a stationary axis, but we find this more difficult to picture than a moving axis. The idea of a moving axis may seem less fantastic if one remembers that the axis must spin out of the parent chromomere in the early stages of meiosis when the loops first make their appearance and, conversely, must spin back in during later stages when the loops regress. When the RNP matrix is dissolved experimentally, the loop axis can be observed to contort, contract, and finally disappear as it becomes amalgamated into the parent chromomere. A continuously moving loop axis thus involves no new assumptions about the capabilities of the chromosome thread. Also, one must consider the fact that most of the DNA of the chromosomes is contained in the chromomeres. We know the loop axis contains DNA from the enzyme experiments of Callan and Macgregor,[7, 14] but the relative amount in the loops must be small. If we imagine that the chromomere contains a long length of wound-up

loop axis, then a moving axis provides a mechanism whereby all of this DNA may be brought into an extended, synthetically active state. On the stationary axis theory, one must assume that the majority of the chromosomal DNA does not participate in RNA formation.

It is important to know whether the behavior of the giant granular loops (and that of the few other loops which also show sequential labeling) reflects some fundamental difference between these loops and others or whether their particular pattern of incorporation merely discloses a general property of all loops. On either theory (2) or (3), we must assume that RNA synthesis in the giant granular loops is restricted to a short region next to the thin insertion. If the zone of RNA synthesis were instead extended along the whole loop, then loops would always label uniformly, and movement of the product, or of the axis and product, would not be detected. This situation is well illustrated by the H^3 phenylalanine incorporation. Protein synthesis in the giant granular loops is *not* limited to the thin insertion, and so the entire loop becomes radioactive at once. If we had only the phenylalanine data on which to go, we should not have suspected movement of materials in the giant granular loops. We think that movement occurs in all the normal loops as well but that it is masked by RNA synthesis occurring simultaneously over their entire lengths. Experiments to prove this assumption (by dilution and transfer) have so far failed. They have, however, supplied information about isotope availability which should help in designing decisive experiments.

We shall next discuss some of the conclusions and difficulties which follow if our alternative (3) is correct and if it applies to normal loops as well as to those few with spatially limited RNA synthesis.

The segment of chromosome thread associated with a single loop site must be extraordinarily long. We can get an idea of its length by comparing the time taken for a point on the loop axis to travel from the thin to the thick insertion with the total time that the loop is in existence. From the information already presented in this paper, we can deduce that under normal conditions total traverse of the giant granular loop must take about 10 days. We also know from the developmental history of ovaries from newts raised in captivity that the chromosomes remain in the lampbrush stage for *at least* six months prior to maturation and ovulation. Since we found no obvious differences between rates of travel of incorporated H^3 uridine in oocytes of different sizes, we will assume that loop movement goes on at a steady rate throughout the period of oocyte growth. The total length of the thread at the giant granular loop site must therefore be at least 18 times the length of the loop itself (180 days/10 days). The loop is usually between 50 and 100 microns long. If we assume the lower figure of 50 microns, then the total site length is roughly one millimeter.

The sum of the lengths of the lateral loops on a haploid set of chromatids of *Triturus viridescens* is roughly 50 cm.[4] A similar figure would hold for *Triturus cristatus*. If each loop site consists of a thread about 18 times the length of the loop itself, the actual length of the haploid set of chromatids would be about 18 $\times$ 50 cm, or about 9 m. Photometric measurements show that the haploid set of newt chromatids contains about 30×10^{-6} μg of DNA. The length of this DNA, when calculated as a Watson-Crick double helical chain, would be about 9.9 m. Despite the wide margin of error inherent in these calculations, it nevertheless

appears plausible to us to suppose that the chromatid consists, insofar as its DNA is concerned, of a single linear array of double-helical molecules.[15, 16]

A striking feature of the chromosome loops is their polarization. This polarization is expressed first of all in the morphological asymmetry of the loop matrix. The available evidence suggests that the direction of asymmetry is constant for a given loop[3]; for instance, the thin insertion of the giant granular loop of *Triturus cristatus* is consistently distal to the thick insertion, relative to the centromere location. The polarization is also evident from the incorporation experiments. If we interpret the incorporation according to hypothesis (2), stationary axis and moving product, then the loop matrix is moving *proximally* along the axis of the giant granular loop, that is, toward the centromere. RNA synthesis would be carried out by only a short segment of the site, near the thin insertion, and this segment would be active all the time. In this case, material produced at one point would pass sequentially over other regions of the chromosome thread and could be successively altered. On hypothesis (3), with a moving axis, each new segment of chromosome thread would be concerned with RNA synthesis for a short time only; and the *activation* would be passing distally along the thread away from the centromere (Fig. 6). Since we cannot distinguish these two schemes at the moment, it does not seem profitable to consider their theoretical consequences in more detail. We feel that closer analysis of the chromosome loops will give us information regarding the geometry of gene-controlled syntheses.

Summary.—We have made an autoradiographic study of H^3 uridine and H^3 phenylalanine incorporation in newt lampbrush chromosomes. In one large pair of chromosome loops, the incorporation of H^3 uridine follows a sequential pattern, starting at the thin end of the loop and proceeding around the loop in about 10 days. The rate of spread of the label is speeded up by prior administration of gonadotrophic hormone.

H^3 phenylalanine shows a different pattern of incorporation, being taken up immediately by all parts of this loop. It is postulated that the total length of a chromosome site is very much longer than the loop itself and that the loop is being continuously unwound at its thin insertion, wound up at the thick. The site possesses a constant polarization, expressed morphologically by the asymmetry of the loop and functionally by the sequential labeling.

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*APPEARANCE OF H-2 AGGLUTININS IN OUTCROSSED FEMALE MICE**

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There have been a number of reports recently of outcrossed female mice becoming tolerant to homografts from the strain of the males with which they have been bred. Several of these reports deal with circumstances where the tolerance is obtained across a relatively weak (non-H-2) barrier,[1-3] but Breyere and Barrett[4, 5] demonstrate that Balb/C females (H-2^d) repeatedly bred with C3H males (H-2^k) will accept and hence be killed by a plasma cell tumor originating in C3H, whereas Balb/C females of the same age and parity, but always incrossed, are completely resistant to the tumor. Thus it appears that continued breeding of females with males of a different strain evokes an immunological response in the female. In this publication we present evidence that one aspect of this response is the production of circulating H-2^d hemagglutinins in multiparous C57Bl/6J (H-2^b) females, bred with DBA/2 (H-2^d) males.

Materials and Methods.—Mouse strains: The breeding population studied here consists of C57Bl/6J female mice, forced-bred with DBA/2J males, all acquired at 4–6 weeks of age from Jackson Memorial Laboratories, Bar Harbor, Maine, by Simonsen Laboratories, Inc., Gilroy, California, and there maintained for the purpose of producing the BDF_1 hybrid. One male and two females are housed together at the start of breeding (at approximately 6–8 weeks of age) and are never separated until retirement at approximately 10 months. Individual breeding records for the females are not available, but the average female produces 29 offspring during her breeding life.

C57Bl/10- H-2^d (B10·D2), C3H/Sn (H-2^k), and C3H- H-2^b (C3H·SW) were used as erythrocyte donors.

Serum collection: Sera from numbered animals is drawn from tail artery of prewarmed mice into individual tubes, allowed to clot and kept at 4°C overnight. The clot is removed and the sera tested either immediately or after being stored frozen at −20°C. There was no observable difference in several sera tested before and after freezing.

Hemagglutination: The hemagglutination method of Stimpfling,[6] as modified,[7] using polyvinylpyrrolidone (PVP) as developing agent was used.

Agglutinations are classified as: strong = titer of 1/80 or more, with the erythrocytes forming large clumps not easily dispersed; weak = variable agglutination with the erythrocytes forming small definite clumps, in some cases somewhat easily dispersed; negative = erythrocytes all loose, coming up in a cloud, making no clumps at all. (Blind retests of sera always give identical classification.)

Experimental.—Of a group of 50 C57Bl/6J females about to be retired from the cross DBA/2J male × C57Bl/6J female, 12 had serum which strongly agglutinated H-2^d erythrocytes, and 15