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A Method for Quantifying Synchrony in Testes of Rats Treated with Vitamin A Deprivation and Readministration[1]

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ABSTRACT

Using a variation of a previously published method for manipulating vitamin A levels, we obtained synchronized rat testes and determined the frequency of stages of the seminiferous epithelium in each rat. In this study, we have demonstrated a method for quantitative analysis of the synchrony. The degree of synchronization was expressed as a fraction of the cycle of the seminiferous epithelium, and thus in terms not influenced by the different durations of the stages of this cycle. The median stage about which the tubules were synchronized was calculated. This method may be used to compare the effects of different synchronizing treatments, which may be subtle, and to study various aspects of spermatogenesis in the synchronized testes. For example, the duration of the cycle of the seminiferous epithelium in synchronized testes is estimated to be 12.5 days.

INTRODUCTION

When an animal becomes deficient in vitamin A, spermatogenesis regresses until only spermatogonia and preleptotene spermatocytes are left, and even these cell types are present in subnormal numbers (Huang and Hembree, 1979). This regression of spermatogenesis is reversible: upon refeeding with vitamin A, the more differentiated cell types reappear in the testis. Vitamin A-refed rats are fertile and have qualitatively normal spermatogenesis (Huang and Hembree, 1979).

More recently, it was observed that if vitamin A-deficient animals are injected with a high dose of vitamin A, spermatogenesis is restored in a synchronous manner (Morales and Griswold, 1987; Morales et al., 1989). In these rats, the 14 stages of spermatogenesis, as distinguished by Leblond and Clermont (1952), are not present in the normal, control frequencies; rather, only a few stages are seen. To further study these synchronized testes, a method of quantifying the degree of synchronization was needed. In our laboratory we have synchronized spermatogenesis in rats and studied several aspects of repopulation and synchronization (described in a subsequent paper). In this paper, we describe in detail our method for analyzing such results and illustrate its application to studies of the synchronization.

MATERIALS AND METHODS

Weanling (20-day-old) male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Three different diets were obtained from ICN (Cleveland, OH); all were custom AIN-76 diets without vitamin A. Diet A was deficient in retinol and retinoic acid; Diet B was identical to Diet A, but 10 mg/kg retinoic acid was added; Diet C was also identical to Diet A, but 30 mg/kg retinol had been added. Upon arrival in our laboratory, rats were fed Diet A to exhaust their reserves of vitamin A. This induced acute vitamin A deficiency, which causes regression of the seminiferous epithelium and has systemic effects. Although these general effects can be reversed by any of the retinoids, only retinol can reverse the effects of vitamin A deficiency on the testes (Coward et al., 1969). Thus, Diet B can relieve the systemic effects of vitamin A deficiency while maintaining this deficiency in the testes.

At 55 – 60 days of age, the rats became critically deficient in Vitamin A as a result of Vitamin A deprivation as judged from their appearance (listless, huffed fur) and weight loss; at 69 or 77 days of age, they were placed on Diet B until they reached 98 days of age. At this point, 4 rats were killed to study the chronic deficient seminiferous epithelium, and 5 rats were in-

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jected s.c. on the back with 5 mg retinol each (retinol acetate, Sigma No. R-3000, St. Louis, MO), dissolved in 0.5 ml 20% absolute ethanol and 80% sesame oil; 24 h later, each received a similar injection of 2.5 mg retinol according to the protocol of Morales and Griswold (1987). At the same time that the rats received their first retinol injection, they were placed on Diet C. Based upon mean food consumption of these rats, this diet provided 0.5 mg retinol per rat per day. Three of these 5 rats were killed 36 days after the first injection of vitamin A (post-vitamin A or PVA), and the other 2 were killed at 128 days PVA.

Testicular material was fixed in Bouin's solution. Tissues were mounted in paraffin and 4-μm sections were cut and stained by the periodic acid-Schiff (PAS)-hematoxylin method. From each rat, one transverse cross section through the testis was prepared for histological analysis. The section was scanned along parallel lines 1 mm apart under a 100× objective; the stage in the cycle of the seminiferous epithelium of each tubule that came in view was determined. The sections obtained at 128 days PVA were scanned twice, the routes being perpendicular.

In total, testes of 6 adult rats, fed Purina Rodent Laboratory Chow (#5001, Purina Mills, Inc., St. Louis, MO) ad libitum, were also investigated to obtain control frequencies for the different stages. The control rats were either 75, 110, or 203 days old (2 rats per age group). There were no significant differences in the distribution of stages over the tubule cross sections in these 6 rats ($\chi^2_{65} = 80.6$; $p>0.05$); hence, the results obtained are independent of age.

RESULTS

In testis cross sections, tubules were scored as to their stage in the cycle of the seminiferous epithelium, according to the system of Leblond and Clermont (1952), or, if that was not possible because of insufficient spermatids, they were scored as underdeveloped. The percentage of tubule cross sections found in each of these 15 categories was calculated. Results for the 5 rats are given in Table 1.

Qualitative Determination of the Degree of Synchrony

Dividing the percentage for each stage in treated rats by the percentage for that stage in control rats yielded the relative occurrence (Table 2). The values of the relative occurrence were plotted on a graph, where the *y*-axis gives the relative occurrence (1.0 representing the control) and the *x*-axis is expressed in stages of the cycle of the seminiferous epithelium, with the width of each stage proportional to its frequency in control rats (thus reflecting its duration). The plots (Fig. 1) demonstrate graphically which stages were enriched and to what degree.

Quantitative Determination of the Degree of Synchrony

The plots of relative occurrence versus stage can be interpreted as the portion of a repeating gaussian-like function that occurs within one cycle of the seminiferous epithelium. Alternatively, these distributions can be presented as the cumulative frequency versus stage of the cycle, starting from the stage at which the relative occurrence is a minimum (Fig. 2). These cumulative frequencies when plotted on a probability scale yielded nearly straight lines, at least for the testes analyzed at 36 days PVA. The positions within the cycle of the various percentile points were determined as follows. To locate the *X*th percentile point, we started at the stage of the cycle *(Y)* that was absent or showed the lowest relative occurrence and determined the point in the cycle *(Z)* at which *X*% of the staged tubules were at stages of the cycle more advanced than point *Y*, but less advanced than point *Z*. The median point of synchronization was given by the 50th percentile point. Also, we routinely determined the 15.9 and 84.1 percentile points. Although it is useful to think of these three points in terms of the normal distribution in which they would mark the mean, plus or minus one standard deviation, the quantification is not dependent on the nature of the distribution. The methods for calculating these points are given below for various situations.

A. Some sequential stages are absent. When some sequential stages are absent, these stages reliably determine the minimum point, or the 0/100% point. Stages that have a short duration, e.g., stages IX, X, and XI, can easily be missed by sampling errors. Therefore, we arbitrarily chose a minimum of 8% of the cycle of the seminiferous epithelium (e.g., stage VIII, or stages IX + X + XI) to be absent before identifying it as the 0/100% point.

The percentile points are most easily calculated from the raw data of the number of tubules found in each stage. We will show this calculation for the first rat, 36 days PVA (Table 3). In this rat, 328 tubules were analyzed, of which 326 could be classified in one of the 14 stages. The 0/100th percentile point is located in

TABLE 1. Frequency of stages of the cycle of the seminiferous epithelium in 5 synchronized rats, and the mean frequency of stages as determined in 6 control rats (mean ± SD).

		36 days PVA			128 days PVA	
Stage	Control	1	2	3	1	2
I	11.9 ± 2.2	0	0.4	0	12.3	2.8
II	7.6 ± 1.8	0	2.2	0.4	7.5	2.3
III	5.0 ± 0.6	0	1.1	1.4	2.0	1.5
IV	5.6 ± 1.7	3.7	3.7	1.4	2.3	1.2
V	4.9 ± 0.8	1.8	2.6	2.5	1.4	2.2
VI	6.6 ± 1.2	37.2	41.3	20.7	1.8	5.5
VII	20.0 ± 1.4	47.6	29.9	51.4	9.4	18.6
VIII	10.4 ± 1.4	8.8	10.3	18.6	9.9	25.0
IX	4.4 ± 1.8	0	3.0	2.5	4.1	6.3
X	2.1 ± 0.7	0.3	3.0	0	2.4	4.3
XI	2.2 ± 1.4	0	1.1	0	3.0	4.6
XII	7.8 ± 1.1	0	1.1	0	15.4	13.6
XIII	6.4 ± 1.6	0	0	0	15.8	9.5
XIV	5.0 ± 1.7	0	0	0	12.8	3.2
U*	– –	0.6	0.4	1.2	– –	– –
N**	2081	328	271	280	709	603

*U:Frequency of underdeveloped tubules.

**N: Total number of tubule cross-sections studied.

stages XI – III (Point Y), which were absent in this rat. The number of tubules between these stages and the 15.9th percentile point is 15.9% of 326, or 51.74 tubules. The number of tubules found respectively in stages IV, V, and VI, is 12, 6, and 122. Thus, the 15.9th percentile point is located in stage VI, at 51.74 – 18 = 33.74 tubular cross sections into stage VI. More precisely, the 15.9% point is located at 33.74/122 = 0.277 of the way through stage VI (point *Z*). Since stage VI encompasses 6.6% of the cycle in control rats, and 0.277 of 6.6% is 1.83%, the 15.9th percentile point is located 1.83% of the cycle of the seminiferous epithelium into stage VI. Since the duration of the stages I – V is (11.9 + 7.6 + 5.0 + 5.7 + 4.9)% (Table 1), or 35.1% of the cycle of the seminiferous epithelium, the location of the 15.9th percentile point can be said to be at 36.9% of the cycle (35.1% + 1.83%) of the seminiferous epithelium.

Any desired percentile point can be calculated this way. In Table 3, the calculation of the location of some other percentile points is demonstrated in an abbreviated form.

The synchronization window in each synchronized testis can be defined as the part of the cycle containing 68.26% of the staged tubule cross sections, distributed equally on both sides of the median (50th percentile point). These 68.26% of the tubules are at developmental stages that are between the points defined by the 15.9th and 84.1th percentile points. By subtracting the location coordinate for the 15.9th percentile point from

TABLE 2. The occurrence of the stages of the cycle of the seminiferous epithelium in synchronized and control rats, relative to control rats.

		36 days PVA			128 days PVA	
Stage	Control	1	2	3	1	2
I	1.0	0	0.03	0	1.03	0.24
II	1.0	0	0.29	0.05	0.99	0.30
III	1.0	0	0.22	0.28	0.40	0.30
IV	1.0	0.66	0.66	0.25	0.41	0.21
V	1.0	0.37	0.53	0.51	0.29	0.45
VI	1.0	5.72	6.35	3.18	0.28	0.85
VII	1.0	2.38	1.50	2.57	0.47	0.93
VIII	1.0	0.85	0.99	1.79	0.95	2.40
IX	1.0	0	0.68	0.57	0.93	1.43
X	1.0	0.14	1.43	0	1.14	2.05
XI	1.0	0	0.50	0	1.36	2.09
XII	1.0	0	0.14	0	1.97	1.74
XIII	1.0	0	0	0	2.47	1.48
XIV	1.0	0	0	0	2.56	0.64

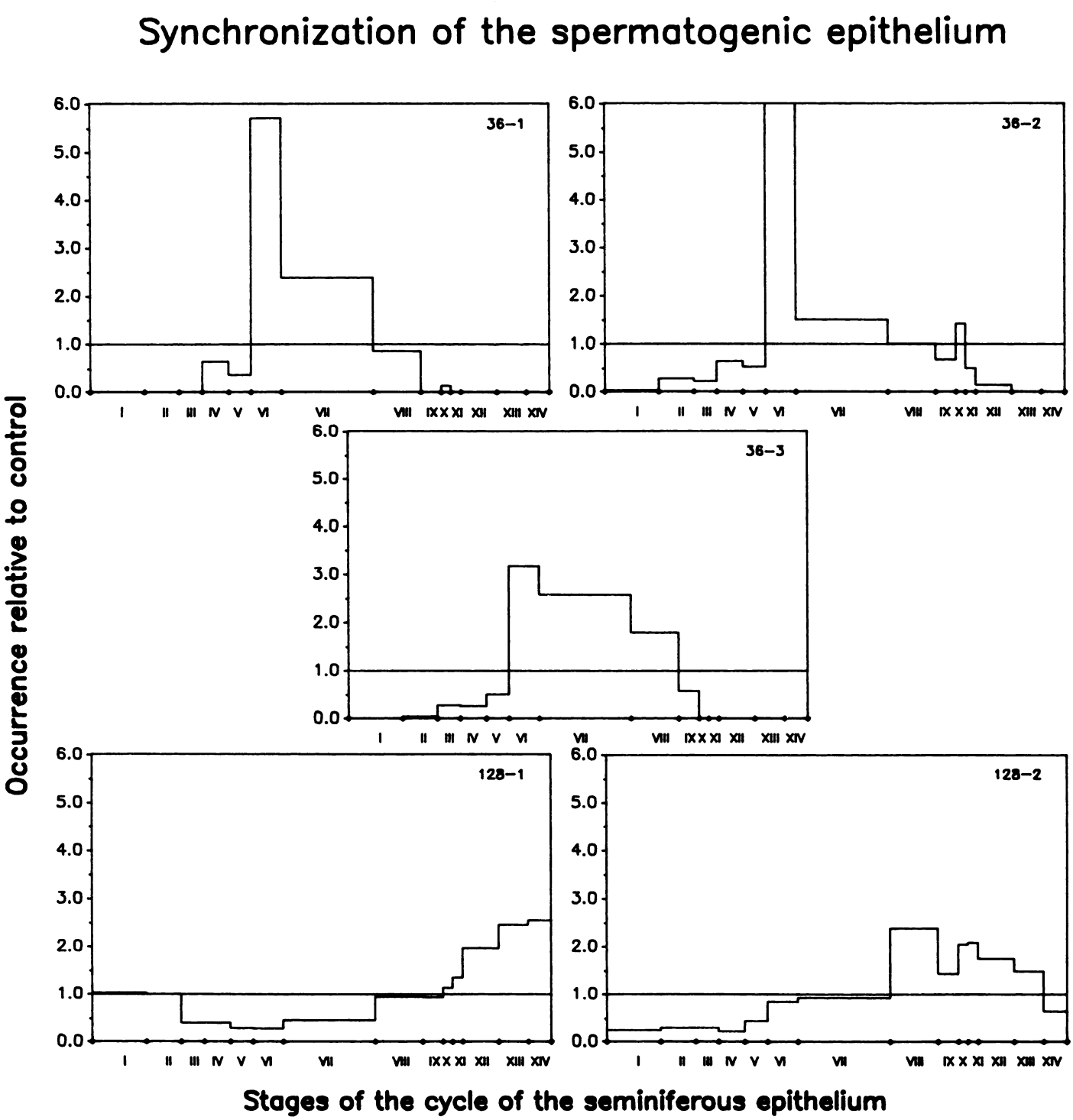

FIG. 1. Graphical representation of the degree of synchronization in testes of rats subjected to vitamin A manipulation. Each plot represents 1 rat, identified by a number of which the first part indicates the time after administration of vitamin A when the rat was studied. Presence and relative enrichment of the various stages of the cycle of the seminiferous epithelium can be seen and compared between different rats and different time points PVA.

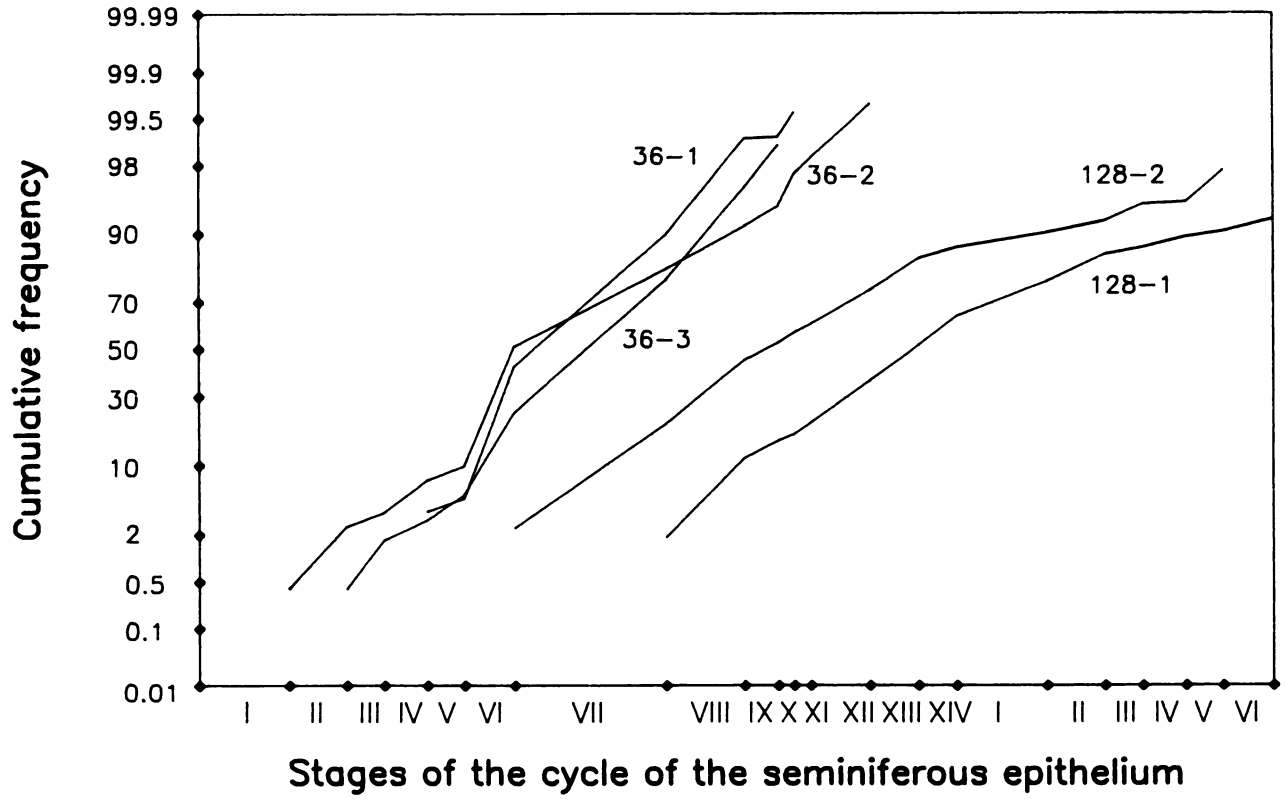

FIG. 2. Cumulative frequency of the stages present in the testes of rats subjected to vitamin A manipulation, plotted on a probit-axis, where the cumulative frequency of a normally distributed variable yields a straight line.

that of the 84.1th percentile point, we obtain the window width, that percentage of the cycle of the seminiferous epithelium that elapses between these two percentile points. For Rat 36-1, the synchronization window encompassed from 36.93% up to 58.92% of the cycle of the seminiferous epithelium (most of stages VI and VII), so the window width was 22.0%. In unsynchronized control rats, 68.3% of the tubule cross sections exhibited stages that comprised 68.3% of the cycle of the seminiferous epithelium; therefore, control rats can be said to have a window width of 68.3%.

We shall introduce an additional term, the synchronization factor, because different investigators might choose different percentile points to calculate window width. The synchronization factor is defined as the window width of control rats divided by the window width of the synchronized rats. For control rats, the synchronization factor is equal to 1; for Rat 36-1, it is 3.1.

B. All stages are present. The separation of a continuous developmental process into a discontinuous system of stages introduces errors into the calculation of the synchronization window. Some error is unavoidable; however, when all stages are present, there is a significant error associated with choosing the minimal point because of the low numbers of tubule cross sections in these stages. This error can be minimized by estimating the synchronization window in a slightly different way, which we will demonstrate on the data of Rat 128-2.

In Rat 128-2, 603 tubule cross sections were staged; 68.26% of 603 is 411.6, which should be the number of tubule cross sections in the synchronization window for this rat. The data in Table 2 show that stages VIII – XIII are enriched, having a relative occurrence of >1.0. The sum of the numbers of tubules in these stages is 382, which is 29.6 tubule cross sections short of 411.6. The next most enriched stage is stage VII, in which 112 tubule cross sections were found. Of these 112, 29.6 tubule cross sections, or 0.264 of these 112 tubule cross sections are needed to fill the synchronization window. In terms of the cycle of the seminiferous

TABLE 3. Calculation of various percentile points in synchronized Rat 36-1.

Percentile point:	15.9	50.0	84.1	100	
Number of tubules:	51.74	163	274.26	326	(A)
Stages needed to approach given[a] percentile:	IV-V	IV-VI	IV-VI	IV-X	(B)
Sum of tubules in stages in (B):	18	140	140	326	(C)
Rough location of percentile point (stage):	VI	VII	VII	XI-III	(D)
Additional tubules beyond those in (B) needed to reach percentile [(A) – (C)]:	33.74	23	134.26	0	(E)
(E)/Number in stage, given in (D):	0.277	0.147	0.861	0	(F)
(F) × (Relative duration of stage (D) (% of cycle):	1.83%	2.94%	17.21%		(G)
Summed relative duration of stages (from stage I), prior to stage (D):	35.1%	41.7%	41.7%		(H)
[(G) + (H)] (% of cycle):	36.93%	44.64%	58.91%		(I)[b]

[a]Latest stage that is absent in the synchronized testes of Rat 36-1 is stage III (referred to as point *Y* in the text).

[b]Line *I* gives the location of the percentile points in percentages of the cycle of the seminiferous epithelium, which is taken to start with stage I. (This location is referred to as point Z in the text).

epithelium, 0.264 of stage VII is equal to 0.264 × 20.0% = 5.29% of the duration of the cycle. Since the duration of stages VIII – XIII is 33.3%, 411.6 tubule cross sections can be found in 33.3 + 5.3 = 38.6% of the cycle of the seminiferous epithelium, which is the window width. The 15.9th percentile point is located at 1 – 0.264 = 0.736 of stage VII, or at 56.4% of the cycle, while the 84.1th percentile point is located at the border of stages XIII and IXV, or at 95.0% of the cycle. The 50.0th percentile point is 411.6/2 = 205.8 tubules away from either the 15.9th or the 84.1th percentile point. We find this at 75.0% of the cycle.

The data from Rat 128-1 demonstrate that when the median of synchronization is located at the later stages, the cycle of the seminiferous epithelium must be considered an unbounded variable extending beyond 100%. Thus, stage I extends from 0% to 11.9% and from 100% to 111.9%; stage II extends from 11.9% to 19.5% and from 111.9% to 119.5%; etc. The 15.9th percentile point is located at 76.5%, and the 84.1th percentile point is located at 118.6%. By expressing it this way, the window width can be calculated as 118.6 – 76.5% = 42.1%. The synchronization factor for this rat is 1.6. Quantitative data on the synchrony observed in the testes of the 5 rats are given in Table 4.

Duration of the Cycle of the Seminiferous Epithelium

As an example of one application of this quantification, the duration of the cycle of the seminiferous epithelium in these synchronized rats is calculated (Table 5). At 36 days PVA, the average median point of synchronization is (44.6 + 41.5 + 50.7)/3 = 45.6%, or at 0.456 of the cycle of the seminiferous epithelium. The duration of the cycle of the seminiferous epithelium in the normal Sprague-Dawley rat has been calculated as 12.9 days (Clermont and Harvey, 1965). Thus, at 36 days PVA, the epithelium should have passed through stage I three times; thus, the median point of synchronization can be expressed as 3 cycles + 0.456 of a cycle = 3.456. Equally, at 128 days PVA, the epithelium should

TABLE 4. Percentile points and other data on 5 synchronized rats presented as percentage of the cycle at which percentile point is located.

Percentile point	Rat #				
	36-1	36-2	36-3	128-1	128-2
15.9	36.9	36.0	38.3	76.5	56.4
50	44.6	41.5	50.7	94.0	75.0
84.1	58.9	64.4	64.7	18.6	95.0
WW (%)*	22.0	28.3	26.4	42.1	38.6
SF**	3.10	2.41	2.59	1.62	1.77

*WW: Window width; location of 84.1th percentile point minus location of 15.9th percentile point.

**SF: Synchronization factor, derived by dividing (84.1 – 15.9) = 68.26 by the window width.

TABLE 5. Calculation of duration of the cycle of the seminiferous epithelium, as derived from the median points of synchronization.

Rat #:	36-1	36-2	36-3		128-1		128-2
Time							
Time PVA:	36.01	36.03	36.07		128.10		128.02
Mean:		36.04				128.06	
Elapsed days:				92.02			
Stage							
Observed	3.446	3.415	3.507		10.940		10.750
Mean:		3.456				10.845	
Elapsed cycles:				7.389			
Elapsed days/Elapsed cycles:				12.45			

have passed through stage I ten times; data on synchronized rats killed at intermediate time points (not shown) indicate that the epithelium actually passes through slightly more than 7 cycles between Days 36 and 128. The average median point of synchronization can be expressed as 10.845. Thus, in 128 – 36 = 92 days, the seminiferous epithelium has completed 10.845 – 3.456 = 7.389 cycles, which means that the mean duration of the cycle of the seminiferous epithelium in these rats is 92 /7.389 = 12.45 days.

We can also estimate the stage from which spermatogenesis appears to be reinitiated from these data. Back-extrapolating, in 36 days, 36/12.45 = 2.891 cycles will be traversed, which indicates that for these rats the average median point of restarting the epithelium was at 3.456 – 2.891 = 0.565, or 56.5% of the cycle. This point correlates with 0.75 of the way through stage VII.

DISCUSSION

In this paper we describe a method to quantify results obtained on rats with synchronized spermatogenesis. Starting from the frequency distribution of stages, we calculate a window width of synchrony. The latter parameter provides two main benefits. First, the degree of synchrony can be represented by a single number, which then may be subject to statistical comparisons.

Second, it accounts for the fact that the stages of the cycle of the seminiferous epithelium have different durations. Thus, a testis synchronized in stages IX-X-XI (window width 5.9%) is in tighter synchrony than a testis in stages IV-V (window width 7.2%), stage I (window width 8.1%), or stage VII (window width 13.7%). Therefore, stating the window width of a synchronized testis gives a better measure of the tightness of synchronization than stating the stages in which it is synchronized. It should be noted that even if the tubules were in perfect synchrony, if the rat was killed when all tubule cross sections were in stage VII, the calculated window width would then only be 13.7%.

Observations

In this paper, data on 5 synchronized rats were analyzed. These analytical methods can provide a means to examine other phenomena, such as loss of synchrony over time, intratesticular variation of synchronized stages, and differences in the duration of the cycle of the seminiferous epithelium, either between animals or within a testis, or as a function of time after readministration of vitamin A.

The difference in the location of the 50th percentile points at similar time points PVA indicates that either there are individual differences between rats in the duration of the cycle of the seminiferous epithelium or spermatogenesis started again from different points in the cycle of the seminiferous epithelium. The differences in the window widths or synchronization factors been the 36-day PVA, and the 128-day PVA rats indicate that, to a certain extent, there is some slight dispersion in the kinetics of spermatogenesis, resulting in a loss of synchrony over time. However, a sample of more than 5 animals will be needed to show whether these results are valid.

The average median points of synchronization suggest that the cycle of the seminiferous epithelium is restarted at 0.75 of stage VII, or in stages VII + VIII. However, this is an extrapolation based upon a duration of the cycle of the seminiferous epithelium of 12.5 days for the first 36 days after injection of retinol.

Duration of Cycle of the Seminiferous Epithelium

The duration of the cycle of the seminiferous epithelium in the normal Sprague-Dawley rat has been calcu-

lated as 12.9 days (Clermont and Harvey, 1965). The duration of the cycle of the seminiferous epithelium in the synchronized rats was estimated by comparing the results obtained at 36 and 128 days PVA. These estimates indicate a cycle time of 12.45 days. Similar results were reported by Morales et al. (1989), who noted that at later times PVA, the observed stages were more advanced than the stages that were expected on the basis of a 12.83-day cycle of the seminiferous epithelium. It has been observed in young mice that the cycle of the seminiferous epithelium is faster than in adult mice (Kluin et al., 1982). This may be a phenomenon related to when spermatogenesis begins, and may occur also in rats when their seminiferous epithelium becomes synchronized.

Distribution of Stages in Synchronized Rats

The distributions of stages in these synchronized rats might give some insight as to the mechanism by which synchrony is induced and the reasons for the lack of better synchrony in some cases. The plot of the distribution of stages on a probit scale (Fig. 2) indicate that distribution is close to a normal distribution. However, it is possible that other models (e.g. probabilistic model and Poisson distributions) might fit as well. Furthermore, the plots can be distorted by several mechanisms, including (1) individual reactions of the rats to vitamin A resupplementation; (2) variation in response to vitamin A or progression of cells in different areas of the seminiferous tubules, resulting in a slight gradient of stages across the testis (van Beek and Meistrich, 1988); (3) sampling errors in determining the frequency of stages; and (4) errors introduced by separating a continuum of spermatogenic development into a discontinuous system of 14 stages.

Definition of "Synchronized"

The data given in Table 4 demonstrate that the 36-day PVA rats show a tighter synchronization than the 128-day PVA rats, as is reflected in the window width or synchronization factor. In rather tightly synchronized testes where some stages are absent, the synchronization factor is relatively independent of the percentile points used to calculate the window width. By stating this dimension, an absolute measure of synchrony can be obtained for comparisons of results between laboratories. In our experience, of 43 rats treated with this or similar protocols that showed a good restoration of spermatogenesis (round or elongating spermatids in more than 75% of the tubule cross sections), 9 had synchronization factors>3, of which 5 had synchronization factors>4.

Comparison with Data from Another Laboratory

We use our method or interpreting data on synchronized rat testes on the data provided by Morales et al. (1989). They reported on 11 rats between 35 and 78 days PVA, which, according to our calculations, showed window widths between 9% and 38% (mean: 16.4%) and synchronization factors ranging from 2.5 to 8.0.

A linear regression performed on the median points of synchronization of these 11 rats indicated a duration of the cycle of the seminiferous epithelium of 11.5 days. This correlates with their observation that at later times PVA, the stages found in the synchronized testes tended to be further developed than predicted. The extrapolation of this regression to the time of injection of retinol, however, points to stage III as the origin of restoration of spermatogenesis, instead of to stage VII-VIII, as expected by these authors (Morales and Griswold, 1987). This may be caused by rounding errors that cannot be avoided when working with the published data from another lab.

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