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Spermatogonial Stem Cell Renewal in the Mouse as Revealed by ^{3}H-Thymidine Labeling and Irradiation[1]

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Spermatogonial Stem Cell Renewal in the Mouse as Revealed by ^{3}H-Thymidine Labeling and Irradiation[1]

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The mechanism of spermatogonial stem cell renewal presented here has unfolded over the past few years as a result of long-term ^{3}H-thymidine (^{3}H-TdR) labeling (Oakberg, '71a; Huckins, '71b,c); cell mapping (Huckins, '71a); cell counts (Huckins, '71a; de Rooij, '73); ^{3}H-TdR labeling in tubule whole mounts (Huckins, '71b,c); cell survival and repopulation studies after irradiation and chemicals (Oakberg, '68; van Keulen and de Rooij, '74); and ^{3}H-TdR labeling in conjunction with irradiation (Oakberg, '71b). These experiments have indicated that the single (A_s) spermatogonium functions as the stem cell. Many questions of stem cell behavior remain unanswered, such as the relationship between the rapid- and slow-cycling components of the population, the kinetics of the long-cycling cells, control of stem cell numbers, and the point of commitment to differentiation. Though the earlier steps of stem cell behavior are not yet clear, the most reasonable interpretation of the data is the model proposed by Huckins ('71a). The A_s spermatogonia divide to form either new A_s cells or a pair of cells with a cytoplasmic bridge. Subsequent divisions of the A_{pr} generate

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chains of A_{al} spermatogonia which differentiate into the A_1. The successive generations of A_2, A_3, A_4, In, and B spermatogonia undergo differentiation and division to form primary spermatocytes.

The above model evolved concurrently with radiation studies designed to characterize and elucidate the behavior of the radiation resistant A spermatogonia. Study of spermatogonial repopulation after 100 R had suggested that some surviving A spermatogonia must have already been "programmed" at the time of irradiation. Furthermore, it was realized that the low mitotic index of A spermatogonia in the irradiated testis could arise through selective survival of cells with an inherently low mitotic rate. Long-term labeling experiments to test these hypotheses already were under way when Clermont and Bustos-Obregon ('68) published their two-stem-cell model of spermatogonial renewal in which there were an acyclic A_0 and a renewing, cycling population of A_1-A_4 spermatogonia. The A_4 were given the role of pivotal cells, forming either more differentiated In or less differentiated A_1 spermatogonia. We repeatedly had demonstrated the high radiation sensitivity of A_1-A_4 spermatogonia (Oakberg, '59) while high radiation resistance would be expected for the acyclic A_0 cells. It was immediately apparent that labeling with ^{3}H-TdR before irradiation would be a critical test of the two-stem-cell model. For if the model were correct, only A_1-A_4 spermatogonia should label, and should subsequently be killed by radiation. The surviving A_0 cells should not be labeled. However, data demonstrating labeled A spermatogonia 5 days after radiation doses as high as 1000 R had been available for some time (Oakberg, '64). Preliminary results from long-term labeling experiments in control mice revealed heavily labeled cells as long as 10 days (more than one cycle of the seminiferous epithelium) after ^{3}H-TdR injection. On the basis of these data, we suggested (Oakberg, '68) that the A_0 spermatogonia were the active stem cells of the seminiferous epithelium. Also, Hilscher ('64) had observed that labeled A spermatogonia persisted for as long as 13 days in the normal adult rat. Morphological analysis and cell-cycle studies of tubule whole mounts have revealed the stem cell role of the A_s spermatogonia of the rat (Huckins, '71a) and the existence of a long-cycling compartment of A_s cells (Huckins, '71c). Also, data demonstrating the survival of labeled spermatogonia in the irradiated rat testis are now accumulating (Huckins, unpublished data).

In our previous studies we relied on sectioned material. For this reason, the present experiments included both sections and whole mounts in order to compare results from the

two techniques. Both methods demonstrate the survival of A spermatogonia labeled with ^{3}H-TdR prior to irradiation, and the survivors are almost exclusively A_s spermatogonia. The data are consistent with the hypothesis that the stem cells are in continuous cycle, and that the total cycle time of some A_s spermatogonia is long. There is no evidence for a noncycling reserve stem cell (A_0) population.

MATERIALS AND METHODS

All mice used in these experiments were 12-week-old F_1 hybrid (101 X C3H) males. Both sections and whole mounts were made from the same animal: one testis was fixed for sectioning, the contralateral testis was used for whole mounts. For sections, testes were fixed for seven hours in Zenker-formol, washed for 12—15 hours in running tap water, embedded in paraffin, sectioned at 5 μm, stained by the PAS technique, dipped in Kodak NTB_2 emulsion, exposed for four weeks at 4°C, developed six minutes in D-170, and counterstained in Ehrlich's hematoxylin. $HgCl_2$ was removed by the procedure of Kopriwa and Huckins ('72). All procedures were rigidly controlled, for proper identification of spermatogonia cannot be made with confidence unless the slides are of excellent quality. For tubule whole mounts, segments of tubules were fixed for two hours in Bouin's fluid, and autoradiographs were prepared by the technique of Huckins and Kopriwa ('69).

Specific activity of the ^{3}H-TdR varied from 2 to 5 Ci/mmol. Dilutions were made in physiological saline to give the desired dose of 17.5 μCi/0.25 ml. All injections were made intraperitoneally.

Radiation exposures of 150 and 300 R were made with a Norelco 300 kV X-ray machine operated at 200 kV, 10 mA, with inherent filtration of 0.2 mm Cu, ∿90 R/min, and a target-testis distance of 53.5 cm. All experiments were run concurrently in order to minimize variability.

Stages of spermatogenesis were classified according to the cohorts of the differentiating spermatogonia. For several reasons, this classification is more meaningful than the 12 stages based on acrosome morphology of spermatids (Oakberg, '56a) which we have used previously: spermatogonial generations span more than one stage of acrosome development, data of sections and whole mounts are more readily compared, and analysis of spermatogonia with a low frequency per cross section is facilitated. These six stages in the cycle of the seminiferous epithelium are defined in table 1.

TABLE 1

Stages in the cycle of the seminiferous epithelium of the mouse as identified by the differentiating spermatogonia

Stage	Spermatogonial type	Comparable stages based on acrosome development	Approximate duration in hours*
1	A_1	Late VI - Late IX	62
2	A_2	Late IX - Late XI	29
3	A_3	Late XI - Early I	29
4	A_4	Early I - Late II	29
5	In	Late II - Late IV	29
6	B	Late IV - Late VI	29

*Calculated from the data of Monesi (J. Cell Biol., 14: 1-18, 1962) and Oakberg (Am. J. Anat., 99: 507-516, 1956).

RESULTS

In sections, the A_S spermatogonia can be identified on the basis of an oval, darkly-staining granular nucleus with poorly defined nucleoli (Figs 1 and 2). In whole mounts, they appear as isolated cells with ovoid nuclei and dust-like chromatin (Clermont and Bustos-Obregon, '68). The morphology of the A_S spermatogonia in controls is identical to that of the cells surviving 150 R, both in sections (Figs 1 and 2) and in whole mounts (Figs 3 and 4). Also, during regeneration, the A_{al} cells reappear at the same time at which they normally are generated (Figs 5 and 6). It is especially important to note that these cells appear in stage 3, in conjunction with A_3 spermatogonia *before* the appearance of the A_4. That this is not an artefact of the sections is demonstrated by the whole mount in figure 7. The morphology of the labeled spermatogonia 8.5 days after ^{3}H-TdR injection in the mouse is the same in both control and irradiated mice (Figs 8 and 9), and rats (Figs 10 and 11).

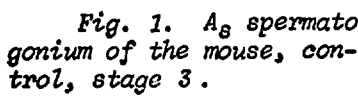

Fig. 1. A_s spermatogonium of the mouse, control, stage 3.

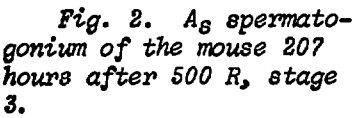

Fig. 2. A_s spermatogonium of the mouse 207 hours after 500 R, stage 3.

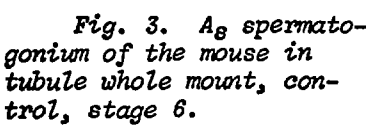

Fig. 3. A_s spermatogonium of the mouse in tubule whole mount, control, stage 6.

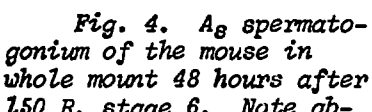

Fig. 4. A_s spermatogonium of the mouse in whole mount 48 hours after 150 R, stage 6. Note absence of B spermatogonia.

In the tubule whole mounts, extensive spermatogonial degeneration is seen in the first day after exposure to 150 R. By two days the spermatogonia have almost disappeared from stages 4 and 5. Of those cells which remain, most are undifferentiated A_s and A_{pr}, although a few short chains persist.

Fig. 5. A_{al} spermatogonia of the mouse in early prophase, stage 3, control.

Fig. 6. Early prophase of A_{al} spermatogonia of the mouse in stage 3, 207 hours after 300 R.

Fig. 7. Whole mount showing A_{al} spermatogonia in association with A_3 spermatogonia.

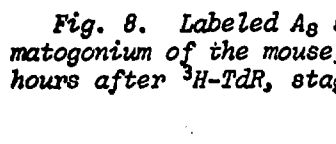

Fig. 8. Labeled A_s spermatogonium of the mouse, 207 hours after ^{3}H-TdR, stage 3.

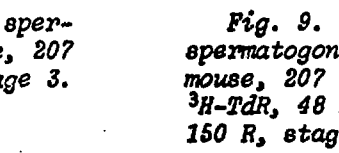

Fig. 9. Labeled A_s spermatogonium of the mouse, 207 hours after ^{3}H-TdR, 48 hours after 150 R, stage 4.

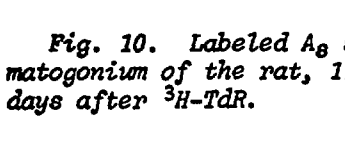

Fig. 10. Labeled A_s spermatogonium of the rat, 11 days after ^{3}H-TdR.

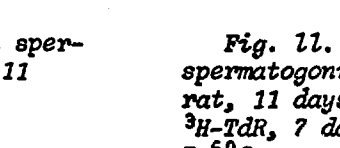

Fig. 11. Labeled A_s spermatogonium of the rat, 11 days after ^{3}H-TdR, 7 days after 330 R ^{60}Co γ rays.

Some of these show overt signs of degeneration. Incorporation of ^{3}H-TdR by some A_s spermatogonia is observed as early as two days after irradiation, and rarely, mitotic figures appear at this interval. [It is important to remember that almost the full complement of spermatogonia associated with preleptotene spermatocytes (stage 1) is still present at

two days, for cell death among A_{a1} and A_1 cells occurs primarily during late interphase or early prophase in the first postirradiation division at the end of stage 1.] Minimal numbers of spermatogonia are reached at three days in stages 4 and 5; they are almost exclusively the undifferentiated A_s cells. There are some pairs, but no chains of cells. A number of these spermatogonia have begun to divide; the mitotic index is 1.98. Interestingly, the total number of 0.64 spermatogonia per frame is comparable to the value of 0.47 A_s spermatogonia observed for control mice (Table 2).

TABLE 2

Number of undifferentiated A spermatogonia in stages 5 and 6 in control and irradiated mice. Data based on analysis of tubule whole mounts

Group dose	Interval (days)	Frames	ΣA	NA*
Control†	—	325	1272	3.50 ± 0.17
150 R-irradiated	2	385	852	1.26 ± 0.14
	3	308	215	0.64 ± 0.05
	5	793	631	0.81 ± 0.08
	8.5	287	696	2.54 ± 0.21
300 R-irradiated	2	155	96	0.60 ± 0.05
	3	355	161	0.51 ± 0.06
	5	424	162	0.37 ± 0.06

*Corrected to 20 Sertoli cells per counting frame (7225 μ^2).

†Counted from stage 6 (among B spermatogonia) only; of the average of 3.50 A spermatogonia per frame, 0.47 are A_s.

Exposure to 300 R gives an effect similar to 150 R, but degeneration is more profound. By two days, only A_s spermatogonia can be seen in whole-mount tubules , and it remains so until five days, when these cells begin to divide. It is of special note that the total number of A_s cells is comparable to the number of A_s spermatogonia in control animals (table 2).

The data of tables 3 and 4 extend the information in table 2 by demonstrating that significant numbers of the surviving A spermatogonia are labeled with ^{3}H-TdR. Stages 1

and 6 were omitted for irradiated mice in table 3 and the 48-hour interval of table 4 because these stages contain many lethally damaged spermatogonia which have not yet divided. Even with these precautions, cell counts of irradiated testes always will give an overestimate of survival because of the mixture of cells which already have begun to divide with those which have not yet degenerated.

TABLE 3

*Frequency of labeled cells 48 hours after 150 R; ^{3}H-TdR given 159 hours before irradiation**

Stage	6 X 17.5 µCi ^{3}H-TdR† Number of cells	6 X 17.5 µCi ^{3}H-TdR† % labeled	17.5 µCi ^{3}H-TdR Number of cells	17.5 µCi ^{3}H-TdR % labeled
Control				
1	103	30.1	111	9.0
2	116	36.2	120	8.3
3	107	41.1	89	14.6
4	104	49.0	117	19.7
5	117	45.3	109	10.1
6	54	37.0	57	1.8
150 R-irradiated				
2	98	27.6	80	16.3
3	42	50.0	44	20.5
4	45	62.2	72	27.8
5	66	51.5	67	7.4

*Total time from ^{3}H-TdR injection to killing was 207 hours, or one cycle of the seminiferous epithelium.

†Injections were given intraperitoneally every 12 hours; interval to irradiation was calculated from time of last injection.

Only rare survivors of A_2-A_4 spermatogonia were seen after 150 R, and they are easily identified. The data of table 3 are almost exclusively A_S spermatogonia. The matching whole-mount data confirm this observation. With a single ^{3}H-TdR injection, frequency of labeled cells was highest at stages 3 and 4, which are the same stages at which A_3 and A_4 spermatogonia are dividing. This confirms previous observations (Huckins, '71b; Oakberg, '71a) that this is a time of

active mitotic activity among the undifferentiated spermatogonia: it also points to the time of origin of the long-cycling spermatogonia. A high frequency of labeling was also observed at stage 5 after multiple injections. The primary difference between single and multiple injections is the higher proportion of labeled cells in the latter. The frequency of labeled cells is usually higher in irradiated mice than in controls because all the A_{al} cells have been killed.

TABLE 4

Frequency of labeled cells 48 and 60 hours after 300 R; ^{3}H-TdR given 24 hours before irradiation

	Time after 300 R			
	48 hours*		60 hours†	
Stage	Number of cells	% labeled	Number of cells	% labeled
Control				
1	120	10.0	97	21.6
2	124	1.6	67	1.5
3	109	21.1	68	7.4
4	121	14.9	73	21.9
5	70	14.3	58	32.8
6	56	32.1	37	35.1
300 R-irradiated				
2	59	6.8	56	10.7
3	36	0.0	35	5.7
4	32	6.3	31	0.0
5	34	55.9	33	33.3
6	—	—	47	40.4

*72 hours after ^{3}H-TdR injection.

†84 hours after ^{3}H-TdR injection.

Only A_s spermatogonia survived 300 R (Tables 2 and 4). Labeling was very strongly dependent upon the stage of the cycle of the seminiferous epithelium. Cells labeled during stages 3 and 4 gave rise to the 56% labeled spermatogonia in stage 5 at 48 hours, and the 40% labeling at stages 5 and 6 at 60 hours. Just as after 150 R (Table 3), this represents the time of peak mitotic activity of the undifferentiated spermatogonia. With 300 R, none of the cells labeling during

stages 1 and 2 in the cycle survived, for no labeled cells were observed either at stage 3 at 48 hours, or in stage 4 at 60 hours (Table 4).

DISCUSSION

The single type A spermatogonia have been identified in both whole mounts and sections as the primary cell type surviving 150 R and they are the only surviving spermatogonial type after 300 R (Tables 2-4). Likewise, Dym and Clermont ('70) identified the A_0 as the radiation-resistant cell, though their counts were made at a tubule stage and time interval that included surviving cells as well as lethally damaged A_{a1} and A_1 spermatogonia which had not yet degenerated. They speculated that the A_0 spermatogonia were stimulated to "break" their dormancy in order to replace the depleted "cycling" spermatogonial population. This is at variance with the results of Withers et al. ('74), which suggested that the kinetics of surviving stem cells was normal as long as 14 days after irradiation. Also, if ^{3}H-TdR is given before irradiation, a significant proportion of the surviving cells are labeled (Tables 3 and 4, Oakberg '64, '71b) and the whole-mount technique clearly reveals these to be single cells (Fig. 4). Therefore, the A_s spermatogonia must have been in cycle *prior* to the radiation insult. Furthermore, many of the surviving cells are labeled even after high radiation doses, where cell survival is very low (Oakberg '64, '71b). Similar data are now being evolved for the rat (Huckins, unpublished data), where labeled A_s spermatogonia survive 330 R of ^{60}Co gamma rays. Furthermore, the surviving cells appear to be long-cycling. This agreement of results in mouse and rat was predictable on the basis of the similarity of stem cell renewal in the two species.

The above data constitute further proof that some of the A_s spermatogonia in both mouse and rat are long-cycling (Huckins, '71c; Oakberg, '71a). They incorporate ^{3}H-TdR at the same time in the cycle of the seminiferous epithelium, labeled cells persist for the same period of time, and the shape of labeling curves is the same in irradiated and control animals. Therefore, one would not expect 100% labeling of these cells in experiments performed to date, for ^{3}H-TdR has not been given for the total cell cycle. Also, the long-cycling population is small, and one would expect to see very few divisions. This is demonstrated by high radiation doses, where the survivors come primarily from the long-cycling population and divisions are infrequent. In the model of stem

cell renewal proposed by Huckins ('71a) and Oakberg ('71b) the stem cells need divide only once during each cycle of the seminiferous epithelium, and therefore we would predict the low mitotic index for A_S spermatogonia reported by Clermont and Hermo ('75).

Since the cycle in the mouse is of 207 hours duration (Oakberg, '56b), scoring at that time permits the observation of cells in the same stage of the cycle of the seminiferous epithelium in which treatment occurred. The data (Tables 3 and 4) indicate a high frequency of labeled cells in the A_S population in both control and irradiated mice after 150 R. This radiation dose is essentially an LD_{100} for A_{pr}, A_{al}, and all generations of differentiating spermatogonia, and morphology of the surviving cells in both sections and whole mounts shows that they are either stem cells or cells recently derived from stem cells. That both controls and irradiated mice show similar labeling indicates comparable stem cell behavior (Oakberg, 71b). The 207-hour interval is especially significant, for, according to the dual-stem-cell concept of Clermont and Bustos-Obregon ('68), the cycling (A_4) spermatogonia should have divided four times in this interval, and the label would have been diluted by 2^4. The fact that labeling is as high as 41—49% in controls, and 50—62% in irradiated mice after multiple injections, and 10—20% and 10—28% after a single injection of ^{3}H-TdR (Table 3) demonstrates that the A_4 spermatogonium cannot have a stem cell role. These data agree with the observation of Huckins ('71a) in the rat and van Keulen and de Rooij ('74) in the mouse that the A_1 spermatogonia are derived from A_{al} cells, some of which divide at the same time as the A_4 division. On the other hand, some A_{al} divisions continue until stage 5 of the cycle. These were observed earlier by Hilscher et al. ('69), and were considered to be A_5 spermatogonia. The data of Huckins ('71a) clearly revealed these divisions as belonging to the A_{al}. In agreement with Monesi ('62), there is no evidence for formation of anything but In spermatogonia by the division of the A_4 cells.

In the two-stem-cell model proposed by Clermont and Bustos-Obregon ('68), the spermatogonia were divided into two populations, the noncycling A_0 (reserve stem cell) and the "renewing stem cells"—which included the A_1-A_4 spermatogonia. The A_4 cells were given the pivotal role, being able to form either the differentiated In or less differentiated A_1 types. Several difficulties with this model have not yet been resolved. Principally these are: (i) that formation of the A_1 from A_4 spermatogonia requires dedifferentiation of a cell well advanced in a developmental se-

quence [in fact, the A_4 spermatogonia were designated as In spermatogonia by Clermont ('62) prior to 1968], (ii) the mechanism by which chains of cells 2^n in length are separated from the syncytium of A_4 spermatogonia has not been explained, (iii) the role of the A_{al} spermatogonia which are intermingled with A_3 and A_4 has not been discerned, and (iv) cell cycle kinetics of undifferentiated spermatogonia is different from that of the differentiated types (Huckins, '71b). It is clear from the studies of Huckins ('71a), however, that the A_{al}, and not the A_4, are the progenitors of the A_1 spermatogonia (Fig. 12). In this model, the A_s, A_{pr}, and A_{al} are indicated as undifferentiated cells on the basis of nuclear morphology, though differentiation probably occurs at the stem cell level. A_1-A_4 spermatogonia are considered to be differentiated cells in contrast to the Clermont-Bustos Obregon ('68) scheme (Fig. 12). Many questions of basic stem cell behavior remain, but the model proposed by Huckins ('71a) provides a logical interpretation of spermatogonial stem cell renewal which is compatible with our understanding of spermatogenesis in the rest of the animal kingdom [see Hannah-Alava ('65) for a review]. Finally, there is reason to expect that all stem cell renewal systems should be basically similar, and the model proposed here also meets this requirement. It implies, however, that at least one cycle of the seminiferous epithelium is required for formation of A_1 from A_s spermatogonia, and that the full duration of spermatogenesis in the adult requires more than four cycles of the seminiferous epithelium.

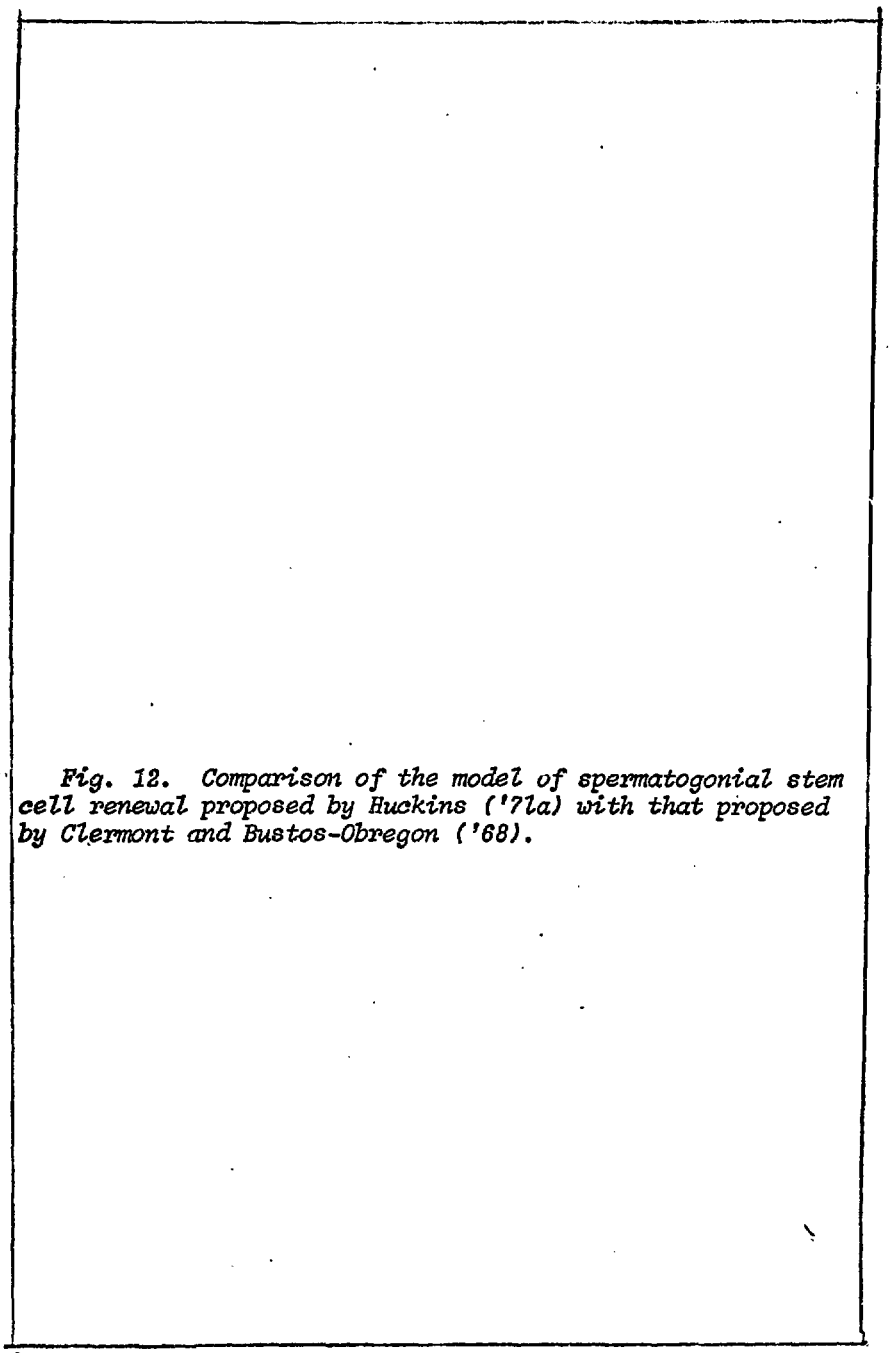

Fig. 12. Comparison of the model of spermatogonial stem cell renewal proposed by Huckins ('71a) with that proposed by Clermont and Bustos-Obregon ('68).

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31104

fig 1

21105

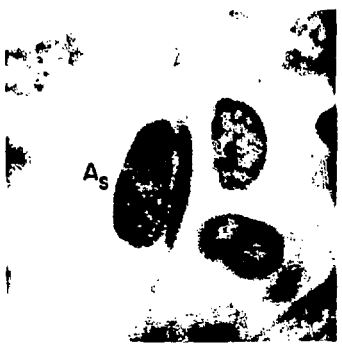

fig 2

31106

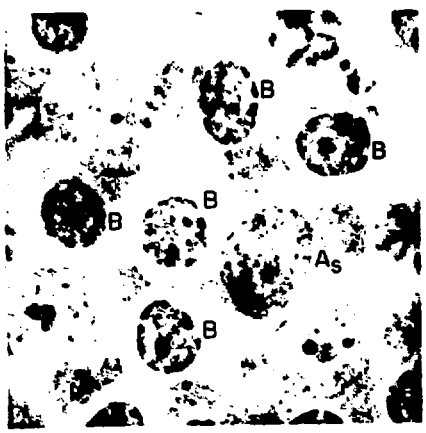

fig 3

31107

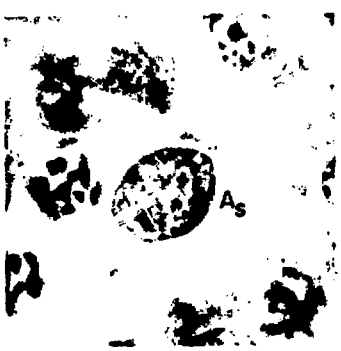

fig 1

31108

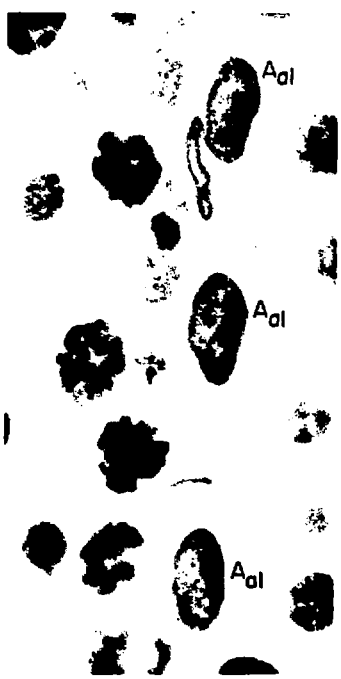

fig 5

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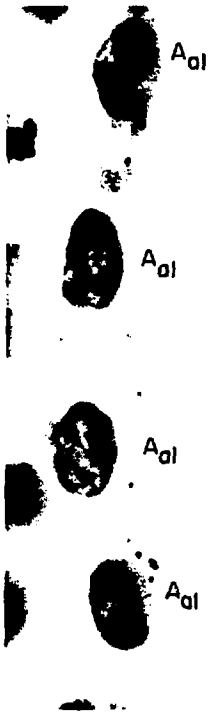


Fig 6

31110

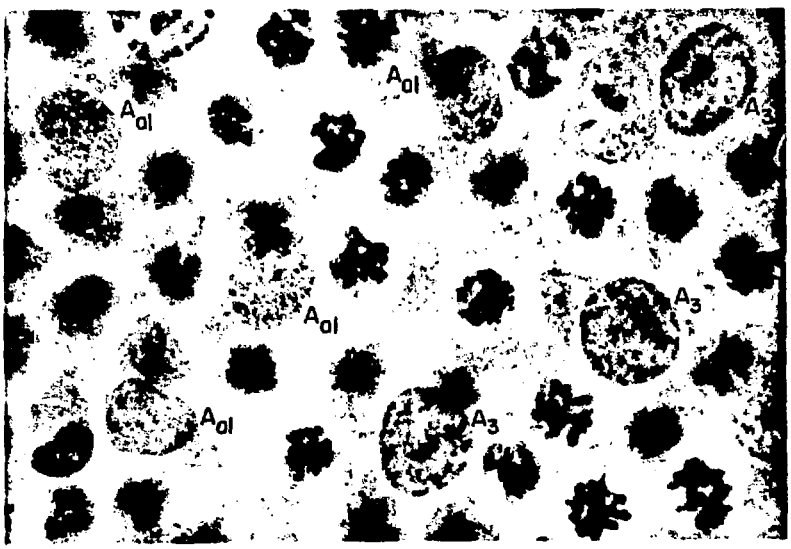
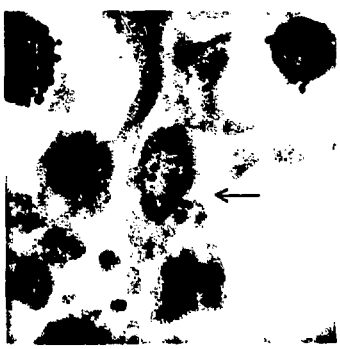


Fig 7

8.4f



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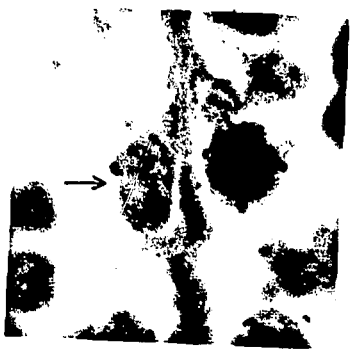

fig 9

31113

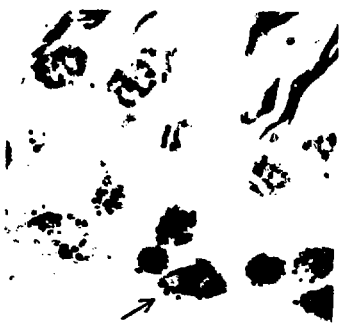

fig 10

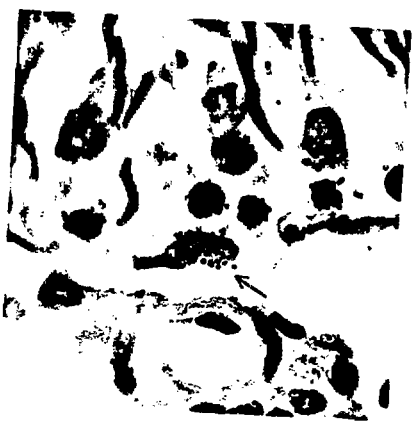

Fig 11

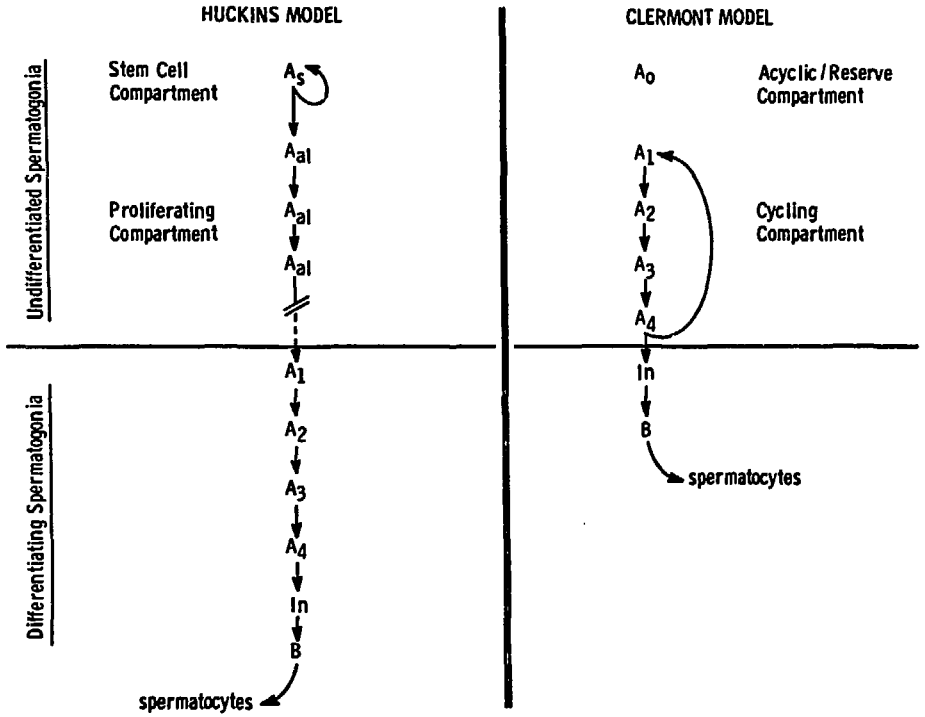
HUCKINS MODEL
CLERMONT MODEL
Undifferentiated Spermatogonia
Differentiating Spermatogonia
Stem Cell Compartment
Proliferating Compartment
A_s
A_{al}
A_{al}
A_{al}
A_1
A_2
A_3
A_4
In
B
spermatocytes
A_o
Acyclic / Reserve Compartment
A_1
A_2
A_3
A_4
Cycling Compartment
In
B
spermatocytes

fig 12