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Tentative Pattern for Renewal of Lymphocytes in Cortex of the Rat Thymus.* (23710)

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It is not known how the 4 cell types present in the thymus (reticular cells, large, medium and small lymphocytes) are involved in lymphocyte production(1-3). In the hope of clarifying the problem, the numbers of these cells and their mitoses were counted in different regions of the organ, and an attempt made to elaborate a pattern of lymphocyte renewal consistent with the experimental data. The present article is a preliminary report on the results and conclusions reached so far.

Methods. Twenty-eight 10-week-old male albino rats were divided into 4 groups of 7 animals each, sacrificed at 10 a.m., 4 p.m. and 10 p.m. during same day, and at 4 a.m. the next morning respectively. After fixation for 2 days in modified Bouin-Hollande (4 g picric acid; 2.5 g copper sulfate; 20 ml neutral formalin; 0.75 ml acetic acid; 0.75 g trichloracetic acid and 100 ml water), the tissues were sectioned at 5 μ and stained by the Dominici technic.

Results. Cytological criteria (Fig. 1). The *reticular cell* has a pale cytoplasm which is little or not visible and a large nucleus (to 11 μ diameter), oval or diamond-shaped, which appears very light. Fine chromatin dots are associated with the nuclear membrane. There is usually one, and sometimes 2-4 large oval nucleoli, the center staining orange-red, the surface light blue. A loose network of fine chromatin threads connects the nucleolus to nuclear membrane.

The *large lymphocyte* has a basophilic, well-limited cytoplasm, arranged into a more or less regular ring around the nucleus. The nucleus is usually round, diameter equal to or greater than 5.9 μ and stains slightly darker than in the reticular cell. Larger chromatin dots than in the reticular cell are associated with the nuclear membrane. There are 1-4 nucleoli of variable shape (oval, stellate, elongated) with an acidophilic core and a more bluish chromatin coat than in the reticular cell. The thread network within the nucleus is slightly denser than in the reticular cell.

The *medium lymphocyte* usually has a small ridge of basophilic cytoplasm, less in amount than in the large lymphocyte. The nucleus which measures 4.6-5.9 μ is often round and the nuclear sap appears blue with a purplish hue. Coarser chromatin masses than in the large lymphocyte are associated

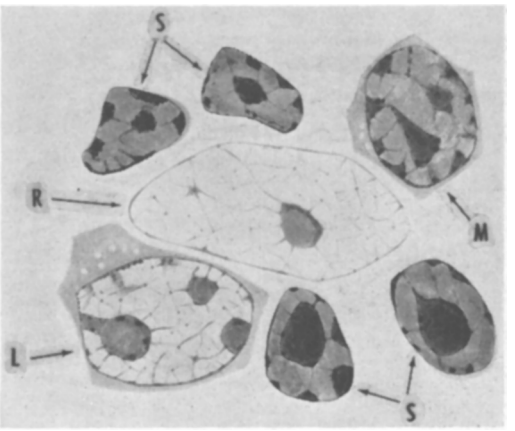

FIG. 1. Drawing of cells of thymus: R, reticular cell; L, large lymphocyte; M, medium lymphocyte; S, small lymphocyte.

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† Fellow of the Nat. Cancer Inst. of Canada.

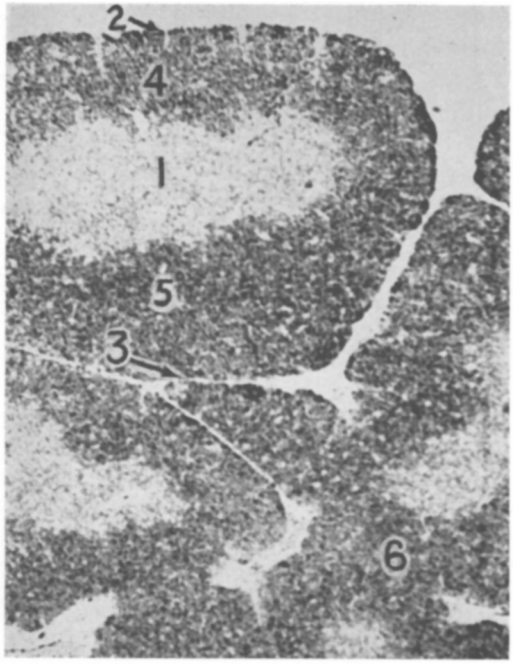

FIG. 2. Microphotograph of a portion of thymus from 10-wk-old male rat showing the 6 zones in which resting and dividing nuclei were enumerated.

with the nuclear membrane. The 1-3 nucleoli are smaller, more regular and have a darker blue coat than in the large lymphocyte.

The *small lymphocyte* has cytoplasm so scanty as not to be visible in most cases. The shape of nucleus is more variable than in the other 2 types of lymphocyte, the diameter is less than 4.6 μ. Many large masses of chromatin are associated with the nuclear membrane. The nucleolus is located towards the center of the cell and measures up to 1.7 μ diameter. It is composed of an acidophilic core and a chromatin coat which appears darker than in any other cell type.

Owing to the small amount of cytoplasm present in lymphocytes, the diameter of mitotic figures is approximately the same as that of resting nuclei. Hence, dimensions given above were used for identification of the mitotic figures of the various cell types.

Cell counts. Resting and dividing cells were counted at magnification of about 1000x using square microscopic fields (25 x 25 μ) in 6 different zones of the thymus (Fig. 2) : 1) medulla, which will not be considered here; 2) subcapsular peripheral cortex (within 25 μ of capsule); 3) subtrabecular peripheral cortex (within 25 μ of trabeculae); 4) subcapsular deep cortex; 5) subtrabecular deep cortex; and 6) intermedullary deep cortex. Resting cells were counted in the first 2 groups of animals but mitoses, including early prophases and late telophases, in all 4.

In each group, zones 2 and 3 yielded approximately the same counts/field for each cell type. Counts from these 2 zones were pooled and recorded for each animal under heading "peripheral cortex." For the same reason, results for zones 4, 5 and 6 were pooled and recorded as "deep cortex." Since the peripheral cortex makes up 9% and the deep cortex 66% of the parenchymal thymus, weighted averages were calculated for each count as follows:

$$\frac{(\text{peripheral cortex count} \times 9) + (\text{deep cortex count} \times 66)}{75}$$

and recorded under the heading "total cortex."

In either the peripheral or deep cortex, the number of cells/field did not vary over a 24-hour period, but mitotic activity showed slight, significant variations. We averaged the figures for all animals available and thus obtained values representative of a whole day. The mean numbers of cells and mitoses/field reported in Table I with their standard errors were thus obtained. In addition, the mitotic index for each cell type, that is, the ratio of number of mitoses over the sum of the numbers of resting cells and mitoses was calculated (Table I).

Basic assumptions. The assumptions which will be used in the discussion, together with a brief statement of the supporting evidence, are listed below:

1) *Four cell types are present.* The separation of cells into 4 types was justified by the fact that, using cytological criteria detailed above, reproducible counts of cells and their mitoses could be obtained.

2) *The 4 cell types are being continuously renewed.* This assumption is upheld since the mitotic indices in the 4 cell types (Table I) greatly exceeded mitotic indices observed in cell populations which are not renewed (under 0.002; see the discussion in ref. 4). Renewal implied that cells of the 4 types di-

TABLE I. Number of Cells and of Mitoses per Field and Mitotic Index in Peripheral, Deep, and Total Cortex of Thymus of 10-Wk-Old Rats.

	Peripheral cortex			Deep cortex		
	No. of cells	No. of mitoses	Mitotic index	No. of cells	No. of mitoses	Mitotic index
Reticular cells	.28 ± .06†	.005 ± .002	.016	.19 ± .03	.003 ± .001	.015
Large lymphocytes	1.07 ± .11	.079 ± .012	.069	.60 ± .08	.041 ± .004	.065
Medium "	1.92 ± .12	.295 ± .020	.133	1.04 ± .05	.156 ± .011	.130
Small "	14.3 ± .45	.379 ± .025	.026	16.0 ± .39	.221 ± .011	.014
	Total cortex*					
Reticular cells	.20 ± .03	.003 ± .001	.015			
Large lymphocytes	.66 ± .08	.046 ± .004	.065			
Medium "	1.15 ± .05	.172 ± .011	.131			
Small "	15.75 ± .39	.240 ± .011	.015			

* Weighted averages for total cortex based on relative volumes of peripheral and deep cortex.
† ± S.E. in all cases.

vided at frequent intervals to reproduce themselves and/or produce cells of another type.

3) *Small lymphocytes arise from medium, which come from large lymphocytes, which in turn come from reticular cells.* This assumption, which has been more or less clearly adopted by many investigators of the lymphocyte(1,5-7), was supported by existence of many transition forms. The progressive increase in numbers of cells and of mitoses from reticular cells to small lymphocytes (Table I) was also in agreement with this assumption.

4) *The life span* (intermitotic time + mitotic duration) *is the same for all cells of a given type.* Since the more frequently cells enter mitosis, the shorter their life span is, it is clear that the mean life span is inversely proportional to the mean mitotic index. Hence, the present assumption requires constancy of the mitotic index for each cell type. This appeared to be the case for reticular cells, large and medium lymphocytes, since they had approximately the same mitotic index in both peripheral and deep cortex (Table I). (Complicating factors with regard to small lymphocytes will be mentioned below.) It must not be forgotten that the existence of diurnal variations in mitotic activity indicates inverse fluctuations in life span and, therefore, the life span under consideration is a mean figure for the 24-hour period. However, it is likely that, even at a given time, the cells of one type have the same life span, since islands formed of similar cells which occasionally undergo mitosis simultaneously were commonly seen in sections.

5) *Mitotic duration is taken to be the same in all cells* of the thymus. This assumption is justified by the results of Widner *et al.*(8), who showed that mitotic duration was approximately the same for a variety of cells in the rat.

6) *Only small lymphocytes leave the thymus in significant numbers,* an assumption based on the predominance of small lymphocytes in lymph and blood vessels.

Further assumptions, which concern the life span of small lymphocytes but do not affect our main argument, will be mentioned later.

Discussion. It has been shown that, when nuclei are counted in sections, the count is higher than the actual number of nuclei(9). Errors are directly related to nuclear size (9), and tend to cancel out when ratios of nuclei of similar size are considered. Accordingly, the data in Table I were used to calculate such ratios for cells with nuclei fairly similar in size. When the ratios of large lymphocytes to reticular cells, and of medium to large lymphocytes were calculated, they were approximately the same for peripheral, deep, and total cortex; therefore, only those ratios obtained for total cortex are reported in Table II.‡

However, the ratio of small to medium

‡ The formula used to calculate the standard errors of the ratios was of the form $\pm \frac{1}{B^2}\sqrt{B^2a^2 + A^2b^2}$ in which A and B are respectively the numerator and denominator of the ratio, and a and b their respective standard errors.

TABLE II. Experimental Ratios of Number of Cells, Mitoses and of Mitotic Indices for Total Cortex of Thymus of 10-Wk-Old Rats.

Cells involved in ratios	Ratios of— No. of cells	No. of mitoses	Mitotic index
Large lymphocytes / Reticular cells	3.3 ± .58†	14.8 ± 3.5	4.3 ± 1.2
Medium lymphocytes / Large lymphocytes	1.8 ± .22	3.8 ± .38	2.0 ± .31
Small lymphocytes* / Medium lymphocytes	7.4 ± .53	1.3 ± .12	.19 ± .02

* In the case of small lymphocytes, only the figures obtained in the peripheral zone of the cortex were used.

† ± S.E.

lymphocytes in deep cortex (15.3) was about twice that in peripheral cortex (7.4), a fact suggesting that some accumulation of small lymphocytes takes place in deep cortex (see end of section on "Relation of small to medium lymphocytes"). It was therefore decided that the counts of small lymphocytes obtained in the peripheral cortex would be used in calculating the ratios in Table II.‡

The purpose of the present discussion is to find a pattern for lymphocyte renewal consistent with the 9 ratios in Table II.

Relation of medium to large lymphocytes. Since the ratio of mitotic indices of medium to large lymphocytes is 2 (Table II), it follows that the life span of medium must be half that of large lymphocytes. It was decided to calculate on this basis what the ratios of the mean numbers of cells and of mitoses of these 2 cell types would be if one generation of medium lymphocytes were to follow either one, 2 or more generations of large lymphocytes. Let us first consider the simplest possibility, that is, single generations of large (L_1) and medium (M_1) lymphocytes, as represented in diagram I of Fig. 3 (in which the vertical dimension is time). Any large lymphocyte would then divide to give rise to 2 medium lymphocytes, which would in turn divide to produce small lymphocytes. Thus, medium lymphocytes would be twice as numerous as large lymphocytes, but since they live only half as long, both cell types would be found in sections in equal numbers and the ratio of the total number of medium to large lymphocytes would be 1 (Table III). It is evident that this ratio is smaller than that recorded in the results in Table II, so that the possibility presented in diagram I may be discarded. If 2, 3 or more generations of large lymphocytes preceded one of medium lymphocytes, similar reasoning would indicate that in each case the ratio of the numbers of cells (Table III) would be much smaller than that obtained experimentally. Accordingly,

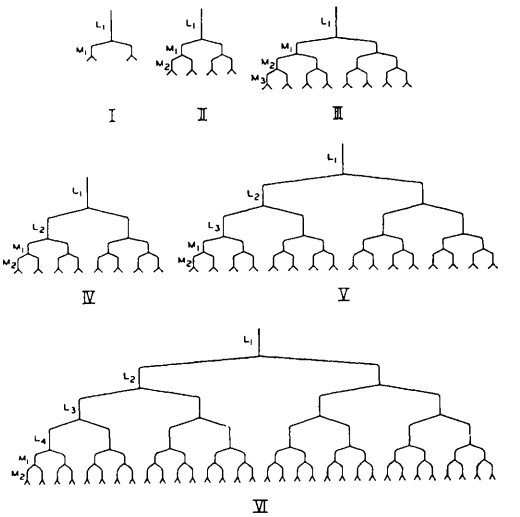

FIG. 3. Diagrammatic representation of possible patterns for the relation of large to medium lymphocytes (1 generation of large to 1, 2 or 3 generations of medium lymphocytes in diagrams I, II and III respectively; 2, 3 or 4 generations of large to 2 generations of medium lymphocytes in diagrams IV, V and VI respectively). The letters L, M and S refer to large, medium and small lymphocytes and the subscript numbers to the cell generation involved. The mitoses are indicated on the diagrams by the junction of a vertical and 2 oblique lines. (Vertical dimension in this and following diagram indicates time.)

TABLE III. Expected Ratios for Number of Medium to That of Large Lymphocytes and for Number of Mitoses of These 2 Cell Types, According to Number of Cell Generations.

Relative No. of cell generations			Ratios of	
Medium lympho-cytes	Large lympho-cytes	Diagram No. (Fig. 3)	No. of cells	No. of mitoses
1	1	I	1.00	2.00
	2		.66	1.33
	3		.57	1.14
	4		.53	1.06
	5		.52	1.04
2	1	II	3.0	6.0
	2	IV	2.0	4.0
	3	V	1.7	3.4
	4	VI	1.6	3.2
	5		1.5	3.1
3	1	III	7.0	14.0
	2		4.7	9.3
	3		4.0	8.0
	4		3.7	7.4
	5		3.6	7.2

In all cases, the mitotic index of medium is twice that of large lymphocytes.

it was concluded that there must be more than one generation of medium lymphocytes.

On the basis of 2 generations of medium lymphocytes following one generation of large lymphocytes (Diagram II, Fig. 3), there would be 1 large lymphocyte (L_1) for either 2 or 4, that is, a mean of 3 medium lymphocytes (M_1, M_2), so that the ratio of the number of medium to large lymphocytes would be 3. Furthermore, the odds of finding medium lymphocytes undergoing mitosis would be 6 times as great as those of finding a large lymphocyte undergoing mitosis, so that ratios of numbers of mitoses would be 6 (Table III). Neither the ratio of the number of cells nor that of mitoses agreed with the experimental findings (Table II). Hence, the possibility illustrated in diagram II was discarded. Calculations were then made on the basis of 2 generations of medium following either 2 (diagram IV), 3 (diagram V), 4 (diagram VI) or 5 generations of large lymphocytes. The data (Table III) revealed that a fair agreement with the experimental results was obtained in each one of the 4 cases. It thus appeared that there were 2 generations of medium lymphocytes, but the number of preceding generations of large lymphocytes remained to be established.

Relation of large lymphocytes to reticular cells. Calculations were then made for 2 to 5 generations of large lymphocytes combined with a variable number of generations of reticular cells. Examination of the ratios of numbers of mitoses (Table IV) indicated that a figure approaching the experimental number (14.8) was obtained only with 4 generations of large lymphocytes. Examination of ratios of numbers of cells (Table IV) revealed that, with 4 generations of large lymphocytes, one generation of reticular cells provided the best fit with the experimental data. However, other possibilities (*e.g.* 0.8 generation of reticular cells for 4 of large lymphocytes) yield ratios which are also fairly close to the experimental figures and cannot be definitely discarded. (The standard errors for number of mitoses of reticular cells were high, Table I. A recount of the mitoses of these cells in 5000 fields gave figures within the range of the errors. Even so, conclusions relating to reticular cells may be considered to be more tentative than those relating to other cell types).

Relation of small to medium lymphocytes. The next step was to investigate the number of generations of small lymphocytes which

TABLE IV. Expected Ratios for Number of Large Lymphocytes to That of Reticular Cells and for Number of Mitoses and Mitotic Indices of These 2 Cell Types According to the Number of Cell Generations.

Relative No. of cell generations		Ratios of		
Large lympho-cytes	Reticular cells	No. of cells	No. of mitoses	Mitotic index
2	.5	.7	3	4
	1	1.5	3	2
	2	3.0	3	1
3	.5	1.2	7	6
	.75	1.7	7	4
	1	2.3	7	3
	1.20	2.8	7	2
	1.5	3.5	7	2
	3	7.0	7	1
4	.80	3.0	15	5
	1	3.7	15	4
	1.33	5.0	15	3
	2	7.5	15	2
	4	15.0	15	1
5	1	6.2	31	5
	2.5	12.4	31	2
	5	31.0	31	1

TABLE V. Expected Ratios for Number of Small Over That of Medium Lymphocytes and Mitotic Indices of These 2 Cell Types According to Life Span of Small Lymphocytes (for 2 Generations of Medium Lymphocytes).

Life span of small lymphocytes (× life span of medium lymphocytes)		Ratios of	
1st generation (S_1)	2nd generation (S_2)	No. of cells	Mitotic index
1	1	4.0	.33
	2	6.7	.20
	2.25	7.3	.18
	2.50	8.0	.17
	3	9.3	.14
2	1	5.3	.25
	1.50	6.6	.20
	1.75	7.3	.18
	2	8.0	.17
	3	10.6	.13
3	1	6.6	.20
	1.25	7.3	.18
	1.50	8.0	.17
	2	9.3	.14

follow the 2 generations of medium lymphocytes. Calculations revealed that with 3 generations of small lymphocytes the ratio of the number of mitoses of small to that of medium lymphocytes would be 4. Only with 2 generations of small lymphocytes was it possible to obtain a ratio of number of mitoses which was in agreement with the experimental data (1.3). Some conclusions may be reached regarding the life span of small lymphocytes in peripheral cortex. The possibilities considered will be limited by the assumption that only second generation small lymphocytes (S_2) migrate out of this zone. The life span of these cells will be considered to end with their exit from the peripheral cortex, whereas the life span of first generation cells (S_1) would end with their mitosis. Table V was constructed for various durations of first and second generations of small lymphocytes. It may be seen that 3 possibilities are consistent with the experimental data given in Table II. The life spans of S_1 and S_2 may be respectively equal to 1 and 2.25, 2 and 1.75, or 3 and 1.25 times the life span of medium lymphocytes. Although it is not possible to decide between these 3 possibilities, it is suggested that the first one may be correct, since the life span would then be the same for S_1 and medium lymphocytes, and such a conclusion implies a continuity between these two cell types. Whereas the argument so far applies to the small lymphocytes in peripheral cortex, it is possible to adapt a similar reasoning to the deep cortex on the assumption of an equal life span of S_1 in deep and peripheral cortex. The life span of S_2 in deep cortex was then calculated to be approximately 4 times as long as that of S_1. Thus, second generation small lymphocytes would spend about twice as much time in deep as in peripheral cortex.

Stem cell renewal theory. Whereas large lymphocytes transform into medium lymphocytes (diagram VI, Fig. 3) and medium into small lymphocytes, reticular cells yield large lymphocytes while preserving their own stock. A pattern fulfilling this requirement is presented in Fig. 4. It may be seen that each division of a reticular cell gives rise to a new reticular cell and to a large lymphocyte, which undergoes a series of divisions, so that 4 generations of large lymphocytes are produced before the new reticular cell under-

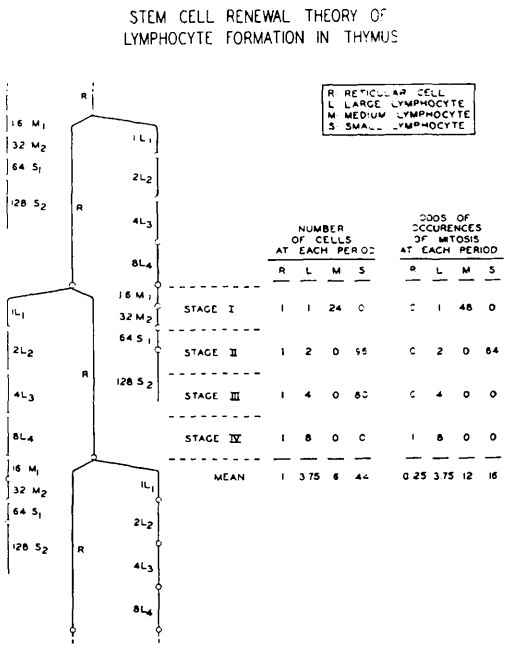

FIG. 4. Most probable pattern for the filiation of thymic cells. The numbers of cells and the odds of occurrence of mitosis at each one of the 4 stages are obtained by inspection of the corresponding portion of the diagram. The means (lower right) are used to calculate the "expected" ratios reported in Table VI.

TABLE VI. Comparison of Experimental Ratios of Number of Cells, Mitoses and of Mitotic Indices with Ratios Expected on Basis of Diagram in Fig. 4.

Cells involved in ratios	No. of cells		No. of mitoses		Mitotic index	
	Exp.	Expected	Exp.	Expected	Exp.	Expected
Large lymphocytes / Reticular cells	3.3 ± .58†	3.7	14.8 ± 3.5	15.0	4.3 ± 1.2	4.0
Medium lymphocytes / Large lymphocytes	1.8 ± .22	1.6	3.8 ± .38	3.2	2.0 ± .31	2.0
Small lymphocytes* / Medium lymphocytes	7.4 ± .53	7.3	1.3 ± .12	1.3	.19 ± .02	.18

* In the case of small lymphocytes, only the figures obtained in the peripheral zone of the cortex were used.

† ± S.E. in all cases.

goes division. Meanwhile, each 4th generation large lymphocyte yields medium lymphocytes, which go rapidly through 2 generations to produce small lymphocytes, which in turn divide to yield a total of 128 mature small lymphocytes. Examination of cells associated with the reticular cell at any given time makes it possible to subdivide the sequence of events into 4 stages (referred to as stages I. II. III and IV in Fig. 4), which follow one another indefinitely. Thus each reticular cell and its progeny would be going through a self-repeating cycle composed of these 4 stages. This observation yields a simple method of calculating the expected ratios of the cells. The number of cells and the odds of occurrence of mitosis were determined at each stage and averaged (Fig. 4). The ratios of the means are reported in Table VI as the "expected" values. It may be seen in Table VI that the expected values fall within the range of standard errors of the experimental figures in all cases (except for the ratio of number of mitoses of medium to that of large lymphocytes, which is on the borderline). It is concluded that the agreement between experimental and expected ratios is close; and. therefore, the scheme appears to be statistically valid. However, eventual refinement of the technic or variations in the assumptions may yield a similar or slightly different scheme which also fits the data. The pattern is considered to be sufficiently close to the observed facts to be used as a tool in future experimental work. It is proposed to refer to this scheme as the "Stem cell renewal theory" of lymphocyte formation in the thymus.

Summary. 1) Counts of resting and dividing cells in the cortex of the thymus of 10-week-old male rats reveal a high mitotic index of the 4 cell types present: reticular cells, large, medium and small lymphocytes. This observation indicates that each one of the 4 cell types is being renewed. Presumably this renewal yields lymphocytes which leave the thymus and enter the circulation. 2) From the mean numbers of cells and of mitoses and the mean mitotic indices of the four cell types over a 24-hour period, a tentative scheme referred to as "Stem cell renewal theory" is presented to account for the continuous production of lymphocytes in the thymus. This theory (Fig. 4) is that each reticular cell at regular intervals yields large lymphocytes which pass through 4 generations and then produce medium lymphocytes, which in turn pass through 2 short-lived generations and then give rise to small lymphocytes, of which there are also 2 successive generations.

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Long Term Endogenous Creatinine Clearance in Man.* (23711)

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Endogenous creatinine clearances are similar to inulin clearances in the normal individual when run *simultaneously* for short periods of time (5-15 minutes)(1-3) and also when run *independently* (no inulin infusion) for long periods of time (1-24 hours)(4-5). Factors that might influence the endogenous creatinine clearance were investigated. Creatinine clearances were determined on 39 normal individuals and the values compared with reported short term inulin clearance values for individuals of the same age group—the determination of inulin clearances on the group of volunteer subjects was not possible.

Methods. All subjects were free from hypertension, muscle or kidney disease. Urine was collected without catheterization, a 1:1000 dilution made and 5 ml removed for analysis. Blood was obtained by venipuncture and placed in a tube containing potassium oxalate. Protein was removed from the plasma by the sodium tungstate-sulfuric acid method of Folin-Wu(6). Creatinine was determined by the method of Van Pilsum *et al.* (7).† Urea was determined by the Van Slyke(8) modification of the Folin-Wu(6) method.

Results. The effect of urinary volume variations on simultaneous creatinine and urea clearances was determined on 2 normal adult males. Clearances were of one or 2 hours duration and were run consecutively. Blood was withdrawn at the beginning and the end of each clearance period and the average of the 2 values was used in the calculation of the clearance. Urea clearance was calculated as standard clearance (C_s) when the rate of flow was less than 2 ml per minute; when greater than 2 ml per minute, it was calculated as maximum clearance (C_m). Creatinine clearance was calculated according to the clearance formula $\frac{UV}{P}$. Immediately preceding and during the clearance period, the subjects assumed normal activities, but avoided vigorous exercise or the ingestion of large amounts of meat. Diuresis was produced by ingestion of water. The results are shown in Table I. The range of variation of 139 to 169 ml per minute (subject 1) and of 123 to 139 ml per minute (subject 2) is of the same order of

TABLE I. Effects of Urinary Volume Variations on Simultaneous Creatinine and Urea Clearances in the Normal Adult Male.

	Urine vol (ml/min.)	Creatinine clearance (ml/min./1.73 m²)	Urea clearance (ml/min./1.73 m²) C_s	Urea clearance (ml/min./1.73 m²) C_m
1.	.88	169	63	56*
	1.08	139	72	69*
	3.00	161		115
	5.00	167		151
	3.00	161		94
	1.53	152	66	74*
2.	1.40	139	59	65*
	1.22	123	58	60*
	1.45	126	60	67*
	2.05	137		80
	9.95	131		114

Standard clearance (C_s) $= \frac{U\sqrt{V}}{P}$ maximum clearance (C_m) $= \frac{UV}{P}$(10).

* Clearances calculated according to the formula $\frac{UV}{P}$, regardless of rate of urine flow.

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† In this method creatinine is degraded to methylguanidine by O-nitrobenzaldehyde in the presence of alkali. The methylguanidine is measured with a modified Sakaguchi color reaction.