ANTIHYPERTENSIVE drugs provide sufficient control of hypertension to avert most acute manifestations of the disease in human beings. This clinical success, however, does not prevent chronic vascular injury. Myocardial infarction and cerebrovascular disease still occur at an accelerated rate and account for premature death. This implies that humoral or hemodynamic changes in hypertension, even with moderate elevations in pressure level, interfere with the function of components of the vascular wall. For example, medial smooth muscle of the hypertensive vessel shows cell growth and connective tissue biosynthesis during the early stages of hypertension. The hyperplastic response is transient, but connective tissue synthesis continues to occur to the degree necessary to compensate for changes in wall tension. The presence of hypertension also accelerates the accumulation of smooth muscle cells to form arterial lesions in lipid-fed animals. Somewhat less is known about the effects of hypertension on the endothelium. We do know that permeability is increased. Studies using fluorescein or Evans blue-labeled serum proteins have indicated the presence of focal regions of increased permeation in arteries of animals with acute renal hypertension. Furthermore, there is evidence of transient increases in endothelial permeability following administration of hypertension-producing doses of angiotensin II. Taken together with the apparent delicate structure of the endothelium, these studies suggest thatcontinuity of the endothelial cell layer is damaged in hypertension.

The objective of this study is to provide a quantitative measure of this injury by examining the ability of endothelium to maintain continuity in normal and hypertensive rats. This is done by examining endothelial cell replication and turnover using tritium-labeled thymidine ($^3$H-TdR) to detect replicating cells. As we will show, rats with acute renal hypertension show a 10-fold increase in replication rate as compared with normotensive controls of the same age. The significance of this increase is discussed in terms of the normal process of cell replication in young and aging rats.

Methods

**ANIMALS**

All experiments were performed on female Sprague-Dawley rats. Except in the aging study, rats 5-6 months of age were used. The aging study used rats ranging from newborn (less than 1 day old) to 1 year of age.

**LABELING SCHEDULES**

Tritium-labeled thymidine ($^3$H-TdR, New England Nuclear, 6.7 Ci/mmol) was dissolved in saline and administered by ip injection. Each dose consisted of 50 /uCi/$^3$H-TdR/100 g. The same dosage (50 /uCi/100 g) was used in all experiments. In all, three different dose schedules were used in the various experiments. The various dosage schedules are summarized in Table 1.

Experiment I was designed to see if labeled cells replicate. In the first group the rats received only a single dose and were killed 1 hour later. A second, comparable group treated at the same time as the first group was killed 24 hours after the same single dose.

Experiment II was designed to determine the effect of age on labeling index. The dosage schedule consisted of three doses of $^3$H-TdR (each dose: 50 /uCi/100 g) at 8-hour intervals. The schedule was chosen to minimize diurnal variation and to provide large enough numbers of cells to permit accurate counting. In addition, if we assume that S phase is about 8 hours long, this schedule provides an estimate of the number of cells which replicate each day.

Experiment III was designed to determine the retention or loss of labeled cells. The rats received the usual schedule of three doses but were not killed until either 24 hours or 24 days after the final dose. The group killed at 24 hours was designed to allow cells to complete the initial cell cycle begun during the labeling interval (as in experiment I). Thus, any further change in thymidine index could be attributed to further cell divisions or loss of cells from the surface.

Experiment IV was designed to assay the effect of hypertension. The same dosage schedule was used as in experiment II.
TABLE 1  Experimental Protocols

<table>
<thead>
<tr>
<th>Dosage Schedules</th>
<th>Experiments</th>
</tr>
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<tbody>
<tr>
<td>dose</td>
<td>24 hours</td>
</tr>
<tr>
<td>1h</td>
<td></td>
</tr>
<tr>
<td>dose</td>
<td>dose</td>
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<td>8h</td>
<td>8h</td>
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<td>dose</td>
<td>dose</td>
</tr>
<tr>
<td>8h</td>
<td>24h</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

θ = fixation by perfusion.

FIXATION PROCEDURES

The procedures for fixation by perfusion with formaldehyde-glutaraldehyde solution at physiological pressures and flow rates were as previously described.8 Under ether anesthesia, a catheter was inserted in the carotid artery. A flowmeter was used to estimate the pressure drop between the perfusion bottle and the catheter tip. This provided an accurate estimate of intravascular pressure. For all rats, including those with hypertension, vessels were fixed at an intraaortic pressure of 90-100 mm Hg and at flow rates of 20-40 ml/min over an interval of 5-10 minutes. The hypertensive rats were fixed at “normal” pressures because of variability in pressures measured in anesthetized rats at time of sacrifice. The procedures for newborn and 2-week-old rats were similar except that the catheter was inserted in the left ventricle.

TISSUE SAMPLES

After removal of the vessel, all aortas except those of newborn and 2-week-old rats were divided into five pieces: A, B and C represented three equal segments of thoracic aorta; D represented the upper portion of the abdominal aorta and E represented the lower portion of the abdominal aorta, including the trifurcation. Aortas of newborn rats were redivided into equal upper and lower segments.

MEASUREMENT OF VESSEL AREA AND CELL DENSITY

Vessels used for measurement of aortic surface area were removed from the rat as described above, except that the aortas were not divided into segments. The aorta was cut just below the subclavian artery and just above trifurcation. This single, large piece was pinned out flat on a Teflon sheet without distending the vessel beyond its fixed dimensions. Measurements of length and width were made directly on the vessel at several points and transferred to graph paper. Area was measured from the drawings on the graph paper.

Cell density (CD), the average number of cells per unit area, was obtained from enface preparations as described below under Collection of Data.

TISSUE PREPARATION

Our procedure for preparation of endothelium for autoradiography has also been described in detail.9 In brief, it consists of three steps: (1) Removal of endothelium: The aorta is opened along the dorsal margin and pinned out flat. The tissue is dehydrated and the endothelium is embedded in a layer of collodion. The collodion film is stripped away from the aorta and removes the endothelial layer as a sheet. (2) Exposure of luminal surface of endothelial cells: At this point, the preparation consists of endothelial cells with their luminal surface embedded in collodion. In order to remove the collodion, we first fix the exposed subendothelium to a glass slide using a layer of gelatin which is then polymerized with formaldehyde. Once the subendothelium is fixed to the slide, the collodion is removed by dissolving it in 1:1 ether/ethanol. (3) Autoradiography: This leaves a sheet of endothelium with the luminal surface directly exposed and available for coating with emulsion for autoradiography. The cells are preserved in their original distribution and most or all cells are included. The preparations are subsequently coated with Kodak NTB-2 emulsion and exposed for 2 weeks. The preparations are then developed, stained with hematoxylin and mounted under coverslips.

COLLECTION OF DATA

Segments from which extensive portions of the endothelium had not been removed by the collodion layer, or in which the endothelium was obscured by folding, were
Results

REPLICATION OF LABELED CELLS

In this experiment, rats were labeled with a single dose of \(^{3}H\)-TdR. Six were killed 1 hour after the dose and six, 24 hours after the dose. As can be seen from Table 2, the thymidine index at 24 hours is approximately double the value at 1 hour. This approximate doubling of the thymidine index at 24 hours is compatible with completion of an initial cell replication by the labeled cells. Furthermore, it seems unlikely that many nuclei are labeled by nonreplicative processes such as DNA repair or changes in chromosome number.

EFFECT OF AGE

This experiment was developed to estimate the contribution of cell growth to the replication rate. Cell replication represents the sum of the rate of cell proliferation (i.e., rate of population increase) and the rate of cell turnover (i.e., rate of replacement of spontaneously lost cells) (Fig. 1). In most tissues of young animals, growth is very rapid and thus the portion of the dividing cell population involved in expansion of cell number constitutes a large portion of the number of replicating cells. The contribution of proliferation to the thymidine index therefore should reach a minimum value coincident with cessation of growth. Other things being equal, the residual replication rate then should represent an estimate of the rate of turnover or replacement.

The thymidine index was examined for rats from birth to 1 year of age. As indicated above, the schedule chosen for thymidine administration consisted of three doses at 8-hour intervals. This schedule is independent of diurnal variation and should provide an estimate of the total daily rate of cell replication. The estimate is high to the extent that cells labeled with the first or second pulses divide prior to measurement. If all the cells labeled by the first pulse divide and no more cells divide, the estimate will be approximately \(\frac{1}{2}\) higher than the true value. This error will, of course, be consistent since the same schedule was used for all rats in the age study and also for the experiments on hypertensive rats.

The thymidine index is maximal at birth and declines to a minimum value between 3 and 6 months of age (Fig. 2). There is also a significant increase in thymidine index between 6 months of age and 1 year (Table 3). The reasons for this are unclear; however, it is of interest to note that normal rats in our laboratory showed spontaneous increases in blood pressure over this time (Table 4).

Finally, an attempt was made to directly measure the residual proliferation in rats over 6 months of age. This was done by determining the total area of the aorta and

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Replication of Labeled Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>Thymidine index*</td>
</tr>
<tr>
<td>1</td>
<td>0.07% ± 0.04</td>
</tr>
<tr>
<td>24</td>
<td>0.17% ± 0.02</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE.

* Based on average of all segments in each rat.
AORTIC ENDOTHELIAL CELL REPLICATION/Schwartz and Benditt

TABLE 3
Thymidine Index as Function of Age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Number of rats</th>
<th>Percent thymidine index</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-6</td>
<td>16</td>
<td>0.15 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. Thymidine index is based on the average for all segments of aorta in each rat.

Table 4
Blood Pressure as Function of Age

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Number of rats</th>
<th>Pressure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-6</td>
<td>26</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>110 ± 4</td>
</tr>
</tbody>
</table>

* Determined by tail-cuff method; mean ± SE.

Figure 1
Proliferation vs. turnover. The progeny of endothelial cell replication may have one of three fates: As shown in A, the progeny may be retained in the population without loss of other cells. This results in proliferation, that is, an increase in total cell number. As shown in B, the progeny may be retained without loss of progeny but with a reciprocal loss of cells elsewhere on the surface producing a form of "turnover" similar to cell replacement in gut epithelium. Finally, as shown in C, the progeny may be produced in "high turnover" areas. In this model, the progeny themselves are more likely to be lost from the surface than are cells from other areas with low turnover.

Figure 2
The purpose of this experiment was to determine the fate of labeled cells. The usual schedule of three doses was given to 12 rats to label all cells entering S phase over a 24-hour interval. Six rats were sacrificed 24 hours after the final dose, because experiment I had demonstrated that labeled cells would have completed an initial cell cycle by this time. Thus any changes in thymidine index after this time would be due either to loss of cells from the surface or to further cell division. The remaining six rats were allowed to survive for an additional 24 days before they were killed. As can be seen in Table 5, the thymidine index at 24 days is only 1/3 of the value found at 24 hours after labeling.

The first conclusion drawn from this experiment is that something has happened to the labeled cells. Either they have been lost from the surface or they have divided sufficiently rapidly to allow dilution of label below the minimum required for autoradiography. It is also evident that the process producing disappearance of cells must be nonrandom. For example, we know that the total number of cells is either constant or increasing. Therefore the average rate of cell loss, i.e., turnover, can be no greater than the number of cells per unit area, the "cell density," in rats of different ages. If cell density were constant in all portions of the aorta, then the total number of cells could be determined as the product of area and cell density. Unfortunately, we found that cell density varies as a function of position along the length of the aorta as is seen in Figure 3. As a result, it is difficult to estimate the absolute number of endothelial cells per vessel. However, cell density distributions at 6 and 12 months are quite similar, both terms of the number of cells per unit area and the distribution along the aortic length. During the same time, the area of aortic surface increases, on the average, by 14% (area in mm² ± 1 SE: 6 months, 378 ± 39; 12 months, 431 ± 31). Thus the rate of growth in total cell number, on the average, is approximately 14%. Similarly, between 1 and 2 years there is, if anything, a decrease in cell density while the average increase in area is 9% (area in mm² ± 1 SE: 24 months, 470 ± 23). Although the variance of the area measurements is quite high, there appears to be little growth in total cell number between age 6 months and 1 year. In the second year of life, with increasing area and decreasing cell density, it is conceivable that the endothelial cell population is constant in number and is being diluted in density by an age-associated increase in aortic surface area. In contrast, total body weight of these rats continued to increase between 6 months and 2 years of age (weight in grams ± 1 SE: 6 months, 293 ± 12; 12 months, 322 ± 13; 24 months, 371 ± 18).

RETENTION OF LABELED CELLS

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that way diluting the label below the minimum required for counting. It might be explained by their dividing several times over the 3 weeks and in replications of the initially labeled cells. That is, the loss of labeled cells with 3 x 10^1 total cells. The formula is again the same, however, this time the rate of loss of labeled cells if the label itself were lost due to serial population based on a constant rate of cell loss of 0.3% /day, again starting from the most cranial segment. If the loss of almost 70% of the labeled cells in 3 weeks is due to cell turnover, then the progeny of the originally labeled cells have added 5% to the total number of aortic endothelial cells over a 3-week interval. This is far in excess of the apparent rate of growth of the aortic endothelium. Thus it is not likely that proliferating cells could replicate enough times to produce the apparent loss of labeled cells.

In summary, the loss of labeled cells must result from cell turnover. This could, however, occur in two ways. The replicating cells could be located in areas of the surface or subpopulations of the aortic endothelium which are subject to a high rate of cell loss (Fig. 1B). Alternatively, the dividing cells could belong to germinal centers, similar to the intestinal crypts, that is, areas of cell growth which provide new cells to replace cells lost elsewhere in the endothelial surface (Fig. 1C).  

**EFFECTS OF HYPERTENSION**

The purpose of this experiment was to obtain an estimate of the daily replication rate in rats with acute sustained hypertension. Rats 5–6 months old, were used in order to minimize the contribution of cell replication to growth (experiment II). The rats were killed within 2 weeks after their blood pressure exceeded a value of 130 mm Hg. Since this usually required about 2 weeks after surgery, the total interval ranged from 4–6 weeks.

Two groups of hypertensive rats were studied. The initial group consisted of 12 age-matched rats and included 4 operated rats, 4 sham-operated rats and 4 unoperated controls. The 4 operated rats showed an increase in thymidine index compared to either unoperated controls or sham-operated rats (Table 6). The second group of rats consisted of a series of 20 additional operated hypertensive rats without paired controls. This larger group was added to the rats in Table 6 to provide more extensive data for comparison with data from normal rats of the same age (experiment 2). As can be seen in Figure 4, hypertensive rats of ages between 5 and 6 months showed a 5- to 10-fold increase in replication rate compared with age matched controls.

Since the observations included cells from the entire aortic surface, data from all hypertensive rats and from controls aged 5–6 months were used also to examine the effect of location on the aortic surface on the rate of

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**Table 5** Retention of Labeled Cells

<table>
<thead>
<tr>
<th></th>
<th>Thymidine index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>0.66 ± 0.13</td>
</tr>
<tr>
<td>24 days</td>
<td>0.20 ± 0.03</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± se.

* Based on average of all segments in each rat.
TABLE 6  Hyperensive Rats vs. Sham and Unoperated Controls

<table>
<thead>
<tr>
<th>Rat no.</th>
<th>Blood pressure*</th>
<th>Thymidine index (%)†</th>
<th>Blood pressure*</th>
<th>Thymidine index (%)</th>
<th>Blood pressure*</th>
<th>Thymidine index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>145</td>
<td>1.8</td>
<td>100</td>
<td>0.05</td>
<td>100</td>
<td>0.18</td>
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<tr>
<td>2</td>
<td>165</td>
<td>2.4</td>
<td>120</td>
<td>0.07</td>
<td>100</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>1.1</td>
<td>100</td>
<td>0.04</td>
<td>92</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>110‡</td>
<td>0.3</td>
<td>90</td>
<td>0.08</td>
<td>100</td>
<td>0.10</td>
</tr>
<tr>
<td>Mean</td>
<td>1.4</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
</tbody>
</table>

* By tail-cuff method.
† Daily replication rate, average of all segments of the aorta in each rat.
‡ Rat 4 did not meet our experimental criteria for hypertension as an elevation to >130 mm Hg. However, this rat was included in the evaluation of data to preserve the pairing with unoperated and sham-operated controls.

endothelial cell replication in normal and hypertensive rats (Fig. 5). The hypertensive rats showed an elevated rate of replication in all segments examined. The usual regional variations seen in nonhypertensive controls, however, were maintained. Thus in both hypertensive and normotensive rats, the replication rate was lower in the abdominal segments of aorta (D and E) than in the thoracic segments. It is interesting to note that the area of the trifurcation (E) showed a lower replication rate than other segments. This was true both for hypertensive and normal rats, although again the replication rate was higher in the endothelium of hypertensive rats.

The presence of a relatively low rate of replication near...
the aortic trifurcation should be noted. Studies on other species have indicated an increased rate of endothelial replication near areas of branching including the trifurcation. This difference in results could be the result of differences in experimental procedure. Our procedure for tissue preparation is more efficient than other procedures in that it preserves most or all of the area of a segment. Thus focal changes in replication near the branched area would be obscured if the overall rate of replication in the remainder of the vessel were sufficiently low. On the other hand, in a previous study we reported maps of the distribution of labeled cells on the aortic surface of the rat. These maps also failed to show any close association of areas having labeled cells with areas of vascular branching.

**Discussion**

Our observations support five conclusions:

1. Endothelial cells of the rat aorta divide within 24 hours of labeling with $^3$H-TdR.
2. The daily replication rate of normal endothelium reaches a minimum value at 3-6 months of age. The total number of cells in the aorta increases by only an average of 14% between 6 and 12 months of age. No increase in cell number occurs between ages 12 and 24 months.
3. About two-thirds of labeled cells disappear from the endothelium within 24 days after rats receive $^3$H-TdR.
4. Hypertension of 2 weeks' duration produces a 10-fold increase in cell replication over the normal values for 5-6 month old rats.

This increase occurs in all areas of the aorta. Despite the increase, normal patterns of regional variation in replication rate remain with a relatively high rate of replication in the thoracic aorta and a low rate in the abdominal aorta.

**ENDOTHELIAL CELL REPLICATION IN THE AORTA, EFFECT OF AGE**

It is important to note that cell replication in any system includes the production of more cells (proliferation) as well as replacement of lost cells (turnover) (Fig. 1). Thus two factors contribute simultaneously to the labeling index in young, actively growing rats. This makes interpretation of data developed in growing animals complex. We attempted to obviate this factor by studying the effect of age on the endothelial replication rate. As Figure 2 shows, cell replication reaches a minimum between 3 and 6 months of age. Further experiments, including experiments with hypertensive rats, all were conducted on animals 5-6 months old. Our data show that the residual replication rate at this age largely represents cell turnover. If there were growth at a low, constant rate of 0.3% growth per day, the aortic endothelial population would double in cell number over 8 months. This is much higher than the rate of growth, as estimated from direct measurement: there is only an increase of approximately 14% in number of cells between age 6 months and age 12 months. It remains possible, however, that some portion of the 0.1-0.3% daily replication represents residual proliferation.

**FOCAL REGIONS OF ENDOTHELIAL CELL TURNOVER**

We attempted to estimate the amount of turnover more directly in experiment III. In this experiment rats were killed 24 hours and 24 days after labeling. The number of labeled cells decreased by 1/2 over the 3-week interval. Unless $^3$H-TdR is toxic to the labeled cells, these data support the idea that the daily replication rate largely represents cell turnover. Moreover, as presented in Results, the data imply that the replicating cells occur as clusters of aortic endothelial cells which are subject to high rates of cell turnover. This is consistent with other data from our laboratory based on maps of the distribution of labeled cells on the aortic surface. These maps showed focal areas where as many as 10-50% of the cells were labeled, while other areas showed few or no labeled cells. It is reasonable to postulate that the pool of rapidly turning over cells which was demonstrated in this study corresponds to the clusters of labeled cells demonstrated in the maps. In summary, the normal aortic endothelium of the adult rat contains focal areas of increased cell turnover.

**EFFECTS OF HYPERTENSION**

The principal effect reported here is a 10-fold increase in the replication rate of endothelium in hypertensive rats. One interpretation of this observation is that, in some way, hypertension causes endothelial cell damage and that this "damage" is reflected by an increased rate of cell turnover. If this is true, then the mean life span of endothelium has gone from 1-3 years to only 100 days (life span = reciprocal of replication rate, assuming no cell proliferation). Moreover, if this increased turnover occurs in clusters, as described above, there should be focal areas where the mean life span is extremely short. This is important since a rapidly turning over endothelium may be unable to maintain itself as a continuous cell layer. Furthermore, even in the presence of a "continuous" endothelium, regenerated cells may be abnormal in structure and function. Our ultrastructural studies have shown defects in the intracellular junctions of regenerated endothelium as late as 6 months after mechanical denudation. Similar physiological studies show increased permeability to plasma proteins even after continuity of injured endothelium has been reestablished. Thus an increase in turnover would be expected to have important consequences for endothelial function.

Increased turnover, however, is not the only possible interpretation of increased replication. The change in replication could equally represent a purely proliferative response. That is, an increase in the number of endothelial cells could occur because of proliferation in compensation for stretching of the wall and the resulting increase in surface area. Similar changes have been described for medial smooth muscle. Wolinsky has shown an increase of 25% in aortic diameter of Goldblatt hypertensive rats 2.5 months after surgery. During this acute phase, smooth muscle cells undergo proliferation and hypertrophy. By 16 months after surgery, however, the blood pressure stabi-
lizes at a new level and smooth muscle cell growth ceases. The result is an increase in wall thickness in proportion to elevated pressure and dilation of the vessel. The increased thickness is adaptive, producing a value for wall stress no different from that of control animals. Once stress has returned to normal, cell growth and connective tissue production also return to normal. Similarly, a 25% dilation of the aortic diameter will produce a 25% increase in the surface area which must be covered by endothelium. By analogy to the behavior of smooth muscle cells, an increased replication rate in the endothelium then may represent a transient hyperplastic response required to reestablish a normal number of cells per unit surface area.

Finally, if the increase in cell replication is due to endothelial injury, it is important to consider possible eliciting factor(s). An increase in turnover might result directly from elevated pressure. However, it is hard to imagine that the endothelial cell is damaged by the relatively small changes in absolute levels of pressure seen in this experiment. Endothelial cells sit inside a rigid tube formed by the elastic layers of the tunica media. It is likely that most of the gradient of pressure drop across the wall occurs in the media rather than across the fragile endothelium. This implies that if hemodynamic parameters are important, parameters other than the absolute pressure level are more likely to cause injury. On the other hand, there is evidence that components of the renin-angiotensin system may cause vascular injury. Elevated levels of renin have been implicated in accelerated atherosclerosis in man and renin or angiotensin can induce increased endothelial permeability in small arteries and the aorta. Moreover, renin is elevated in the early stages of renal hypertension, although it falls to normal in later stages. Thus it is possible that the increased replication seen here represents a response to the transient elevation of renin secretion. If this is so, then, as in the case of increased proliferation in response to dilation, one would expect to find a normal rate of replication in operated animals with chronic, stable hypertension. Experiments to test these possibilities are now under way.

References

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S M Schwartz and E P Benditt

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