Circadian Activity Rhythm of the Deer Mouse, Peromyscus: Effect of Deuterium Oxide

Abstract. *Chemical modification of a vertebrate activity rhythm, the circadian (approximately 24-hour) rhythm of* Peromyscus leucopus noveboracensis, *has been demonstrated. Activity in a running wheel was used to measure the rhythms of mice kept individually in conditions of continuous darkness. Deuterium oxide was presented in the drinking water. The length of the periods of rhythm increased directly and linearly with the increase in concentration of deuterium oxide. There is no threshold for this effect, and the maximum concentration of deuterium oxide (30 percent) that was presented resulted in a 6 percent lengthening of the period of circadian rhythm. The mice reverted to rhythms similar in period length to control values after deuterium oxide was withdrawn from their drinking water.*

Biological rhythms whose periods approximate 24 hours have been found in numerous species and in many cases have been demonstrated to be endogenous, requiring no environmental cueing to maintain the rhythms. Such innate oscillations, referred to as "circadian" (*circa,* about; *dies,* day) rhythms, are maintained by as yet undescribed physiological mechanisms. These "physiological clocks" are responsible for the timing of many processes that are reflected in the organism's metabolic and behavioral patterns.

Although the period and phase of circadian rhythms are maintained within narrow limits under conditions of continuous light or continuous darkness and constant environmental temperatures, they have in some instances been changed through alteration of the organism's external or internal chemical environment. Several of the many anesthetics and metabolic inhibitors studied have proved effective in shifting the phase of circadian rhythms (*1*). Other chemicals have been demonstrated to lengthen the period of plant and protist circadian rhythms [Bünning (*1*)]. Important to the present study are reports showing that cultures of *Euglena* adapted to growth in 45 and 95 percent D_2O (deuterium oxide) through long-term exposure to the medium showed a 3- to 5-hour lengthening of the period of the circadian rhythm of phototactic response. The period of circadian rhythm of the bean plant (*Phaseolus*) is also lengthened by continuous provision of 50 percent D_2O in the water supply (*2*). A similar effect of heavy water has now been found for a vertebrate species.

The eleven animals used in these experiments were either live-trapped or first generation laboratory-born deer mice (*Peromyscus leucopus noveboracensis*). All animals used had been selected, without regard to age, weight,

Table 1. Analysis of all animals given D_2O in drinking water. Period lengths (T) are listed ± one standard deviation. The value at the asterisk (*) is the same as the one listed directly above. $\Delta T/T$ was calculated from the difference between the period lengths for sequences with water and D_2O solution divided by the period length of the control (water) sequence. The significance of the difference between the two period lengths was established with P values of $< .001$ for all cases except the one marked with a dagger (†) in which the P value was $< .05$. Sequences A through E correspond to those in Fig. 1.

Mouse and sequences	Length of period (T) given water (hr)	Av. consumption of water per day (ml)	No. of days analyzed	Length of period (T) given D_2O sol. (hr)	Av. consumption of D_2O sol. per day (ml)	No. of days analyzed	Conc. of D_2O sol. (%)	$\Delta T/T$ (%)
1 A-B	23.63 ± 0.02	5.5	7	25.20 ± 0.08	5.5	5	30	6.64
B-C	23.61 ± .01	5.5	9	*			*	6.73
C-D	*			24.58 ± .02	5.7	8	20	4.11
D-E	23.35 ± .01	5.3	14	*			*	5.27
2 A-B	23.56 ± .01	3.5	10	24.43 ± .03	4.2	6	20	3.69
B-C	23.48 ± .04	3.5	5	*			*	4.05
C-D	*			24.32 ± .01	3.7	9	20	3.58
D-E	23.22 ± .02	3.5	8	*			*	4.74
3 A-B	24.06 ± .01	6.0	9	24.34 ± .01	5.9	6	5	1.16
B-C	24.19 ± .02	7.4	9	*			*	0.62†
4 A-B	23.61 ± .06	4.3	6	25.28 ± .04	3.6	6	30	7.07
B-C	23.97 ± .04	4.3	7	*			*	5.46
5 A-B	23.68 ± .02	5.4	6	23.90 ± .01	5.9	11	5	0.92
B-C	23.55 ± .01	6.4	9	*			*	1.48
6 A-B	23.73 ± .01	4.9	9	24.31 ± .01	4.7	10	10	2.44
B-C	23.67 ± .02	4.7	10	*			*	2.70
7 A-B	23.96 ± .01	5.6	10	24.28 ± .01	5.8	8	5	1.33
B-C	24.02 ± .01	6.3	7	*			*	1.08
C-D	*			24.48 ± .03	5.7	12	10	1.91
D-E	23.97 ± .02	5.5	14	*			*	2.12
8 A-B	24.07 ± .02	4.2	8	24.82 ± .01	5.0	11	10	3.11
B-C	24.27 ± .01	5.0	10	*			*	2.26
9 A-B	23.87 ± .02	4.2	9	25.76 ± .05	3.8	8	30	7.91
B-C	24.41 ± .02	3.9	12	*			*	5.53
10 A-B	24.19 ± .01	4.9	12	25.31 ± .02	5.1	19	20	4.63
B-C	24.13 ± .02	6.1	16	*			*	4.89
11 A-B	23.68 ± .01	7.5	8	25.17 ± .07	5.9	4	30	6.29
B-C	23.90 ± .01	3.9	11	*			*	5.31
C-D	*			24.23 ± .01	3.5	9	10	1.38
D-E	23.88 ± .01	3.7	7	*			*	1.46
E-F	*			24.61 ± .03	2.8	10	20	3.05
F-G	23.84 ± .01	3.1	7	*			*	3.22

or sex, for accurate and persistent free-running activity rhythms. For the selection and for subsequent testing, each mouse was placed in a cage and was allowed free access to a running wheel. The cage was located in a ventilated closed box and activity was recorded under conditions of continuous darkness. Food and water were available at all times. Each revolution of the activity wheel was recorded on an Esterline Angus event recorder. The records of four of the mice are shown in Fig. 1.

Water was supplied from a 125-ml bottle at the top of each cage; this quantity of water was consumed in about 3 weeks. Drinking water containing D_2O was supplied from a 40-ml bottle, and this volume was consumed in from 7 to 10 days. It was thus possible to maintain a mouse on D_2O undis-

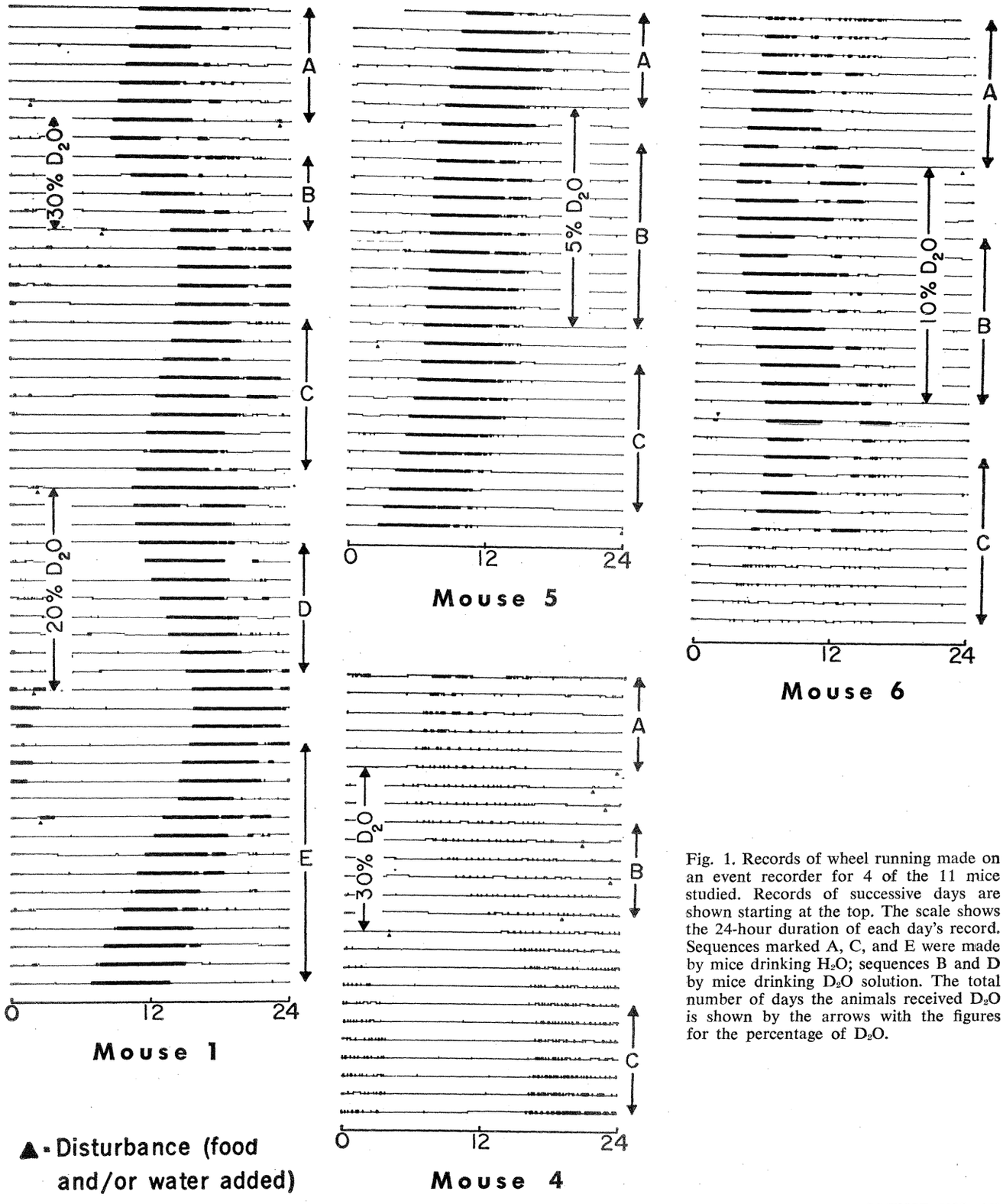

Fig. 1. Records of wheel running made on an event recorder for 4 of the 11 mice studied. Records of successive days are shown starting at the top. The scale shows the 24-hour duration of each day's record. Sequences marked A, C, and E were made by mice drinking H_2O; sequences B and D by mice drinking D_2O solution. The total number of days the animals received D_2O is shown by the arrows with the figures for the percentage of D_2O.

turbed through at least seven active periods (*3*).

An activity record of 6 to 15 days was made before replacing the tap water with a solution of D_2O. Concentrations of D_2O ranged from 5 to 30 percent by volume and this drinking water was given until the activity record showed a rhythm of fairly constant period length. The D_2O solution was then removed and normal tap water provided. A number of days of water consumption, resulting in a return to periods of constant length, preceded any further presentation of D_2O in the drinking water. Water consumption was calculated at each D_2O–H_2O change.

Two methods of data analysis were used. The first, and most accurate, involved calculating the circadian period as represented by the onset of activity [Rawson (*1*)]. Such calculations were made for each H_2O and D_2O run, but were limited to days during which the onset of activity indicated a relatively constant period length (Fig. 1) (*4*). A standard error of the period length was calculated for each run and the mean period lengths of adjacent runs were compared by Student's *t*-test for the difference between two means (*5*). A second method involved periodogram analysis of the data (*6*). This analysis derives root-mean-square values of total activity for each of more than 70 possible periods ranging from 18 to 30 hours in length. For a given run, the highest root-mean-square value indicates the period length that best fits the data presented. Data used for this analysis were the same as those used for the onset of activity analysis.

Lengthening of the period of the activity rhythm of *Peromyscus* was discernible in most cases with the first period of activity after D_2O was administered (Fig. 1). The period length continued to increase for several days until it reached a stable value that remained constant for the remainder of the time D_2O was administered. The period of rhythm began to shorten within one or two active periods after D_2O was removed from the drinking water, and, within 4 days, it had usually reached a constant length.

In each of the 16 cases in which animals were presented with D_2O the length of the period was increased. The percent increase varied linearly with the concentration of D_2O in the drinking water; Fig. 3 is a summary of the percent changes in period length for each concentration of D_2O. Each run gave two values of period change—one calculated with the period length before D_2O was given, and another with the period length after D_2O was removed. The linear curve of Fig. 3 was fitted to the data by the method of least squares and passes through the origin, thus showing no evidence for a threshold in the response of the rhythm to D_2O. Periodogram analysis (*6*) of the data showed changes in period length of the same direction and linearity and closely similar in magnitude to those described by analysis of the onset of activity (see Fig. 2).

The percentage increases in period length are listed in Table 1. In 12 of the 16 cases in which animals were presented with D_2O, the period of the run after D_2O was presented differed from that of the run before D_2O presentation by less than 1.0 percent, while the average percent change in period length resulting from the strongest D_2O solution (30 percent) was 6.45 percent. Fluid intake during treatment with D_2O did not vary consistently from intake during control periods. The mice (average body weight, approximately 20 g) obtained between 0.28 and 0.30 ml of D_2O per day from a 5 percent solution, while those drinking 30 percent D_2O obtained between 1.08 and 1.65 ml.

The multiple effects of deuterium in chemical and physical reactions (*7*) make it difficult to identify the processes that control the effect of period lengthening. There are, nevertheless, several well-defined effects of deuterium isotopes which might through further study be found to be the basis of the

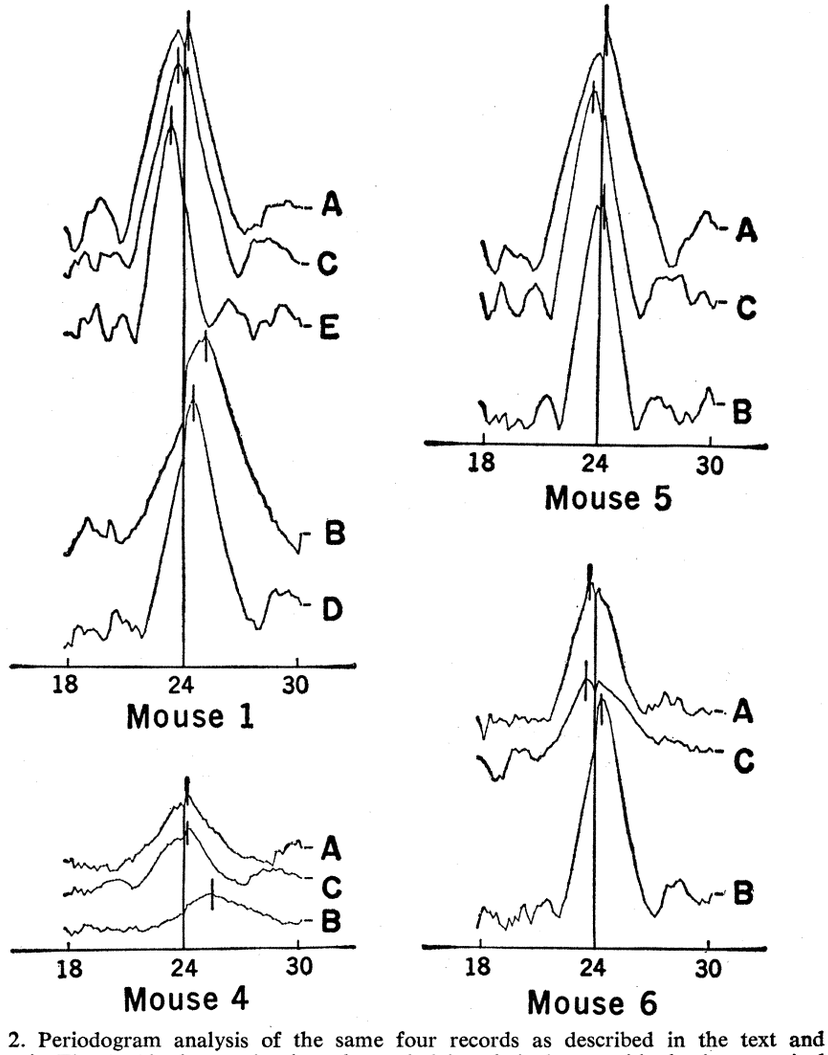

Fig. 2. Periodogram analysis of the same four records as described in the text and shown in Fig. 1. Abscissa scale gives the period length in hours with the long vertical line at 24 hours. Ordinates of all graphs are to the same scale but with base lines displaced. The peak of each graph is marked by a short vertical line. Mice and sequences are identified as in Fig. 1.

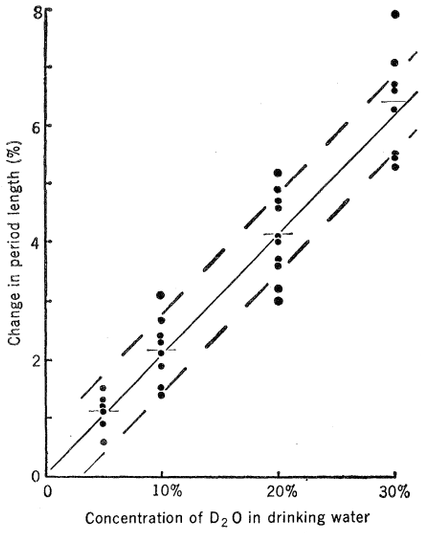

Fig. 3. Relation of D_2O concentration to period length of *Peromyscus* circadian rhythms. Solid line has been fitted by the method of least squares. Dashed lines represent ± one standard deviation. Short horizontal lines show average values.

effect of period lengthening in *Peromyscus*.

Much of the search for a "site" of the physiological clock of various organisms has centered around the nucleus and the nucleic acids [Bünning (*1*)]. The possibility that DNA, RNA, or protein synthesis is responsible for circadian rhythmicity was suggested by the work of Karakashian and Hastings with puromycin and actinomycin D (*1*). Dicken and Bray (*8*) found that DNA synthesis decreased in mammals drinking D_2O. Bray found a linear dosage effect in which the amount of P^{32} incorporated into rat liver DNA varied inversely with concentrations of D_2O up to 30 percent in the body water. Decreases in DNA synthesis were an order of magnitude greater than the changes found in this study with *Peromyscus*, which suggests that the effect of period lengthening on the circadian rhythm is not directly related to DNA synthesis.

The linear relation, without threshold, shown in Fig. 3 is consistent with variations in some of the physical properties of mixtures of D_2O and H_2O (*7*). Viscosity and positive ion mobilities match the data closely while changes in specific gravity and vapor pressure do not. That some physical property of H_2O or D_2O might control the length of the period of rhythm is in accord with the small, though measurable, effects of tissue temperature on these rhythms (*1*, *9*).

Although the effects of D_2O do not as yet identify any single mechanism as determining the period of circadian rhythms, D_2O should prove to be a useful tool for the further analysis of the physiological basis of these rhythms.

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2. V. G. Bruce and C. S. Pittendrigh, *J. Cell. Comp. Physiol.* **56**, 25 (1960); E. Bünning and J. Baltes, *Naturwissenschaften* **50**, 622 (1963).
3. Mice were disturbed in order to clean the cages, replenish food and water, and to repair equipment failures. Such disturbances were usually made during the inactive phase of the activity cycle, when dim light has a negligible effect on the phase or period of the circadian rhythm [Rawson (*1*)]. When disturbances occurred during the active phase, continuous darkness was maintained. The adequacy of the conditions of isolation was shown by "free running" circadian rhythms that had different period lengths for each animal and gave no evidence for synchronization of one mouse with another or with an exact 24-hour day.
4. Days at the beginning of each run, in which the period of oscillation was clearly changing, were not included in the analysis since it was the "equilibrium" period length that was of interest.
5. P. G. Guest, *Numerical Methods of Curve Fitting* (Cambridge Univ. Press, London, 1961). A straight line was fitted to the onsets of activity by the method of least squares in accord with the suggestion of C. H. Lowe, *et al.* [*Science* **156**, 531 (1967)]. Their criticism of the previous method used [Rawson (*1*)] is of theoretical importance only as the differences in calculated period lengths by the two methods were less than 2.5 minutes per period, or 0.16 percent, for *all* of the 43 period lengths analyzed in this study (Table 1).
6. J. T. Enright, *J. Theoret. Biol.* **8**, 426 (1965). Our computer program used a 10-minute interval in scanning a range of period lengths. This limited the accuracy of the method to ± 5 minutes, which was less accurate than the derivations from onsets of activity. Consequently this periodogram analysis is not reported in detail, although it is the analytic method of choice when a precise measure such as the sharp onset of activity cannot be obtained.
7. J. F. Thomson, *Biological Effects of Deuterium* (Macmillan, New York, 1963); A. Farkas, *Orthohydrogen, Parahydrogen and Heavy Hydrogen* (Cambridge Univ. Press, London, 1935).
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9. Deuterium oxide may also directly inhibit the activity of enzymes. Thomson (*7*) reports that catalase activity, as measured in vitro by the rate of decomposition of perborate, decreases linearly with increasing concentrations of D_2O. In 30 percent D_2O there is less than 10 percent inhibition, which is similar in magnitude to the change demonstrated for the period length of the *Peromyscus* circadian activity rhythm.
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Mixed Lymphocyte Reaction: An in vitro Test for Antilymphocytic Serum Activity

Abstract. *When Lewis rats were exposed to antilymphocytic serum, produced in rabbits, to Lewis lymphoid cells, the transformation of their thoracic duct lymphocytes in response to foreign lymphoid cells was markedly reduced in comparison with that in response to similar cells from donors treated with saline or normal rabbit serum. It is suggested that the mixed lymphocyte reaction may be used as an in vitro test for the in vivo activity of antilymphocytic serum.*

Allogeneic skin graft survival may be prolonged by pretreatment of a recipient with heterologous antiserum produced against the recipient's lymphoid cells or against cells from another donor of the same species or strain (*1*). This prolonged survival is accompanied by an inability of the animal's lymphoid cells to initiate graft-versus-host reactions and normal lymphocyte transfer reactions (*2*). The normal lymphocyte transfer reactions are produced by injecting lymphoid cells intradermally into genetically dissimilar recipients (*3*). In addition, antilymphocytic serum usually induces a circulating lymphopenia (*4*), although this is unnecessary for prolonged graft survival (*5*). It has also been reported that when lymphoid cells from two genetically dissimilar donors are cultured together, some of the small lymphocytes in the mixture will transform into large, immature-appearing blast cells. This mixed lymphocyte reaction represents an in vitro homograft reaction which correlates with the immunological status of the donors, as measured by the ability to induce runting or normal lymphocyte transfer reactions (*6*). Therefore, it seemed desirable to determine whether the mixed lymphocyte reaction (MLR) was effected by pretreating the cell donors with antilymphocytic serum. Results of the present study indicate that the mixed lymphocyte reaction was depressed and, hence, may serve as an in vitro test for antilymphocytic serum activity.

The antilymphocytic serum was produced in six adult, male New Zealand white rabbits by the intravenous injection of 30 to 80×10^6 thoracic duct lymphocytes obtained from male Lewis rats. Six injections were made over a period of 7 weeks, and 34 days after the