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Use of Sodium Pentobarbital for Repeated Anesthesia in the Rabbit

*By V. Everett Kinsey**

The need in this laboratory for an anesthetic which could be safely administered to small laboratory animals over periods of approximately one year gave rise to the present investigation. It was desired to find an anesthetic which could be easily given, required no further attention, and especially one which would be relatively safe and leave the animal in an apparently normal physiological state even though it be repeated every other day over a period of months.

Of the many anesthetics available it appeared that one of the medium to short-acting barbiturate derivatives would come nearest to meeting all of the above requirements. Many previous studies on the action of this series of compounds have been concerned with detoxification, elimination, the effect of single doses on various organs of the body and, particularly, with the hypnotic and minimal lethal dose. Tatum (1) has written a review of the status of the whole barbiturate problem. Investigations dealing with repeated administration of various barbiturates are adequately referred to here, by Masuda, *et al.* (2), and by others, some of whose papers will be discussed in more detail later. Most workers concur in the opinion that several barbiturates produce appreciable tolerance after administration for relatively short periods of time (days, not weeks) as measured by a lessening of sleeping time. Few of the workers, however, have subjected the animals to weeks or months of treatment and little stress has been placed on the important question of survival, either at the time of injection or later, although deaths presumably due directly to the anesthetic are recorded in several instances.

The choice of barbiturate appeared to be limited to those compounds which would produce sleep in small laboratory animals for periods not greatly in excess of two hours when given every other day. Several preliminary experiments confirmed this and showed that when, after repeated anesthesia, animals were kept unconscious even for two hours following a single injection of the barbiturate, long periods of depression followed. The debilitating effect of the latter apparently led to the extraordinarily high mortality which was observed. Because of these discouraging results, a shorter acting compound, sodium pentobarbital (Nembutal)[1] was chosen for this investigation, even though two or sometimes more separate injections were required to produce anesthesia for two-hour periods.

It is the purpose of the present study to review the whole question of repeated intraperitoneal injections of sodium pentobarbital in the rabbit from the viewpoint of the in-

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[1] Kindly furnished by the Abbott Laboratories, North Chicago, Ill.

vestigator who has need of frequent recurring anesthesia. Particular emphasis will be placed on such practical considerations as dosage, tolerance (as evidenced by reduced sleeping time) and mortality.

MATERIALS AND METHODS

Powdered sodium pentobarbital was weighed out accurately at intervals and dissolved in sterile salt solution (0.9 per cent NaCl) containing 10 per cent ethyl alcohol[2] in concentrations of 65 mg./ml. In this medium the Nembutal stayed permanently in solution and when kept at room temperature in bottles stoppered with rubber diaphragm caps, remained sterile with ordinary precautions over the period of time needed to use all of the particular lot—usually about a week.

Every injection was given intraperitoneally with a one-half inch gage 27 needle. It is likely that an occasional dose would be injected subcutaneously or possibly into the rather thick scar tissue which eventually formed in most of the animals, and no doubt, too, now and then, the intestine would be punctured, although no known peritoneal infection resulted from the latter. In view of these possibilities, the occasional animal which would fail to go to sleep on one day was disregarded in figuring the average sleeping times.

The abdomens of the rabbits were clipped periodically, and the site of the injection was thoroughly wet with 70 per cent alcohol before administering the anesthetic. Only two local infections were found and these may have resulted from the bites of other rabbits. All of the animals were weighed before every injection.

Two criteria were used to measure the sleeping time of the rabbits: (*a*) the time during which an animal would not move when infrared radiations were applied to the eye,[3] and (*b*) the time during which the righting reflex was lost. Although many comparative tests showed that the sleeping times as judged by the two criteria were the same, the first criterion only was used in judging groups A, B and C while the second was used for all of the other rabbits considered.

EXPERIMENTAL

The investigations with rabbits involved two essentially separate problems. The first was concerned with the effect on the sleeping time of repeated injections of a particular dose of Nembutal, while the second dealt with the dose of Nembutal which would be necessary to produce a particular sleeping time.

About equal numbers of healthy young, adult Dutch and Chinchilla rabbits (no albinos) were selected from stock and acclimated to life in a small well-ventilated animal room where they were kept in fairly large individual wire bottom cages. They were fed alfalfa hay every morning, a cupful of soaked oats and bran mixture during the late afternoon and carefully selected greens between two and four times weekly. That the animal quarters and diet were quite adequate is evidenced from the following: (*a*) the average weight of the rabbits increased from 2300 to approximately 2700 Gm., notwithstanding the repeated anesthesias to which they were subjected; (*b*) the mortality, other than those animals dying at the time of anesthesia or killed deliberately, was less than 10 per cent for a year and (*c*) the ability to reproduce and the subsequent rapid growth of the young was observed on non-treated rabbits.

The question of the variation of sleeping time following the repetition of a uniform dose of Nembutal was first investigated. Fitch and Tatum (3) among others, have shown that the M. L. D. (*i. e.*, dose which kills 50 per cent of the animals) for

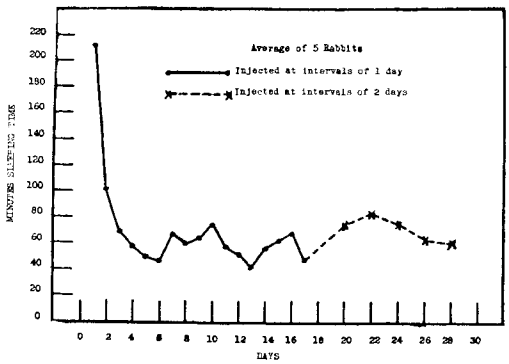

Fig. 1.—Showing the average sleeping times of five rabbits when injected repeatedly with 44 mg. per Kg. of Nembutal dissolved in 0.9 per cent NaCl containing 10 per cent ethyl alcohol. Alcohol received was equal to 0.055 Gm. per Kg.

intraperitoneal doses of sodium pentobarbital is equal to 65 mg. per Kg. They further pointed out that a dose of 39 mg. per Kg. was followed by a sleeping period of 2 hours and 48 minutes. Kohn (4) studying the individual susceptibility of rabbits to intravenous injections of Nembutal found that 70 per cent of the M. L. D. usually produced no untoward effect. Accordingly, it was decided to administer a dose of 44 mg. per Kg. daily over a period of several weeks. The results from such an experiment performed on five rabbits appear in Fig. 1. The period during which the righting reflex was lost was used as the criterion of the sleeping time. The individual variation of sleeping times ranged between 165 and 243 minutes the first day and thereafter, between 0 and 186 minutes. As mentioned previously, the zero sleeping times were disregarded in figuring the averages, although including them would only add slightly to the effect observed, *i. e.*, tolerance.

[2] As supplied commercially for veterinary use.

[3] Five-gram calories per minute were used. The results of this study will be published elsewhere.

Following seventeen daily doses of Nembutal a three-day rest period was allowed and then the injection period was increased to two days. Unlike the work reported by Masuda *et al.* (2), where the drug was given intravenously, only a small portion of the tolerance was lost during the three-day interval, as shown by the broken line in Fig. 1. It should be mentioned, however, that they observed less tolerance in the first place, since the average sleeping time after development of tolerance amounted to only about 70 per cent of the original. The explanation for this apparent discrepancy will be given later in this paper.

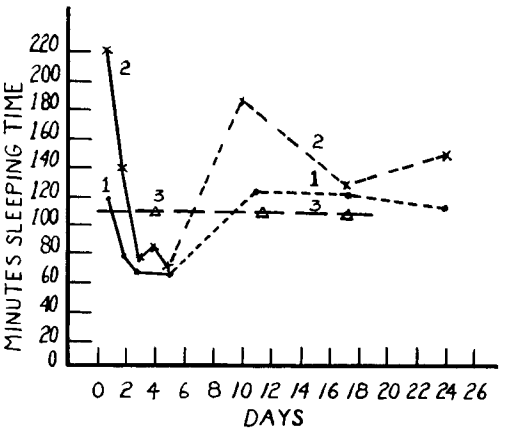

Fig. 2.—Showing the average sleeping times of rabbits when injected repeatedly with 44 mg. per Kg. of Nembutal dissolved in 0.9 per cent NaCl under the following circumstances:

Curve 1. Average of 6 animals. Solid line, no alcohol until the fifth day. Broken line, 0.055 Gm. per Kg. of alcohol daily until the seventeenth day with Nembutal on the eleventh and seventeenth days only. No injections until the twenty-fourth day at which time alcohol and Nembutal were given.

Curve 2. Average of 3 animals. Solid line, +0.055 Gm. of ethyl alcohol for four days. No alcohol on the fifth day. Broken line, same as the broken line in curve 1.

Curve 3. Average of 5 animals. 0.055 Gm. of ethyl alcohol daily for three days with the Nembutal made up in alcoholic saline on the fourth, eleventh and last days only.

Although Dille and Ahlquist (5), who studied the synergism between intravenous injections of ethyl alcohol and Nembutal in rabbits, found that doses of alcohol as low as 0.055 Gm. per Kg. of body weight (the amount used throughout this study) did not increase the sleeping time of these animals appreciably, it was thought advisable to check these results.

One group of rabbits (I) composed of six animals was given a dose of Nembutal made up in 0.9 per cent NaCl while a second group of three animals (II) was given a similar injection of Nembutal dissolved in 0.9 per cent NaCl containing 10 per cent ethyl alcohol. The average sleeping times of these two groups of rabbits were 117 and 220 minutes with an extreme range of 98–138 minutes and 201–245 minutes, respectively (Curves 1 and 2, Fig. 2). The average sleeping time of group II corresponds quite closely to the larger group of similarly treated animals reported upon above. (Fig. 1.) In view of the rather marked synergism observed, a few further experiments were conducted to evaluate the effect of the alcohol on repeated anesthesia.

The same doses were continued for four days and on the fifth day the group (I) which had received no alcohol was given Nembutal made up in 10 per cent alcoholic saline and the group (II) which had received Nembutal and alcohol was injected with Nembutal solution containing no alcohol; in other words, the solutions were given in reverse order. It will be seen from Fig. 2 that not only was the sleeping time almost identical by the third and fourth day, but when alcohol was included in the injection for the first time (the fifth day) for group I, it then did not potentiate the action of the Nembutal at all. Furthermore when 0.055 Gm. of alcohol per Kg. was given to both groups of rabbits at daily intervals until the tenth day and then Nembutal in 10 per cent alcholic saline solution was given, the average sleeping times increased only to about the original time observed for the Nembutal without alcohol. This was confirmed by repeating the alcohol only, until the seventeenth day and again giving Nembutal and alcohol. After this the average sleeping times of both groups following abstinence of any injections for a week was determined and found to be 109 minutes.

Thus, not only does alcohol alone prevent the return of any potentiation but no sign of potentiation is observed following complete freedom from injections for a week.

Since it was shown that Nembutal alone would overcome the synergism, one further experiment was performed to see whether previous injections of alcohol alone would reduce the sleeping time when Nembutal was given in alcoholic saline. A group (III) of five rabbits was injected daily for three days with 0.055 Gm. of alcohol per Kg. and on the fourth day they were given 44 mg. per Kg. of Nembutal as well as the alcohol. Their average sleeping time was 110 minutes. After two seven-day intervals during which they were not treated they again received Nembutal and alcohol and the average sleeping time was 107 minutes both times (Curve 3 of Fig. 2).

From the above results it would seem that there is a marked initial potentiation between alcohol and Nembutal, even for the relatively low doses of alcohol studied, and, moreover, that this synergism disappears after the first several repetitions of the two drugs given either together or separately. However, from the standpoint of repeated anesthesia the alcohol would not appear to alter the results as measured by sleeping time.

One reason for the fact that Dille and Ahlquist report less potentiation from the 0.055 Gm. of alcohol per Kg. of body weight than was observed

in the present investigation may be that—as they state—"Habituation was avoided by allowing at least two days and usually longer between each injection." Since the experiments reported above show that even a week's rest period is not sufficient to allow the reappearance of potentiation, accurate results—at least at low alcohol levels—can probably be obtained only with fresh animals.

The reason for the apparent discrepancy between the work of Masuda *et al.* and that reported in Fig. 1 now becomes evident, namely, the alcohol greatly increased the initial sleeping time. If instead of using the data from Fig. 1 for comparison with the data presented by the above workers, the non-alcoholic injection times of Fig. 2 are used, the results then agree satisfactorily.

With the foregoing information available the second problem, *viz.*, the amount of anesthetic needed to produce a particular sleeping time could be considered. Thirty-seven rabbits were divided into three groups *A*, *B* and *C* containing nine, sixteen and twelve animals, respectively. The injections were carried out in such a way that each animal received an anesthetic every other day for a two-week period and rested one week, after which the cycle was repeated. Treatment days were alternated, so that one group of animals only was injected every day. The one week of rest out of every three probably lessened the number of animals dying as a result of too frequent anesthesia and, no doubt too, decreased somewhat the amount of anesthetic required.

It was desired to anesthetize the animals in group *A* for one hour and those in groups *B* and *C* for two hours. Obviously, the sleeping time for any one animal would vary considerably around these exact periods. After gaining some experience, especially in adjusting the quantity for additional injections in groups *B* and *C*, it became possible to obtain fairly consistent average sleeping times.

From Fig. 1 it was seen that the duration of anesthesia in rabbits from Nembutal injections dropped off quickly following the first two repetitions of the drug, and that once tolerance had developed, and the frequency of injection had been reduced to one dose very other day, the average sleeping time was about 70 minutes. It was further learned that the length of sleeping time could not then be materially augmented by unrestricted increase of the dose without greatly endangering the life of the animal. In other words, after several injections of Nembutal, the sleeping time decreased much faster than the resistance of the animal to larger and larger initial doses increased. Accordingly, it was necessary to administer two or more separate injections to increase the sleeping time to the two-hour period. Unlike the other barbiturates used, Nembutal did not produce any signs of prolonged depression after the rabbits first aroused from total anesthesia. In fact complete recovery as measured by ability to hop, eat, etc., appeared to take place almost as rapidly after two or even three doses as after one injection.

The first injection of from 40 to 50 mg. per Kg. of sodium pentobarbital was given each day while second and third doses varied in size from about ten to twenty-five mg. per Kg. of body weight, depending upon the previous record of the particular animal. An exception was made to these dosages in the case of the first several days of treatment because the animals would then sleep considerably longer than the desired periods. In these cases slightly less anesthetic was employed. Caution governed all of the decisions regarding dosage as the continued life of the animal then was the main objective.

The reactions of different animals varied considerably and even those of any one animal seemed to change from week to week. Several examples fairly typical of the dose of Nembutal used and the resulting sleeping time for the two periods studied are given in Table I. This table illustrates the need for two and sometimes three injections in the case of the two-hour period animals. The second injection was given after 5 or 10 minutes in the cases where the animal would not go to sleep, as for example the ninth and eleventh day for animal *Ch*.

Altogether over 3000 injections were given the

Table I.—Showing Examples of the Sleeping Time for One Complete Cycle (Not the First) in Response to Repeated Injections of Sodium Pentobarbital in Animals being Treated Every Other Day for Two Weeks Following a Week's Rest Period

One-Hour Period			Two-Hour Period		
Day (Animal *GC*)	Dose, mg./Kg.	Sleeping Time, Minutes	Day (Animal *W*)	Dose, mg./Kg.	Sleeping Time, Minutes
1	43	75	1	41	118
3	43	55	3	41 + 11	77 + 45 = 122
7	43	70	7	41	105
9	44	57	9	41 + 22	59 + 81 = 140
11	44 + 19	15 + 58 = 73	11	41 + 22	65 + 55 = 120
		Average 66			Average 121
(Animal *Ch*)			(Animal *Q*)		
1	41	58	1	45 + 27	72 + 53 = 125
3	41	65	3	47 + 27	65 + 71 = 136
7	41	98	7	47 + 22	85 + 40 = 125
9	41 + 12	0 + 55 = 55	9	47 + 24 + 16	70 + 27 + 35 = 132
11	41 + 15	0 + 47 = 47	11	50 + 8 + 27 + 19	0 + 30 + 55 + 40 = 125
		Average 65			Average 131

Table II.—Showing the Amount of Sodium Pentobarbital Required to Produce Repeated Anesthesia for Two Periods: Group *A*—75 minutes—and Groups *B* and *C*—Approximately 2 Hours

Group	Number of Anesthesias	Average Dose, mg./Kg.	Average Sleeping Time, Minutes	Sleeping Time per mg./Kg., Minutes	Number of Animals, Orig.-Final	Period of Treatment, Days
A	319	44.6	74.5	1.66	9–4	242
B	553	63	122	1.93	16–5	221
C	306	68	123	1.80	12–3	201

three groups of rabbits throughout a period of almost a year. With the exception of the first several weeks, during which period the maximum sleeping time was not recorded, the observations from these anesthesias have been assembled and are summarized below.

The distribution of the dosage having been considered, there will now be discussed the amount of anesthetic required on the average to produce sleep for (*a*) slightly over an hour, and (*b*) a two-hour period. The average dose of the barbiturate and the average sleeping times for all of the anesthesias have been determined, and from these the sleeping time per mg. per Kg. of body weight has been calculated. These data are set forth in Table II.

It will be seen from this table (column 5) that relatively more anesthetic is required to produce sleep for the shorter period. This was to be expected, and merely is evidence that a threshold dose must be given before sleep is produced at all. Thus, when the animal awakens, about half the original dose will produce anesthesia for a time approximately equal to the first period.

It is obvious that the averages appearing in Table II are only valid when there has not been a material or progressive change in susceptibility. To check this point the average daily sleeping time per unit dose of anesthetic was determined and plotted against the day the anesthetic was given. (Fig. 3.) An arbitrary dose of 44 mg. per Kg. was selected for the unit dose for illustrative purposes as this was the dose used previously for the determination of tolerance. (Fig. 1.) Thus the sleeping times of rabbits in group *A*, which actually received 44.6

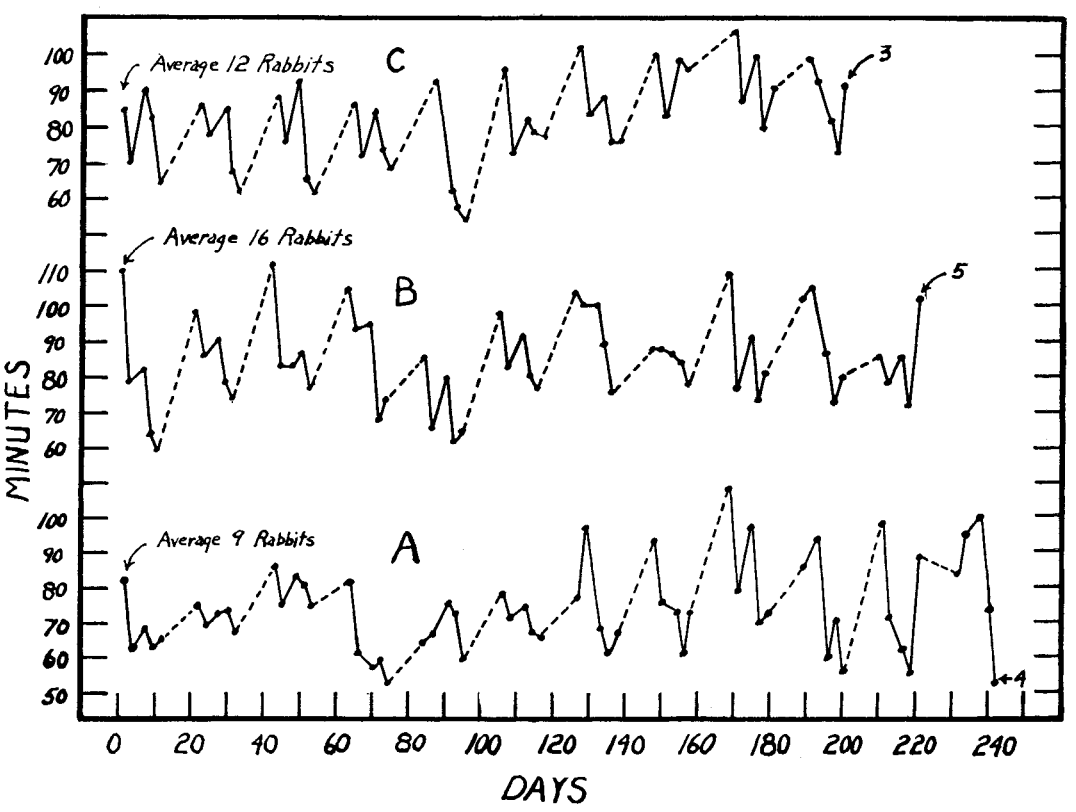

Fig. 3.—Showing the relative average sleeping times of three groups of rabbits injected repeatedly with Nembutal.

The dose given each group was different (see Table II), but the sleeping times have been adjusted for a standard dose of 44 mg. per Kg. The anesthetic was given on Tuesday and Thursday of the first week, and Monday, Wednesday and Friday of the second week, then a week's rest period (dotted line) was allowed.

mg. per Kg., are the only ones which required little mathematical adjustment, and hence, they represent the only case where the comparative sleeping time is practically identical with the actual sleeping time. Groups *B* and *C*, on the other hand, received on the average 63 and 68 mg. per Kg. as reported in Table II and thus, for comparative purposes, required a proportionate correction to the lower dosage of 44 mg. per Kg. As reported above, the sleeping time does not strictly follow the dosage. Therefore the corrected values are comparative only and tend to be high. In any case, however, the values shown represent correctly the condition of the rabbits in regard to susceptibility; and moreover the absence of a continued slope for each group of animals indicates that there has been no change in this characteristic. The lack of increased tolerance after long periods of administration demonstrate an absence of habituation to the drug—which is of interest from a clinical point of view.

treating animals over a period of approximately one year. How well this objective was accomplished may be seen from Fig. 4 which shows the mortality of the rabbits in groups *A*, *B* and *C*. Regarding the cause of death the following breakdown will be of interest. Two of the five animals which died from causes not associated with the anesthetic were killed after 48 weeks of repeated anesthesia; one died from a testicular infection; and two died from unknown causes. Of the remaining thirty-two rabbits, twelve were still living after 50 weeks of treatment, while twenty died of the usual respiratory collapse without regaining consciousness, shortly after receiving an injection of Nembutal. Deaths, rather oddly, occurred with about equal frequency on each of the five treatment days, and would sometimes follow a dose of the barbiturate which two days previously would scarcely put the same animal to sleep. On the other hand, a rabbit would occasionally become extremely refractory, and after giving it three injections without producing sleep, the animal would be returned to its cage for that day. Kohn has made similar observations on the change, from time to time, in susceptibility of individual rabbits. Only once did a rabbit succumb to any injection following the initial daily dose of Nembutal,

Table III.—Comparing the Lengths of Sleeping Time of Male and Female Rabbits when Injected Intraperitoneally with Sodium Pentobarbital. All of the Rabbits Have Been Subjected to Repeated Anesthesia

Sex	Date	Number of Rabbits	Average Dose, mg./Kg.	Average Sleeping Time, Minutes	Average Sleeping Time per mg./Kg., Minutes	[a]Mean Average Sleeping Time per mg./Kg., Minutes
Males	March 29	19	70.0	118	1.68	1.59
	March 31	19	76.0	114	1.50	
Females	March 29	13	64.5	105	1.63	1.54
	March 31	13	71.0	103	1.45	

[a] The dates used in this table represent the fourth and fifth treatment days of the three week cycle.

In general, the variation in sleeping time for any one period appears to parallel the frequency of the anesthesias. Thus the general trend seems to be from a rather long sleeping time to a somewhat lesser one as a result of the Tuesday and Thursday doses. Then a longer sleeping time is seen as a result of the four-day interval until Monday, with a continued lessening of the duration of anesthesia following the Wednesday and Friday injections. Finally, a return to a fairly constant level occurs with the week's rest period. This cyclic behavior appears to be more evident in groups *B* and *C*.

Another important factor is the relative response of male and female animals to repeated anesthesia. Neither Kohn nor Fitch and Tatum were able to demonstrate any difference in the sleeping periods between the two sexes following a single injection of Nembutal in rabbits. That their finding is confirmed for repeated anesthesia is apparent from the results appearing in Table III. Here the average sleeping times of male and female rabbits are seen to correspond to within 3 per cent. There were equal numbers of one-hour period animals of each sex included in this tabulation. Still further confirmation was found in a smaller series of animals, where the average sleeping times following the first injection of Nembutal (44 mg. per Kg.) were 197 and 193 minutes for male and female rabbits, respectively.

Finally, attention is called again to the significant question of survival. It will be remembered that the prime objective of this study was to find an anesthetic which would be fairly innocuous when

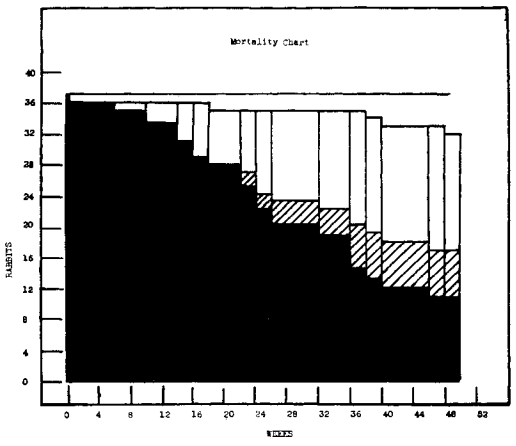

Fig. 4.—Showing the mortality of the rabbits given long repeated anesthesias.

Solid black. Animals living.

Cross hatch. Animals dying at the time of anesthesia but suffering from "snuffles."

Open block. Animals dying at time of anesthesia.

Open. Animals killed deliberately or dying from causes not associated with the anesthetic.

and no death was associated with loss in weight. It may be that decreasing the amount of hay in the rations increased the mortality.

On several occasions, animals were saved from death by artificial respiration, in some cases supplemented with coramine. Better results could probably have been obtained with the use of the other analeptics, metrazole or picrotoxin. However, with a limited personnel and with four animals being used simultaneously, the close observation required to see every case of failing respiration in time for prophylactic measures was impossible.

While it was hardly to be expected that the periodic examinations made of various parts of the eye would reveal any pathology, there was a possibility of damage to other bodily organs as a result of the frequent repetition of the anesthetic. Dr. Kohn-Richards kindly made a histological study of several of the tissues from three of the rabbits which had received injections of Nembutal; his report is as follows:

"Rabbit *V*, injected from February 20 to November 17—killed November 18. Received 8.6 Gm. of sodium pentobarbital total. Liver, cloudy swelling, no fat. Kidney, severe cloudy swelling, hyperemia, no fat. Heart, cloudy swelling.

"Rabbit *Ch*, injected from December 31, 1938 to November 16, 1939—killed November 18. Received 8.3 Gm. of sodium pentobarbital total. Kidney, no fat, cloudy swelling. Liver, moderate fat infiltration, possibly a normal limit, marked cloudy swelling, some round cell infiltration around the bile ducts which may not be significant.

"Rabbit *B*, injected from January 12 to November 29—killed December 8. Suffered from 'snuffles' for from 4 to 6 weeks before being killed. Liver, fat moderately present, hyperemia, distension of sinusoids, marked cloudy swelling. Heart, cloudy swelling. Kidney, no fat, tubuli epithelium markedly swollen and indistinct, severe cloudy swelling, some places showing early necrosis.

"The last animal undoubtedly shows the most pronounced changes. However, since according to the protocols, the animal suffered from snuffles, it is doubtful if the changes can be attributed to the drug or solely to the drug. The changes in the other two animals are of a nature that one might expect the organs to return to the normal status and do not seem to indicate irreparable damages."

CONCLUSIONS

1. As a result of several daily intraperitoneal injections of 44 mg. per Kg. of Nembutal, dissolved in 0.7 ml. of 0.9 per cent NaCl containing 10 per cent ethyl alcohol, the sleeping time of rabbits decreases abruptly from approximately $3^1/_2$ to $1^1/_4$ hours. When the alcohol is omitted from the solution the first one or two sleeping times only are shortened.

2. The use of Nembutal intraperitoneally for long-repeated anesthesia in rabbits is practical if some degree of care is used in adjusting the dosage to the requirements of the individual animal.

3. There is no evidence of habituation of rabbits to Nembutal even when the drug is given repeatedly over a period of one year.

4. To obtain anesthesias in rabbits of one to two hours' duration, with minimal risk, it is necessary to give repeated small doses of the drug.

5. An average initial dose of 40–44 mg. per Kg. intraperitoneally is satisfactory for this type of anesthesia in rabbits.

6. The sex of the rabbits does not alter the sleeping time for either single or repeated injections of Nembutal.

7. The liver, heart and kidney appear to suffer mild but not irreparable injury as a result of long-repeated anesthesia.

The author wishes to express his gratitude to Miss Barbara Petty for her assistance throughout this work and to Dr. Charles F. Kutscher who made the study possible through his liberal support of this laboratory.

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NOTICE

There has been some adverse criticism of some of the illustrations which have appeared in the JOURNAL recently. Authors can assist in allaying this criticism by adhering strictly to the directions on the preparation of papers given under "Notice to Authors."