spot, corresponding to the position of the authentic unlabeled sample, was obtained. There was no tailing in the hexane–ethyl acetate system, but there was a small amount of tailing in the hexane–acetic acid system.

Determination of Specific Activity—A 10 mg./ml. solution of 1,1-bis(*p*-chlorophenyl)-2-nitropropane-^{14}C in methanol was used to determine the specific activity of the compound.

Five samples were prepared by pipeting 100 μl. of the solution into five scintillation vials containing a liquid scintillation cocktail composed of 0.14% PPO (2,5-diphenyloxazole) and 0.01% dimethyl POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene] in equal volumes each of toluene and 2-ethoxyethanol. These samples were cooled in a Packard TRI CARB liquid-scintillation spectrometer, model 3003, for 0.5 hr. The window width of 50–100° was chosen. Utilizing an internal standard of toluene-^{14}C (toluene-^{14}C, 5.01×10^5 dis./min./ml., R-21 Tracerlab), the observed counts per minute were converted to disintegrations per minute and corrected for quenching and counter efficiency. A specific activity of 13.85 ± 0.11 μc./mmole was obtained.

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Development of Tolerance to Pentobarbital

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Abstract □ Tolerance to pentobarbital developed 4 hr. after the first injection and reached a peak at 17–22 hr., after which it decreased to nonsignificant levels by 48 hr. The duration and frequency of administration of pentobarbital affected the degree of development of tolerance to pentobarbital. PTI (Percentage Tolerance Index) decreased progressively with the increase in the number of injections. When four injections were given within the span of 28 hr., the animals showed a greater degree of tolerance on the third injection which was administered 24 hr. after the initial injection. Tolerance was also present on the fourth injection but to a lesser degree when compared with the third injection. The experimental data from this study suggest that tolerance to pentobarbital does develop and is the result of the pentobarbital stimulating its own metabolizing enzyme.

Keyphrases □ Pentobarbital—tolerance development, rats □ Drug tolerance—pentobarbital, rats □ Tolerance, pentobarbital—rats

Animals can be shown to develop tolerance to barbiturates, pentobarbital and thiopental (1). Gruber and Keyser found that dogs tend to become tolerant to a variety of barbiturates (2). As a criterion of tolerance, they used the reduction of the sleeping time elicited by the same dose after it had been repeated several times. According to Goodman and Gilman, tolerance to barbiturates has developed when, after repeated administration, a given dose produces a decreasing effect or, conversely, when increasingly larger doses must be given to obtain true barbiturate effects obtained with the original dose (3). Tatum *et al.* provide the classic definition of tolerance as "a phenomenon characterized by the fact that more drug must be used to produce equivalent effects" (4). Jaffe and Sharpless have shown that some degree of physical dependence can be produced in as little as 20 hr. after pentobarbital administration (5). Singh has also shown that tolerance to pentobarbital and thiopental is developed within 24 hr. (1). The purpose of this paper is to report that: (*a*) a certain time lapse occurs before the tolerance is developed, and (*b*) this developed tolerance reaches a peak and then declines.

EXPERIMENTAL

Female albino rats, random by breed (Caeserian Drive One) and weighing 125–175 g., were used. Pentobarbital sodium was dissolved in distilled water. The volume of each injection was kept constant, *i.e.*, 1 ml./kg. All injections of pentobarbital (25 mg./kg.) were given intraperitoneally. The sleeping time (difference between loss of righting reflex and regain in righting reflex in minutes) was determined.

Animals were divided into five major groups:

Group 1—Pentobarbital was administered to 34 animals at zero time and at an interval of 24 hr.

Group 2—Eighteen subgroups were composed of 10 to 15 animals each. In these subgroups, the first injection was given at zero time and then the second injection was given at intervals of 2, 3, 4, 7, 9, 13, 17, 18, 19, 20, 21, 21, 22.5, 22.5, 24, 48, 72, and 168 hr. Some experiments (21 and 22.5 hr.) were duplicated.

Group 3—Pentobarbital, 25 mg./kg., was administered daily to 10 animals at intervals of 24 hr. for 16 days.

Group 4—Three subgroups were comprised of 10 to 15 animals each. To each subgroup, pentobarbital, 25 mg./kg., was administered at intervals of 0, 2, 24, 26; 0, 3, 24, 27; and 0, 4, 24, 28 hr.

Group 5—Clinical signs, *i.e.*, water consumption, urine output, food consumption, and growth, were observed in animals that had developed tolerance to pentobarbital, 25 mg./kg. Each animal was housed separately in a metabolism cage, and initial observations on each animal were taken. Each animal was given two injections of pentobarbital after the initial observations. Then the animals were allowed to recover on the third and fourth day. Statistical methods used were those of Snedecor (6).

Because most work on the development of tolerance to barbiturates has been done on male rats, guinea pigs, mice, and dogs, in this project the authors decided to study tolerance to pentobarbital in female white rats. Percentage tolerance index (PTI) was computed as follows (1):

$$\frac{\text{hypnotic effect of first injection}}{\text{hypnotic effect of second injection}} \times 100$$

If PTI is unity, *i.e.*, 100%, it indicates no tolerance. PTI greater or less than unity indicates tolerance or cumulative effect. Before

Table I—Effect of Pentobarbital (First and Second Injections) on Sleeping Time (S.T.)

Mean S.T. of First Injection, min. (A)	Interval of Second Injection, hr.	Mean S.T. of Second Injection, min. (B)	Difference in S.T.	% Change between A and B
102 (10)[a]	2	140 (10)	+38[b]	+37.3[c]
100 (11)	3	112 (11)	+12	+12.0
88 (12)	4	72 (12)	−16	−18.2[c]
67 (12)	7	53 (12)	−14	−20.9[c]
63 (12)	9	50 (12)	−13	−20.6[c]
57 (13)	13	24 (13)	−33	−57.8[c]
47 (10)	17	15 (10)	−32	−68.0[c]
62 (14)	18	18 (14)	−44	−70.9[c]
70 (14)	19	16 (14)	−54	−77.2[c]
57 (12)	20	12 (12)	−45	−78.9[c]
64 (12)	21	15 (12)	−49	−76.6[c]
55 (12)	21	14 (12)	−41	−74.5[c]
64 (12)	22.5	20 (12)	−44	−68.7[c]
73 (12)	22.5	23 (12)	−50	−68.5[c]
57 (14)	24	24 (14)	−33	−57.8[c]
88 (15)	48	72 (15)	−16	−18.2[c]
88 (10)	72	80 (10)	−8	−9.1
70 (10)	168	64 (10)	+1	+1.4

[a] Sample size is given in parentheses. [b] (+) or (−) indicates increase or decrease in S.T. [c] $p < 0.05$.

computing PTI, the data of the first and second injections were analyzed by means of differences (6).

RESULTS

Group 1—Significant tolerance ($p < 0.05$) was developed in female albino rats when the second injection of pentobarbital was administered at an interval of 24 hr. after the initial injection.

Group 2—Effect of pentobarbital (first and second injections) on the sleeping time is computed from Table I and given in Fig. 1. Significant cumulative hypnotic effect was present during the first 3 hr. The animals began to show tolerance after 4 hr., reaching a peak between 17 and 22 hr., then declining and was not evident at or after 48 hr.

Group 3—Table II shows the effect of continuous administration of pentobarbital on the development of tolerance. In this group, four injections of pentobarbital were given to the same animals at different time intervals. No tolerance was exhibited at 2 and 3 hr. However, tolerance was present if the third and fourth injections were given at 0, 2, 24, 26, 0, 3, 24,27; and 0, 4, 24, 28 hr. Comparison is made between the first two injections (0, 2; 0, 3; 0, 4) and the last two injections (24, 26; 24, 27; 24, 28). Significant ($p < 0.05$) tolerance is developed at and after 24 hr. Significant ($p < 0.05$) tolerance was present when the fourth injection was given at 26, 27, and 28 hr. following the initial injection. However, the degree of developed tolerance was significantly ($p < 0.05$) less when compared with the third injection.

Group 4—The effect of continuous administration of pentobarbital daily for 16 days is shown in Table III. Significant ($p < 0.05$) tolerance was developed after 24 hr. PTI decreased progressively with the increase in the number of injections.

Group 5—Clinical signs, *i.e.*, urine output, water consumption, food consumption, and weight, were not affected during the development of tolerance to pentobarbital. However, on the 3rd day, 24 hr. after the second injection, a significant ($p < 0.05$) increase in water consumption and urine output was observed; food consumption was not altered over the period of 5 days. Weight remained constant in the treated rats. However, a significant ($p < 0.05$) increase in weight was observed in the control animals.

DISCUSSION

The hypothesis of the authors was that the development of tolerance to pentobarbital can be influenced by interval and frequency of administration of the drug, duration of administration of the drug, and metabolism in the liver.

Interval and Frequency of Administration of Drug—Singh has shown that tolerance to barbiturates, pentobarbital, and thiopental

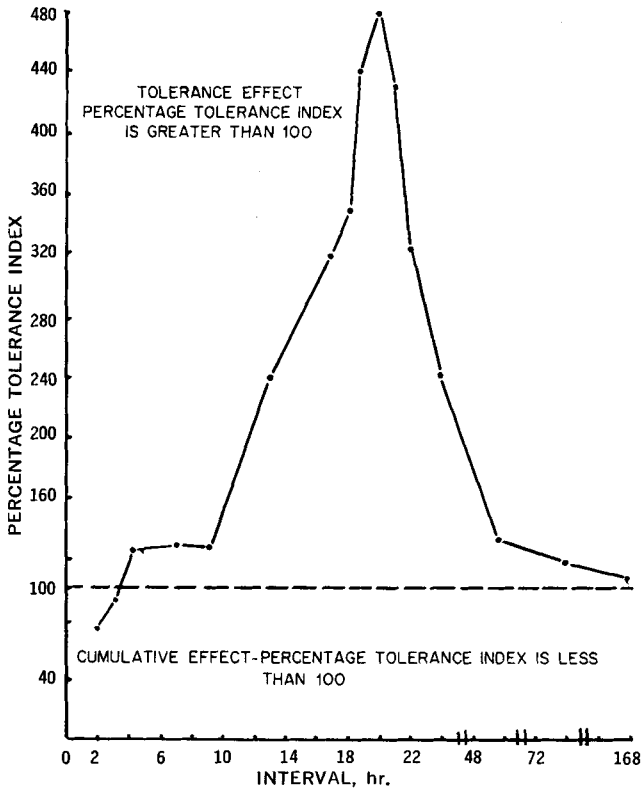

Figure 1—*Effect of pentobarbital, 25 mg./kg., on the percentage tolerance index of female albino rats.*

is developed in 24 hr. (1). Results of the present experiment also indicate that a minimum of 4 hr. is necessary for the tolerance to be induced (Table I). Maximum tolerance is induced in an animal if the second injection is given between 17 and 22 hr.; then it declines. Nonsignificant tolerance is present after 48 hr. In less than 4 hr., animals showed cumulative effect instead of tolerance.

Duration of Administration of Drug—Duration of administration of pentobarbital will also affect the degree of development of tolerance to pentobarbital (Table III). PTI was greater on the second and subsequent administrations. In these experiments, all the injections were given daily for 16 days. On the other hand, when four injections were given within the span of 28 hr., animals showed a greater degree of tolerance on the third injection administered at 24 hr. after the initial injection (Table II). Tolerance was also present on the fourth injection but to a lesser degree when compared with the third injection.

Metabolism in the Liver—The liver plays an important role in the duration of action of barbiturates (3). Duration of action of barbiturates is decreased once tolerance is developed to barbiturates (Tables II and III). Anesthesia induced by barbiturates can be

Table II—Effect of Continuous Administration of Pentobarbital (25 mg./kg.) on Development of Tolerance[a]

Subgroups		Intervals, hr. 0	2	24	26
1	S.T.	76.4 (10)	123.0 (10)	27.1 (10)	59.4 (10)
	PTI[b]		62.0[c]	281.9[c]	128.6[c]
		0	3	24	28
2	S.T.	87.9 (10)	105.5 (10)	21.1 (10)	41.8 (10)
	PTI		83.3[c]	416.5[c]	210.0[c]
		0	4	24	28
3	S.T.	78.9 (10)	52.3 (10)	27.2 (10)	33.5 (10)
	PTI		150.8[c]	290.0[c]	235.5[c]

[a] $N = 10$. The same animals are used in each group. [b] PTI = percentage tolerance index. [c] $p < 0.05$.

Table III—Effect of Continuous Administration of Pentobarbital (25 mg./kg. i.p.) on Development of Tolerance

No. Injections	Injection Interval, Days[a]	Mean S.T., min.[b]	Tolerance Index, %
1	1	46.4 (8)	
2	2	26.7 (8)	173.0[c]
3–7	3–7	—	
8	8	36.0 (8)	128[c]
9	9	31.8 (8)	145[c]
10–14	10–14	—	
15	15	38.2 (8)	121[c]
16	16	41.6 (8)	111

[a] Injections were given daily to the same animals for 16 days. [b] The sleeping time was determined on 1, 2, 8, 9, 15, and 16 days. Sample size is given in parentheses. [c] $p < 0.05$.

prolonged by hepatic injury (7). Plaa *et al.* found a positive relationship between the doses of several hepatotoxins and the prolongation of pentobarbital sleeping time (7). Singh and Boyd found that tannic acid, which causes centrilobular liver necrosis, prolonged the sleeping time of thiopental (8). This effect became evident at 72 hr. after the administration of tannic acid. Balazs and Grice also reported that administration of carbon tetrachloride can prolong the sleeping time of pentobarbital (9).

Pentobarbital is metabolized in the liver (3). Conney *et al.* have shown that pentobarbital is an enzyme inducer (10). One explanation of the development of tolerance to pentobarbital is that pentobarbital possibly stimulates its own metabolizing enzyme and thus results in increased pentobarbital metabolism on repeated administration and decreased sleeping time (Tables I–III). This leads the authors to suggest that in the development of tolerance to pentobarbital, the liver possibly is involved.

Clinical Signs—During the development of tolerance to pentobarbital, neither water consumption nor urine excretion was affected. However, on the day after the tolerance had been induced, a significant increase ($p < 0.05$) was noticeable in water consumption and urine excretion. Singh reported similar clinical findings with thiopental also (11).

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Sterility Testing of Insulin by Membrane Filtration: A Collaborative Study

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Abstract □ A membrane filtration procedure was devised for testing the sterility of insulin zinc suspensions solubilized in ascorbic acid diluting fluid. A collaborative study showed that the filtration procedure afforded significant improvements over the direct method of sterility testing.

Keyphrases □ Insulin, sterility—membrane filtration □ Membrane filtration—sterility testing, insulin zinc suspensions □ Sterility testing—insulin, membrane filtration □ Collaborative study—membrane filtration, insulin sterility

In 1941 the Federal Food, Drug, and Cosmetic Act was amended to establish a certification service to ensure the safety and efficacy of insulin-containing drugs by testing each lot prior to distribution. The official analytical methods and standards of quality and purity are described in the USP (1), the NF (2), and the *Code of Federal Regulations* (3). Since insulin must be administered by parenteral injection, these official compendia require all insulin preparations to be sterile.

The official USP XVII/NF XII method for the sterility testing of insulin requires that 20 containers from each "filling operation" be tested in thioglycollate broth for detecting bacteria and in Sabouraud fluid medium for molds and yeasts. The solution or suspension of insulin is transferred with a sterile syringe and needle directly to tubes of media. Tubes containing thioglycollate medium are incubated for not less than 7 days at 30–32° and those containing fluid Sabouraud for not less than 10 days at 22–25°. After incubation the media are examined for the presence or absence of microbial growth.

The principal objection to the USP XVII/NF XII method is that a precipitate is formed by the insulin suspensions in the culture media. Macroscopically the precipitate is indistinguishable from microbial growth;