elevation of deep body temperature acts additively with a high dose of radiation to reduce survival time.

In an earlier work, Crile (6) showed that exposure to moderate heat (circa 44°) and radiation were effective in destroying various transplantable tumors in mice. It is of interest that such synergestic, or additive, effects may be operative on a local level, e.g., tissues and organs, as well as tumors, and on the whole animal level, as demonstrated using hamsters.

The present experiment is initial in nature and caution is advised in extrapolation to possible results with other species. However, because of growing possibilities of accidental exposure (e.g. industrial or warfare) to ionizing radiation in combination with environmental heat stress this overall area warrants considerable additional effort.

Summary. Adult male hamsters were placed in a hot room, 34–35°, and heat acclimated for 2 months. Controls were maintained at 24°. Each animal was given a single whole-body exposure to ionizing radiation from a 60 cobalt source. Half of the heat-acclimated animals were then transferred to 24° and half returned to the hot room. Half of the controls were placed in the hot room after irradiation. Comparisons of survival times were made; heat exposure either before or after irradiation, or both before and after irradiation lowered the survival time to less than 50% that of the controls, i.e., hamsters which were irradiated but not heat exposed.

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Antibody Formation in Hibernating Ground Squirrels (*Citellus tridecemlineatus*)* (33408)

J. M. MCKENNA AND X. J. MUSACCHIA

Departments of Microbiology and Physiology, School of Medicine, and the Space Sciences Research Center, University of Missouri, Columbia, Missouri 65201

While the literature on various aspects of the physiology of mammalian hibernation is voluminous, surprisingly little has been published concerning the immune mechanisms of mammals in this state. Andjus *et al.* (1) injected rabbit erythrocytes intravenously into hibernating ground squirrels, and assayed the sera for agglutinins for periods up to 40 days of hibernation. They reported that, as long as the animals were hibernating, no circulating agglutinins were formed. Jaroslow and Smith (2) gave a single intraperitoneal injection of ^{131}I-labeled bovine serum albumin (BSA) *before* placing the ground squirrels at 5°, and studied antigen disappearance. They concluded that there was no net synthesis of antibody.

It is the opinion of the few workers in this field that the hibernating mammal is not capable of producing circulating antibodies while in hibernation. This opinion has been based almost exclusively on studies of the primary response to a few antigens using relatively insensitive methods for detecting antibodies. Furthermore, it has not been established whether the essential machinery to invoke an anamnestic response is operating in hibernating mammals.

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The relative sensitivity of various serologic methods for detecting antibodies has been summarized by Marrack (3) and Kwapinski (4). The minimal amount of antibody N detectable by red cell agglutination is given as 0.1 μg. The measurement of the sensitivity of antigen disappearance or immune clearance methods is not as straightforward as other serologic techniques. It is reasonable to assume that the usual ranges of antigen–antibody combining ratios occurring *in vitro* also apply *in vivo* as suggested by Dixon *et al.* (5). The ratios found in rabbits following primary immunization with BSA were 1:5.5 (5) thus indicating a sensitivity of not less than 3–5 μg of N.

It is possible that the negative results reported to date are largely the result of using relatively insensitive methods for detecting antibody. Pilot experiments in these laboratories during the winter of 1966-67 suggested that circulating antibody could be produced at low levels by hibernating mammals provided the assay system was adequately sensitive.

It is the purpose of this paper to show that hibernating mammals do form circulating antibody following primary immunization and that the secondary response is operational in these animals.

Materials and Methods. Animals. Ground squirrels (*Citellus tridecemlineatus*) were collected in the late spring, 1967 in the vicinity of Fort Leavenworth, Kansas. The animals were housed 2/cage for 8–10 months before use and during experimentation, 1/cage. While in the active, nonhibernating state, animals were maintained on an *ad libitum* diet of Wayne lab Blox animal feed, and a weekly supplement of lettuce. Water was available at all times. Experimental animals for hibernation procedures were placed individually in lucite plastic containers without food or water and were provided with San-i-cel corn cob bedding. The hibernaculum temperature was 5–7°. Control animals were kept at 22 $\pm$ 1° and all studies were carried out during the winter months (1967–1968).

Antigen. Influenza A virus vaccine (Strain PR_8) was supplied through the courtesy of Dr. E. S. Barclay, Merck, Sharp and Dohme, Inc., West Point, Pennsylvania.

Immunizations and assay system. For studies of the primary response to PR_8 vaccine, ground squirrels which had been in hibernation for at least 2–4 days were each given a single intraperitoneal injection of 1 ml of vaccine diluted with 0.85% NaCl to contain 5×10^3 chick cell agglutinating (CCA) units. Care was taken to minimize disturbance of the hibernating animals, with the result that they were not visibly awakened by the injections. Control animals (i.e., active) were given identical doses of antigen. One week later all animals were sacrificed by decapitation and the blood was collected. The sera were allowed to separate in the refrigerator overnight, were centrifuged and stored at —20° until assayed. For studies of the secondary response, each animal was given a second injection of 5×10^2 CCA units in 1 ml intraperitoneally 1 month after the initial injection. These animals were sacrificed 5 days after the second injection of antigen. For a measure of the secondary response, one group of animals was allowed to awaken at room temperature immediately following the second injection. One other group was allowed to awaken for 2 hr at room temperature before sacrifice.

All sera were tested for the presence of antibodies to PR_8 by hemagglutination inhibition (HI) as described by Schmidt and Lennette (6). Appropriate controls showed the efficacy of the KIO_4 treatment of the sera to remove nonspecific inhibitors. Statistical determinations of levels of significance were carried out by Student's t test.

Results. Dose response curves established that ground squirrels responded well on both primary and secondary immunization to PR_8 vaccine. However, it was noted that an antigen mass of 5×10^3 CCA units was required to elicit a measurable response with a narrow range in all animals following primary immunization.

The results, given in Table I, indicate that hibernating ground squirrels did produce circulating antibody on primary immunization, and further, that a secondary response may have been obtained. However, the range of titers observed in the hibernating animals

TABLE I. Geometric Mean Titer (GMT) Hemagglutination Inhibition (HI) of *Citellus tridecemlineatus* to Influenza A Virus Vaccine (PR_8).[a]

	Primary (7 day)	Secondary (35 day)	p
Normotherms	128 (64–256) (11 animals)	4096 (2048–8192) (11 animals)	ND[b]
Hibernators	9.8 (4–32) (11 animals)	45 (4–1024) (8 animals)	.02

[a] Titers are expressed as the reciprocal of the serum dilution showing complete hemagglutination inhibition. (Range of titers is given in parentheses.)

[b] Not done.

was considerably greater than that in the control group.

The data in Table II give a composite picture of all the hibernating animals used. It was originally felt that the geometric mean titer (GMT) of the group C animals was of a secondary type since it was significantly different from the GMT of group A animals. (cf. Table I). The GMT of group B was determined after these animals were allowed to awaken at room temperature for 2 hr before sacrifice. This GMT is also significantly different from the GMT of group A. When animals were allowed to regain activity at room temperature for 5 days after the second injection, following 30 days in hibernation, a true anamnestic response was seen (group D).

It is well known that during the hibernation period animals undergo partial awakening from time to time. In a series of independent studies, using skin surface thermistor probes and a Yellow Springs telethermometer apparatus, Musacchia and Westhoff (unpublished) have noted that during winter the hibernation periods ranged from about 10–15 days in *Citellus tridecemlineatus*. In the current investigation, repeated daily observations (3 times/day) of postural changes and wakefulness were recorded. This provided a suitable index of wakefulness. Each event of wakefulness was considered to be 1 day out of hibernation for a given animal. There was no apparent relationship between the titer of any given animal and its periods of wakefulness. This was true of all groups of hibernating animals.

The time courses of both primary and secondary responses in all the animals studied are shown in Fig. 1. The time course of active animals was typical of the usual mammalian response to primary and secondary antigenic stimulation. On the other hand, the animals kept in hibernation for the full 35 days after primary stimulation showed an increasing HI titer from 7 to 35 days. The difference between the GMT at 35 days for group 3 ($\log 2 = 4.5$) was not significant when compared to that of group 4 ($\log 2 = 5.5$).

Whether the antibody formed by the hibernating animals was immunoglobulin M or immunoglobulin G has not yet been determined. Lack of sufficient serum remaining after antibody titrations has deferred these experiments until a future hibernating season.

Discussion. The present findings at first glance appear to be somewhat at variance with previously reported results on antibody

TABLE II. Geometric Mean Titer (GMT) of Hibernating *Citellus tridecemlineatus* to Influenza A Virus Vaccine (PR_8).[a]

Group	Hibernation[b] (days)	Primary titer	Days awake after hiber.	Secondary titer
A	7 (11)	9.8 (4–32)	—	—
B	35 (15)	23 (16–64)	—	—
C	35 (8)	—	—	45 (4–1024)
D	30 (15)	—	5	3072 (64–8192)

[a] Titers are expressed as the reciprocal of the serum dilution showing complete hemagglutination inhibition. (Range of titers is given in parentheses.)

[b] Number of animals is given in parentheses.

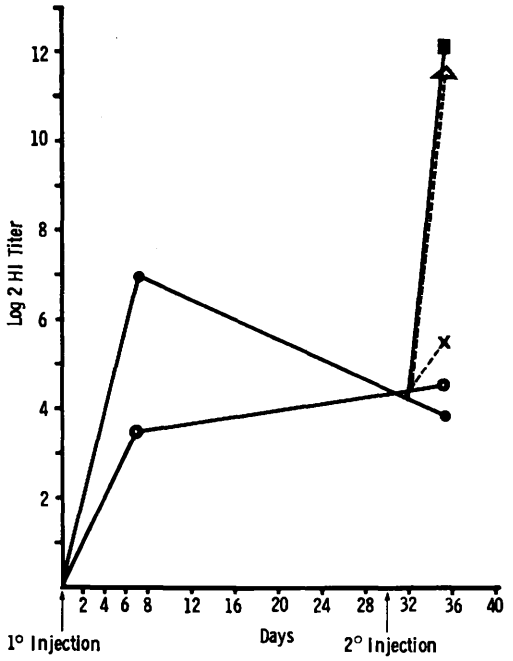

FIG. 1. Time course of primary and secondary antibody responses in all animals studied. Group 1. ●—● 1° active; 2. ■—■ 2° active; 3. O—O 1° hibernating; 4. ×—× 2° hibernating; 5. △—△ 2° hibernating—then active.

formation in hibernating mammals (1, 2). It was pointed out in the introduction that relatively insensitive assay methods for the detection of antibody were used in those earlier works. In the HI technique, detections on the order of 10^{-3} μg of antibody N can be made (7). Therefore, it may well be that earlier negative results were due to insensitive assay techniques rather than failure of the hibernating animals to synthesize antibody. Jaroslow and Smith (2) interpreted their findings, using immune clearance, to mean that the events of the latent period can occur during hibernation, but that if the animal remains in hiberation, the ability to synthesize antibody is lost. Subsequently, all the steps leading to antibody formation, namely, induction, maturation, and proliferation, would have to be reinitiated. It is of interest to note in their work that, as hibernation progressed, more animals showed an immune clearance of antigen, or had no latent period. This finding agrees with the data of Andjus *et al.* (1) which we consider to show a progressive shortening of the latent period as hibernation progressed. Our interpretation is evidenced from the progressive steepening of the titer curves to peak titers as hibernation periods were increased.

If the interpretation of Jaroslow and Smith (2) is correct, and the events leading to antibody production are lost in prolonged hibernation, then it should not be possible to demonstrate an anamnestic response in hibernating mammals. Our data (Table II) suggest that hibernating ground squirrels are fully capable of an anamnestic response, the preliminary events of which were initiated during hibernation. The GMT of animals allowed to awaken and remain active for 5 days following reimmunization compared favorably to the secondary response of control animals not in hibernation.

Whether the GMT of 45 (Table II, Group C) is truly a secondary response is not certain. Further, it is not clear whether the GMT of 23 (Table II, Group B) is really primary or may indeed by a pseudosecondary response. At the cellular level, the secondary antibody response is characterized by two phases. Initially, there is a proliferation and differentiation of lymphoid cells with the net synthesis of DNA (8). Soon cellular multiplication ceases and antibody synthesis becomes the dominant feature (8). It is quite likely that extensive cellular proliferation does not occur during hibernation (9). However, Dutton (10) was able to inhibit antibody synthesis *in vitro* by irradiation with tritiated thymidine selectively in those lymphoid cells which were actively dividing. Resting cells were unaffected, and only those cells producing antibody were dividing. Furthermore, Urso and Makinodan (11) showed clearly that antibody-containing cells divided at a significantly higher rate than incompetent cells during the log phase of activity. It is conceivable that the antigen might seek out those cells which were synthesizing DNA at the normal metabolic rate, since it was shown by Manasek *et al.* (12) that the reduction in the net synthesis of DNA by lymphoid cells of hibernating hamsters was due to a reduction in the *number* of cells performing

this function, and not to a reduced synthesis of DNA by a given cell.

Richter and Haurowitz (13) showed that antibody synthesis to innocuous protein antigens continued unabated in rabbits for several months following either single or multiple injections. These antibodies were of the nonprecipitating type, but were detectable by the agglutination of antigen-coated red cells. This continued formation of antibody may be the result of retained antigen or antigenic fragments over prolonged periods of time as suggested by Campbell and Garvey (14). With the prolonged retention of antigen which is degraded very slowly, for example in a hibernating animal, a pseudosecondary response in animals hibernating for 1 month is not unreasonable.

Summary. Specific antibody formation to influenza A virus vaccine (strain PR_8) has been demonstrated in hibernating *Citellus tridecemlineatus*. Although only a small amount of hemagglutination-inhibiting antibody was formed on primary antigenic stimulation, it was detectable. A true anamnestic response was shown in animals given a booster dose of antigen 1 month after the initial injection when these animals were awake at room temperature for 5 days before sacrifice. Whether a true secondary response was obtained entirely in hibernation is not ascertainable from our data, although the GMT was significantly different from the primary GMT. A possible pseudosecondary response was also noted following primary antigenic stimulation when the antigen presumeably was retained in the hibernating animals.

Note added in proof: Since the submission of this paper, Jaraslow (Proc. Nat. Acad. Sci., **61**, 69, 1968) has shown the development of the secondary response in hibernating animals of the same species as we used.

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Endotoxin Induced Hypothermia and Tolerance in the Rat* (33409)

JAMES P. FILKINS AND N. R. DI LUZIO[1]

Department of Physiology and Biophysics, University of Tennessee Medical Units, Memphis, Tennessee 38103

Lipopolysaccharides extracted from gram-negative bacteria, i.e., endotoxins, are often termed pyrogens due principally to their ability to induce fever in humans, rabbits and dogs (1). In addition, tolerance to endotoxin febrility has generally been demonstrated in animals chronically pretreated with daily in-

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[1] Present address: Department of Physiology, Tulane University Medical School, New Orleans, Louisiana 70112.