Relative Effects of Fighting on Bound and Unbound Corticosterone in Mice.* (29781)

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Crowding of mice has been implicated as an important variable in many diverse areas of biological experimentation; to mention a few: resistance to disease(1,2) and endoparasitism(3), reproductive efficiency(4), and behavior of unborn young when mature(5). Such effects are thought to be directly or indirectly related to increased activity of the pituitary-adrenocortical axis which is, in turn, related to fighting (more accurately, to the psychological aspects of social subordination) (6). Most investigators have relied on alterations of adrenal weight or histology as indicators of increased adrenal activity following crowding or defeat in a fighting situation and, in actuality, little is known about the relationships between circulating corticosteroid levels and the social environment of mice except by such indirect indicators. This paper describes the relationship between unbound and protein-bound corticosterone in the plasma of mice during a 24-hour period after either the first or the fourth daily exposure to defeat by trained fighting mice.

Materials and methods. In brief, mice were individually exposed to a trained fighter once a day for either 1 or 4 days, blood was collected by decapitation at 1 of 6 times after such exposure, and plasma was analyzed for corticosterone with and without $ZnSO_4$ precipitation (Table I). Controls consisted of animals maintained in isolation and killed for blood collection on the same time schedule as those mice exposed to fighters.

In more detail, 270 C57BL/6J male mice were isolated at weaning (21-28 days) and used at 85-95 days of age. Exposure to a trained fighter of the same strain(7) took place in the fighter's home cage, lasted for 15 minutes, and always began at 10 a.m. All mice remained isolated in their home cages except when actually being exposed to a fighter. Three blood samples, each containing pooled blood from 5 mice, were collected at each of the 6 post-fighting (or control) times for a total of 90 mice in each of the 3 types of treatments. Blood was collected at 20 minutes, 1, 3, 6, 12 or 24 hours after exposure (*i.e.*, post 10:15 a.m.). Isolated controls were killed on the same time schedule. One exception must be noted: all mice scheduled to be autopsied at 24 hours post-fighting (post 10:15 a.m.) were actually autopsied just before the next daily series of fights began at 10 a.m. in order to avoid the disturbance accompanying the fighting procedures.

Corticosterone was analyzed fluorimetrically by the procedures outlined by Guillemin *et al*(8) both with and without protein precipitation by $ZnSO_4$(9). Following the method of Knigge and Hoar(9), the corticosterone fraction left in the supernatant after $ZnSO_4$ precipitation of the protein will be referred to as "unbound"; The difference between this amount and that determined with no protein precipitation ("total") will be referred to as "bound."

All treatments and blood collections took place in the same room, which was entered only at 10 a.m. for the daily series of fights or when required for autopsy. Collections of blood samples were kept as random as possible with respect to treatment and time of analysis. On no day were more than 3 samples of blood (15 animals) collected and, when this maximum occurred, the samples were separated in time as much as possible. At autopsy time, the room was entered and all mice killed by decapitation within 15 seconds after removal of a cage from its rack. A low, relatively constant, vibrating noise was supplied at all times in an effort to mask footsteps, door openings, etc. Lighting was from 5 a.m. through 7 p.m. The autopsies scheduled at 10:15 p.m. (12 hours post-fight-

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TABLE I. Mean Plasma Corticosterone Levels (μg/100 ml) Following Daily Exposure to Trained Fighting Mice for Either 1 or 4 Days (1 Fight per Day) or Kept in Isolation and Killed on the Same Time Schedule as Fighter-Exposed Mice. Means represent the average of 3 plasma samples, each pooled from 5 mice.

Corticosterone fraction	No. of fights	Time after last fight (hr)*					
		⅓	1	3	6	12	24
Unbound	1	13.7 (11–18)†	10.7 (9–13)	6.0 (4– 7)	6.7 (4– 9)	2.3 (2– 3)	3.7 (0– 6)
	4	7.7 (7– 8)	8.3 (2–18)	0	0	.7 (0– 2)	0
	None	0	0	0	1.7 (0– 4)	0	.7 (0– 2)
Bound	1	7.3 (6– 9)	6.6 (6– 8)	8.3 (7–10)	8.6 (8– 9)	7.6 (7– 8)	8.3 (7– 9)
	4	7.6 (6– 9)	9.0 (8–10)	10.0 (9–11)	9.7 (9–10)	8.3 (8– 9)	8.3 (6–10)
	None	7.6 (6– 9)	7.0 (6– 9)	9.3 (8–11)	8.6 (8– 9)	8.3 (8– 9)	8.6 (7–10)
Total	1	21.0 (17–27)	17.3 (15–19)	14.3 (12–17)	15.3 (13–18)	10.0 (9–11)	12.0 (9–14)
	4	15.3 (14–17)	17.3 (11–28)	10.0 (9–11)	9.7 (9–10)	9.0 (8–11)	8.3 (6–10)
	None	7.6 (6– 9)	7.0 (6– 9)	9.3 (8–11)	10.3 (8–13)	8.3 (8– 9)	9.3 (7–11)

* All fights ended at 10:15 a.m.

† Range in parentheses.

ing) were accomplished by the light of a 5-watt red light left burning in the room when the lights went out at 7 p.m. All tests of significance were t-tests.

Results. Prolonged elevation of total corticosterone in the plasma followed the first exposure to a trained fighter while the response was of a much shorter duration after the fourth such exposure (Table I). Total plasma corticosterone was significantly (.05 or .01 level) higher in animals exposed to fighters for 1 day than in isolated controls at 20 minutes, and at 1 and 3 hours after treatment but not significantly different thereafter. Total plasma corticosterone was significantly increased only at 20 minutes after exposure in animals exposed to 4 daily fights when compared to isolates. The levels found at 1 hour after fighting in the 4-day group were excessively variable. When total corticosterone is compared in mice exposed to 1 or 4 days of fighting, significance is found only at the 6-hour time interval ($P<.05$) but is approached ($P<.06$) at 3 hours.

There were no significant differences in any of the data concerning bound corticosterone (Table I). Isolated controls averaged 8.3 μg ($\pm$ S.E. of 0.3) of bound corticosterone per 100 ml of plasma across the 24-hour period while animals exposed to 1 or 4 days of fighting averaged, respectively, 7.8 ($\pm$ 0.3) and 8.8 ($\pm$ 0.3) μg/100 ml.

The differences found in total corticosterone levels are almost entirely reflected by alterations in that fraction of the hormone not zinc-precipitable (Table I). Unbound corticosterone was present only as traces at 6 and 24 hours (4:15 p.m. and 10 a.m.) in isolated controls while it was present at all times, and in large amounts just after fighting ended, in animals exposed to the first defeat by a fighter. The response is again of a characteristically curtailed duration in mice exposed for 4 days; unbound corticosterone disappearing almost entirely by 3 hours after exposure.

Discussion. The data for both isolated and experimental animals undoubtedly were influenced by two non-experimental factors: (a) the 24-hour cycle in circulating corticoid levels(10) and (b) the disturbance in the experimental room due to either the fighting procedures or to collecting blood. The 24-hour cycle is represented in isolates, to some extent, by the high point at 6 hours post-fighting (total corticosterone), the same relative time of day but a much smaller peak than previously demonstrated for mice(10). Entering the room daily to treat or kill animals, despite the noise generator (and possibly this factor itself), probably played a major role in damping out the 24-hour cycle in the isolated animals.

The stimulus acting on mice to produce adrenal responses after fighting is associated with defeat and is primarily psychological in nature(6). In the present study, exposure to a trained fighter resulted in, at most, 2-5 minutes of actual fighting or chasing; the remainder of the time the mice were sub-

jected to the stimulation of the presence of the fighter but with no overt aggression apparent. It must be noted that the fighting procedure used here consisted of transport to and from a fighter's cage as well as defeat by a fighter. Smelik(11) has shown that cage switching stimulates ACTH production or release but it is known that the effect of transport to another cage results in relatively small effects on the adrenal when compared to fighter-exposure(12). Therefore, the adrenal responses found are largely, but not entirely, due to defeat by a fighter.

The hormone circulating in the plasma in mice is almost entirely zinc-precipitable during continual isolation. This is in agreement with the findings for control guinea pigs and rats by Knigge and Hoar(9). These same workers found that increased peripheral corticosteroid levels following acute drug anesthesia or chronic cold-exposure were due to increases in unbound corticosteroid in the former and bound in the latter. It is obvious from Table I that the response to short daily exposures to fighting is much more like the acute drug response since all increases are in the unbound fraction and there is no buildup of bound corticosterone even after 4 days of fighting.

The first exposure to a fighter was followed by more than 24 hours of elevated (unbound) corticosteroid levels while the response lasted less than 3 hours after the fourth such exposure. Physiologically, this could reflect an alteration in the adenohypophyseal response as demonstrated in rats(13) or at any other level along the axis including alterations in corticosteroid synthesis or degradation(14). The curtailed response of the fourth day was probably not due to alterations in binding(15) since there were no real differences in any of the data for bound corticosterone. There are sound behavioral reasons for predicting the observed difference between the first and fourth exposures: (a) the amount of fighting among mice normally decreases with time and (b) more importantly, due to their rearing in a non-social and non-handled situation, the first exposure undoubtedly represented a radical alteration in the environment of these mice. These facts, then, would argue for the differential effects of the first and fourth exposures being accounted for, at least in part, by differences in the level of neurotropic stimuli.

Different responses could be predicted for fighting in a crowded situation, the usual experimental condition, since the defeated mice would be subjected to additional stimulation due to the continued presence of the winner as well as other mice. Fighting rather than crowding was chosen for the experimental situation in the present study for the obvious reason of greater ease of control.

Summary. Circulating corticosterone is almost entirely zinc-precipitable in isolated C57BL/6J (adult male) mice. Mice maintained in isolation except for daily 15-minute exposures to trained fighting mice show a profound increase in circulating corticosterone which is entirely traceable to increases in unbound (non-zinc-precipitable) hormone following fighter-exposure. Mice exposed to the first defeat by a fighter were characterized by more than 24 hours of elevated unbound corticosterone with no alteration in that fraction bound to protein. Adaptation to the experimental situation is rapid since, by the fourth exposure to a fighter, the elevation of unbound hormone lasts less than 3 hours; again, with no change in the level of protein-bound corticosterone.

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Oxidation of Free Fatty Acids by Dog Liver.* (29782)

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It has been shown in several laboratories (1-5) that the liver takes up large quantities of free fatty acids (FFA) and that hepatic uptake is dependent on plasma FFA concentration(6). The perfused rat liver has also been found to remove FFA and to release esterified fatty acids(7-9). In the present study the oxidation of FFA to CO_2 was investigated in dogs under *in vivo* conditions employing isotopically labeled substrate.

Materials and methods. Seven dogs of either sex, weighing about 20 kg, were used after 16 hours of fasting. Simultaneous blood samples (1-6 per dog) were collected from the femoral artery, portal vein and hepatic vein. The latter 2 vessels were catheterized after laparotomy under manual guidance. Palmitate-1-C^{14} or oleate-1-C^{14} was infused as described previously(10).

Plasma FFA concentrations were determined by the method of Dole and Meinertz (11). After completion of the FFA determination, the bottom phase of the titrated mixture was maintained in an alkaline state by addition of NaOH. The top phase (designated as neutral lipids) was removed, the bottom phase washed 3 times with ligroine or hexane and the washings added to the withdrawn top phase. The remaining bottom phase (containing FFA) was acidified, washed 3 times with hexane and the washings were pooled. Both FFA and neutral lipids were counted in a Packard liquid scintillation detector using 0.5% 2,5-diphenyloxazole and 0.05% 2-p-phenylenebis-4-methyl, 5-phenyloxazolone in toluene as scintillation fluid.

Blood was collected for $C^{14}O_2$ and CO_2 determinations under mineral oil into a mixture of saponin and oxalate. Analysis of $C^{14}O_2$ was performed according to the method of Passman, Radin, and Cooper(12). Concentration of CO_2 was determined by the Technicon AutoAnalyzer.

Results and discussion. The results are summarized in Table I. The portal supply of blood to the liver was asumed to be 2 parts portal vein to 1 part hepatic artery (13). The mean portal supply of FFA was .229 mEq/l. The liver removed 39% of this amount. A consistent production of $C^{14}O_2$ by the liver was also evident. The oxidation index indicated that on the average .02 mEq/l of FFA, or about ¼ of the removed amount was oxidized. Thus, about 10% of the arterial FFA content is oxidized by the liver through one passage. A portion of the labeled fatty acid was recirculated in the form of neutral lipids, as judged by the frequent release of net counts in the neutral lipid fraction. Assuming that the average FFA has 17 carbon atoms, the oxidation of FFA would account for approximately 38% of the CO_2 produced by the liver.

The present data obtained in dogs under *in vivo* conditions are not unlike those described for the perfused rat liver, where 6.27% and 18.00% of the C^{14} labeled palmitic acid was oxidized to CO_2 in 4 hours by the fed and starved livers respectively(8).

Summary. The hepatic oxidation of FFA to CO_2 was investigated in dogs. Thirty-nine per cent of the mean portal supply of FFA was removed by the liver and about ¼ of this amount was oxidized to CO_2. This amount of CO_2 could account for approxi-

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