lease of additional FFA from adipose tissue after a fasting period of 30 hours cannot, therefore, be attributed to a change in its responsiveness to catecholamine stimulation. In the light of Leduc's(13) report of increased excretion of epinephrine and norepinephrine in cold-stressed rats, which lasted for many days, lack of hormone is probably not a factor in the diminution of the cold effect. Apparently the fast, alone, if it is sufficiently prolonged, produces a change in the activity of the adipose tissue which is not further modified by exposing the donor to cold. These findings suggest the presence of more than one source for the FFA, one of which responds rapidly to cold stimulation. The other, apparently less affected by cold, still has a reserve which is responsive to stimulation by the catecholamines, *in vitro*.

The probable existence of more than one fatty acid pool has been suggested several times(8,17,18). The above data suggest that the cold effect is not altogether mediated through stimulation of the lipolytic enzymes (19), but to what other origin the temporarily greater output of FFA by the adipose tissue may be attributed is not apparent.

*Summary.* Rats deprived of food at the beginning of the experiment were exposed to a cold environment at 3-5°C for periods varying from 4 to 30 hours. The release of FFA by epididymal adipose tissue *in vitro* was compared to that from animals maintained at room temperature of 25°C. FFA was released in significantly greater amounts by the tissue of the animals exposed to cold until the fast had progressed for 30 hours, at which time there was no difference between the two groups. Following a fast of 18 hours, the adipose tissue from the cold-exposed group and from the warm room group responded similarly to epinephrine and norepinephrine.

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## Effect of Fasting or Feeding on Release of Free Fatty Acid by the Adipose Tissue of the Cold-Acclimated Rat.* (29647)

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Studies relating the metabolism of adipose tissue through release of free fatty acid (FFA)† to the need for increased energy production during exposure to cold have been

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† Free (unesterified) fatty acid is referred to as FFA in this report.

concerned with the effects of cold on plasma FFA levels(1,2), on the release of FFA by adipose tissue, *in vitro*(1,2,3), on the rate of turnover and oxidation of FFA(4,5), and on lipogenesis by adipose tissue(6). The property of norepinephrine to stimulate nonshivering thermogenesis in cold-acclimated rats(7) and to cause release of FFA by adipose tissue(8), together with its reported excessive excretion by cold-stressed rats(9) have led to attempts to relate this hormone and lipid metabolism to the increased energy requirements necessitated by a cold environment.

Experiments with both rats(10) and dogs (11) have documented the increased food ingestion which occurs to meet the energy needs of animals exposed to cold. An earlier report from this laboratory(12) demonstrated the dependency of the response of adipose tissue during acute cold stress, as measured by release of FFA *in vitro,* on the state of the animal in relation to the last food intake before the adipose tissue was removed for incubation. The present report is concerned with the effect of feeding or fasting prior to sacrifice on FFA release, *in vitro,* by adipose tissue from cold-acclimated rats.

*Methods.* Male albino rats from the Holtzman colony weighing approximately 200 g at the start were used throughout the experiments. The animals were sacrificed by an occipital blow and the epididymal fat pad was quickly removed and placed in buffer for trimming and sampling. The thin, distal portion of the fat pad was used routinely. Pieces weighing 50 to 100 mg were placed in 3 ml of Krebs-Ringer phosphate buffer which contained bovine plasma albumin (Armour's crystallized) at a level of 5%. The pH of the medium was 7.4. The tissue was incubated for 2 hours at 37°C in a Dubnoff metabolic shaker in an oxygen atmosphere. FFA release to the medium was determined by the method of Dole and Meinertz(13). FFA is expressed as μMoles per g per hour calculated as palmitic acid. Animals used for exposure to cold were selected at random and were housed in individual cages in a cold room at 3-5°C. Animals used as controls were maintained at 24-25°C. All were allowed Purina laboratory chow, *ad libitum,* unless otherwise noted.

As a background for better understanding of the effects of cold on adipose tissue, the food consumption of animals housed at 3-5°C was compared to that of rats kept at room temperature. Food was weighed into containers and the amount consumed was determined daily. As has been reported(10), food consumption increased gradually and reached a maximum at about 14 days, at which time the rats ate about 40% more food each day than did those maintained at 24-25°C.

*Experiment 1.* Animals were placed in the cold environment (3-5°C) and groups of 6 were sacrificed at intervals up to 8 days. FFA release from adipose tissue was measured without preliminary fasting. One group continued in the cold for 20 days before measurements of FFA release were made.

*Experiment 2.* The experimental rats were kept at 3-5°C for 34 to 36 days, at the end of which time they were considered to be acclimated. Animals used as controls were from the same group, maintained under the same conditions but at 24-25°C. When norepinephrine was used to test its effect on adipose tissue from cold-acclimated rats, it was added to the incubation medium as the bitartrate at levels of 0.05 μg/ml or 0.15 μg/ml, calculated as free norepinephrine. The animals were not fasted before sacrifice.

*Experiment 3.* After being in the cold environment for 34 to 36 days, the animals were fasted for 18 hours beginning at 4:00 P.M. before they were sacrificed. Again, adipose tissue release of FFA was measured with and without addition of norepinephrine to the medium.

*Results. Experiment 1.* The results of Exp. 1 are recorded in Table I. After one day in the cold room, and without preliminary fasting, the release of FFA by adipose tissue *in vitro* had reached a level which was maintained at substantially the same value as exposure was prolonged up to 20 days. The lowest value observed was 3.91 μMoles per g per hour after 2 days in the cold room,

TABLE I. Release of FFA by Epididymal Adipose Tissue from Non-Fasted Rats Exposed to Cold.

| Days in environment | FFA, μmoles/g/hr* Animals at 24-25°C (4)† | Animals at 3-5°C (6)† | P‡ |
|---|---|---|---|
| 1 | .93 ± .27 | 3.92 ± .15 | <.01 |
| 2 | 1.27 ± .27 | 3.91 ± .40 | <.01 |
| 3 | 1.48 ± .44 | 4.58 ± .54 | <.01 |
| 4 | 1.27 ± .49 | 3.96 ± .63 | <.02 |
| 6 | 1.37 ± .26 | 4.37 ± .33 | <.01 |
| 8 | .86 ± .36 | 3.99 ± .24 | <.01 |
| 20 | 1.99 ± .61 | 4.74 ± .55 | <.02 |

* Mean ± S.E.
† Numbers in parentheses indicate No. of animals in each group.
‡ Significance of difference between warm- and cold-acclimated animals by Fisher's "t" test.

and the highest 4.74 μMoles per g per hour after 20 days. The values at the progressively longer exposure times varied above and below those of the one-day exposure and the differences are not significant. These values compare with 0.86 to 1.99 μMoles per g per hour FFA released by tissue from non-fasted animals maintained at room temperatures of 24-25°C. The differences between the warm-room and cold-room animals are highly significant at each time period investigated, $P < .02$ on days 4 and 20 and $P < .01$ at all of the other periods by Fisher's "t" test.

*Experiment 2.* After 34-36 days at 3-5°C, the adipose tissue from these rats produced significantly more FFA than did that from rats maintained at room temperature, 7.81 ± 1.09 compared to 2.37 ± 0.34 μMoles per g per hour. The result might be attributed to the fact that the tissue is depleted of fat after prolonged cold exposure, hence each unit of weight would represent more "active" protoplasm than the same unit from an animal not acclimated to cold. However, the results of Exp. 3 (below), and of earlier work(12), which showed no difference between release of FFA by adipose tissue of control and cold-stressed rats when they were fasted for a sufficiently long period prior to sacrifice of the donor animal, tend to negate such a possibility. As expected, the tissues from both groups of animals responded to norepinephrine with a markedly accelerated output of FFA, and, both with 0.05 μg/ml and 0.15 μg/ml, the response of the tissue from cold-acclimated rats was significantly greater than that from rats not exposed to cold. The results are recorded in Table II.

*Experiment 3.* The animals in Exp. 3 were acclimated to cold. In this experiment, however, these, as well as the control animals, were fasted 18 hours before being sacrificed. The fast began at 4:00 P.M. In contrast to Exp. 2, there was no significant difference between the release of FFA to the medium by adipose tissue *in vitro* between the two groups. Again, norepinephrine stimulated FFA release in both groups. The difference was not significant when 0.05 μg/ml was

TABLE II. Effect of Norepinephrine *in vitro* on Free Fatty Acids Released from Adipose Tissue of Non-Fasting Warm- and Cold-Acclimated Rats.

| Environment* | Group | No. of rats | Additions† | FFA, μmoles/g/hr‡ | P§ |
|---|---|---|---|---|---|
| Warm room | 1 | 5 | None | 2.37 ± .34 | 1-2 <.01 |
| | 2 | 5 | Norepinephrine, .05 μg/ml | 3.77 ± .30 | 1-3 <.01 |
| | 3 | 5 | Norepinephrine, .15 μg/ml | 6.23 ± .41 | 2-3 <.01 |
| Cold room | 4 | 8 | None | 7.81 ±1.09 | 4-5 <.01 |
| | 5 | 5 | Norepinephrine, .05 μg/ml | 13.49 ± .93 | 4-6 <.01 |
| | 6 | 5 | Norepinephrine, .15 μg/ml | 17.65 ± .78 | 5-6 <.05 |
| | | | | | 1-4 <.02 |
| | | | | | 2-5 <.05 |
| | | | | | 3-6 <.05 |

* Warm room animals were maintained at 24-25°C and cold room animals at 3-5°C for 34-36 days.
† Norepinephrine was added to the medium as the bitartrate calculated as the free base in the amounts shown per ml of medium.
‡ Mean ± S.E.
§ Significance by Fisher's "t" test.

TABLE III. Effect of Norepinephrine *in vitro* on Free Fatty Acids Released from Adipose Tissue of Warm- and Cold-Acclimated Rats Fasted for 18 Hours.*

| Environment† | Group | No. of rats | Additions‡ | FFA, μmoles/g/hr§ | P‖ |
|---|---|---|---|---|---|
| Warm room | 1 | 6 | None | 4.16 ± .26 | 1-2 <.01 |
| | 2 | 7 | Norepinephrine, .05 μg/ml | 5.54 ± .18 | 1-3 <.01 |
| | 3 | 7 | Norepinephrine, .15 μg/ml | 7.12 ± .61 | 2-3 <.05 |
| Cold room | 4 | 5 | None | 4.09 ± .38 | 4-5 <.02 |
| | 5 | 7 | Norepinephrine, .05 μg/ml | 6.44 ± .59 | 4-6 <.01 |
| | 6 | 7 | Norepinephrine, .15 μg/ml | 9.55 ± .65 | 5-6 <.05 |
| | | | | | 2-5 >.05 |
| | | | | | 3-6 <.01 |

* Food was removed from the cages at 4:00 P.M.

† Warm room animals were maintained at 24-25°C and cold room animals at 3-5°C for 34-36 days.

‡ Norepinephrine was added to the medium as the bitartrate calculated as the free base in the amounts shown per ml of medium.

§ Mean ± S.E.

‖ Significance by Fisher's "t" test.

added to the medium, but it was significant when 0.15 μg/ml was used. The total output reached about half the magnitude exhibited by the tissue from non-fasted animals. The results are recorded in Table III.

*Discussion.* When adipose tissue from animals fasted for 30 hours at 3-5°C was compared to tissue from animals fasted for the same period at 24-25°C, the *in vitro* release of FFA was of the same order(12). The same similarity of response was observed in the present experiments when tissue from rats exposed to 3-5°C for 35 days and then fasted for 18 hours was compared to that from room temperature animals also fasted for 18 hours (Table III).

In marked contrast was the FFA release by adipose tissue from non-fasted cold-stressed rats. As is well recognized, adipose tissue from fed animals kept at room temperature releases very small amounts of FFA (14 and Table I). However, tissue from non-fasted rats exposed to cold for periods ranging from 24 hours to 20 days released 3 or more times more FFA than did tissue from their room temperature controls (Table I). This same effect of cold stress was seen when the donor animals had been acclimated for 35 days at 3-5°C. The response of adipose tissue as it is conditioned by cold exposure seems, therefore, to depend in part upon the nutritional state of the animal at the time the tissue is removed for studies *in vitro*.

The relation of the time since the last ingestion of food to adipose tissue response has been emphasized in experiments designed to establish optimum conditions for use of the tissue for bio-assay of insulin by Marques *et al*(15). These authors observed a greater response, but a less consistent one, of adipose tissue from animals which had been fasted 24 hours than from those which had been fasted only 5 hours before the effect of insulin on $CO_2$ production was evaluated. Release of FFA by adipose tissue has been shown to be markedly accelerated by exposure of the donor animal to cold for periods up to 40 hours(3). The animals were allowed food during exposure. The authors point out that "the adipose tissue responds almost instantaneously to the increased energy requirement of the organism." The results of present experiments with fed animals support this concept, but when food is withdrawn, either from animals subjected to acute cold stress or from animals acclimated to cold, the observable effects of cold stimulation on the adipose tissue is limited to 20-30 hours after fasting begins. These findings indicate that the adipose tissue may serve as a processing station for ingested foodstuffs in order to present FFA to the organism as a substrate for oxidation. Patkin and Masoro(6) suggested that the increased lipogenesis from acetate observed in adipose tissue of cold-acclimated

rats might serve to furnish substrate for increased tissue oxidation.

Comparison of the *in vitro* effects of norepinephrine on the adipose tissue (Table II) revealed that the tissue from fed, cold-acclimated animals without addition of the hormone released more FFA than did the tissue from warm-room animals even when stimulated by 0.15 $\mu$g of norepinephrine per ml of medium. With addition of norepinephrine the FFA release was 3-4 times greater by tissue from cold-acclimated animal as compared to the warm-room controls. This response occurred in spite of the visible depletion of fat from the adipose tissue of the cold-acclimated animal. Such a response indicates a greater "sensitivity" of the tissue from the cold-acclimated animal. However, the response is more complicated than these results indicate, inasmuch as the difference in response was largely eliminated by a fast of 18 hours before the adipose tissue release of FFA was measured *in vitro*. The state of the metabolic pool as influenced by food ingestion prior to the *in vitro* experiment seems to have an important bearing on the response of the adipose tissue both with and without exogenous hormonal stimulation. Hannon and Larson(2) observed a greater release of FFA from epididymal fat to be induced by cold acclimation which was further stimulated by norepinephrine. Their observations on R.Q. changes also indicated an increased oxidation of FFA induced by cold and by norepinephrine.

Thus, the participation of FFA from adipose tissue and of norepinephrine in nonshivering thermogenesis seems to be an established metabolic response to cold exposure. Increased food intake, measured by Cottle and Carlson(10), plays a major role in meeting the increased caloric requirements of cold acclimation. Several days are required, however, for food ingestion to reach a maximum. During this period, the adipose tissue of the fed animal releases FFA after 24 hours at a rate which is not significantly exceeded after 20 days. This is in contrast to tissue from the fasted, fully acclimated animal which shows no greater release than tissue from the fasted, warm-room control animal. Therefore, the role of the adipose tissue in cold acclimation should probably be considered in relation to its participation in processing ingested foodstuffs.

*Summary.* White rats were exposed to a temperature of 3-5°C for periods varying from 24 hours to 35 days. Adipose tissue from animals so exposed from 1 to 20 days or for 35 days produced 3 to 4 times more FFA than did tissue from animals maintained at room temperature. However, if the animals were fasted before the tissue was removed, these differences virtually disappeared. It is evident that studies of participation of the adipose tissue in the adjustment of the rat to a cold environment should be interpreted relative to the nutritional state of the animal at the time the studies are made.

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