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ADVENTITIOUS FACTORS AFFECTING THE CONCENTRATION OF FREE FATTY ACIDS IN THE PLASMA OF RATS

BY

A. M. BARRETT

From the Research Department, Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

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The concentration of free fatty acids in the plasma of rats has been shown to be affected by the sex and body weight of the animals, their external environment, the availability of food and water, and the time of day and season of the year.

It is now known that under many conditions leading to mobilization of body fat, the fatty acids are released from adipose tissue in the free form and are carried as such into the blood stream in combination with the plasma albumin. The biological half-life of radioactive palmitic and oleic acids has been shown to be about 2.5 min, which indicates a very high rate of turnover of plasma free fatty acids (Laurell, 1957). It is apparent that relatively small changes in the rate of release or utilization of free fatty acids can lead to marked changes in their arterial concentration. The considerable individual variation of plasma free fatty acids observed in rats complicates the quantitative assessment of free fatty acid mobilizing substances in this species. This paper considers some of the factors contributing to the variation and makes suggestions for keeping it to a minimum.

METHODS

Rats, of Wistar origin, were obtained from the colony of specific pathogen-free albinos maintained at Alderley Park. They were fed on a cubed diet *ad libitum* unless otherwise stated, with tap water freely available at all times, and were housed in wire cages ($28 \times 32 \times 15$ cm) at five per cage in one of two animal houses for at least 2 weeks before experiment. One house, maintained at 21° C, also housed rabbits and mice; the other was kept at 27° C and housed rats only. Neither house had controlled lighting but, since little natural light penetrated into the house kept at 27° C, it was usually artificially illuminated from 8 a.m. to 6 p.m.

Arterial blood samples were withdrawn from the abdominal aorta of stunned or anaesthetized rats into a heparinized syringe and the plasma separated within 15 min of collection. The plasma was immediately extracted and the concentration of free fatty acids was determined by the method of Dole (1956). His method was modified in that the thymol blue indicator solution was prepared according to the British Pharmacopoeia (1958) and diluted 1:10 with absolute alcohol when required for use.

Anterior pituitary glands were obtained from freshly decapitated male rats. After being crushed with sand and a glass rod, the glands were extracted with 0.01 N-hydrochloric acid in 0.9% in saline (0.9 ml./gland) for 1 hr. The extract was centrifuged and the supernatant

solution recovered. Dilutions of this solution were prepared so that any desired fraction of a gland could be injected in a volume of 1 ml./100 g of body weight. The extracts were used immediately after preparation and dilution.

RESULTS

Effect of method of blood sampling. Groups of six male rats were bled after a sudden blow on the back of the head or whilst anaesthetized with ether or sodium pentobarbitone. The plasma free fatty acid was determined; the results, summarized in Table 1, demonstrate the lack of any statistical significance between

TABLE 1

EFFECT OF METHOD OF BLOOD SAMPLING UPON PLASMA FREE FATTY ACID (FFA) CONCENTRATION OF MALE RATS

Plasma free fatty acid concentrations are means with standard errors. Pentobarbitone sodium (60 mg/kg) was injected intraperitoneally

Treatment before withdrawal of blood sample	No. of rats	Mean time taken for sampling (sec)	Plasma FFA concentration (μequiv./l.)
Stunning	6	51	369±41
Ether anaesthesia	6	110	371±29
Pentobarbitone	6	238	391±31

any of the means of the three groups. The time taken in obtaining the blood sample had no apparent effect. Difficulty was experienced in obtaining sufficient blood from stunned rats for individual determinations. Since, in contrast to the present findings, it has been reported elsewhere that barbiturates may depress plasma free fatty acid levels whereas ether anaesthesia is without effect (Fodor & Grafnetter, 1960), in all the further experiments blood samples were obtained from rats anaesthetized with ether.

Effect of sex. Consistent differences were found between the concentrations of free fatty acids in the plasmas of fed male and female rats (Table 2). Not only

TABLE 2

EFFECT OF SEX ON PLASMA FREE FATTY ACID (FFA) CONCENTRATION OF RATS

Sex	No. of rats	Mean body weight (g)	Plasma FFA concentration	
			Mean±s.e. (μequiv./l.)	Range (μequiv./l.)
Male	10	167	297±26	174–434
Female	10	151	443±58	244–820

was the level in the females significantly higher than that in the males ($P<0.05$) but the range of observed values was much wider.

Effect of fasting. Groups of male and female rats were deprived of food for 6, 12, 24 and 48 hr starting at 10 a.m. Free access to drinking-water was allowed during the period of the fast. There was a progressive rise in plasma free fatty acids accompanied by a progressive loss in body weight (Table 3). The initial plasma levels of free fatty acids in the males were again significantly lower than those in the females ($P<0.01$) but no significant difference was found between the sexes at any time after the withdrawal of food.

TABLE 3

EFFECT OF FASTING ON PLASMA FREE FATTY ACID (FFA) CONCENTRATION OF RATS

Plasma free fatty acid concentrations are means with standard errors. M, male; F, female

Duration of fast (hr)	Sex	No. of rats	Mean initial body weight (g)	Mean loss in weight during fast (g)	Plasma FFA concentration (μequiv./l.)
0	M	16	167	—	302±16
	F	16	151	—	446±26
6	M	6	158	3·5	677±46
	F	6	144	4·1	657±52
12	M	6	175	8·7	835±80
	F	5	150	9·0	1,134±61
24	M	6	165	10·0	956±96
	F	6	141	10·7	999±45
48	M	5	166	25·0	1,376±116
	F	6	141	22·7	1,368±63

Effect of noise and change in external environment. Substantial increases in plasma free fatty acids were found to result from the noise and disturbance caused by cleaning out the animal rooms before experiments were started or by the transference of animals from animal house to laboratory. Accidental deprivation of water, even in the presence of adequate food, caused a rise in the concentration of the plasma free fatty acid, perhaps because, without water, rats fail to eat.

During the course of a series of experiments designed to detect free fatty acid mobilizing activity in anterior pituitary extracts, it became apparent that steps involved in bleeding one group of animals could affect the free fatty acid level in the plasma of subsequently bled groups. Graded doses of extract were injected intraperitoneally into groups of five rats. A control group received saline. After 6 hr the groups were bled one after the other, by chance the control group being the last. The individual results, plotted in Fig. 1, *a*, indicated that small doses of pituitary extract depressed free fatty acid levels but that as the dose was increased the effect disappeared. The experiment was repeated but this time the control rats were injected and bled first. The results, shown in Fig. 1, *b*, demonstrated an apparently normal dose/response curve in which the greater the dose of extract administered the greater the plasma free fatty acids 6 hr later. The experiment was repeated again when, instead of bleeding the animals in groups, one was bled from each group in turn, the animal being selected at random. There was no significant difference between the mean plasma free fatty acid level of the control group or that of any of the groups injected with extract (Fig. 1, *c*). The conclusion drawn from these experiments is that no fatty acid mobilizing activity could be detected in the pituitary extracts tested under these conditions, but that apparently minimal environmental disturbance could produce misleading results.

Effect of housing conditions. Rats which were housed in the same room as mice and rabbits showed consistently higher and more variable plasma free fatty acid

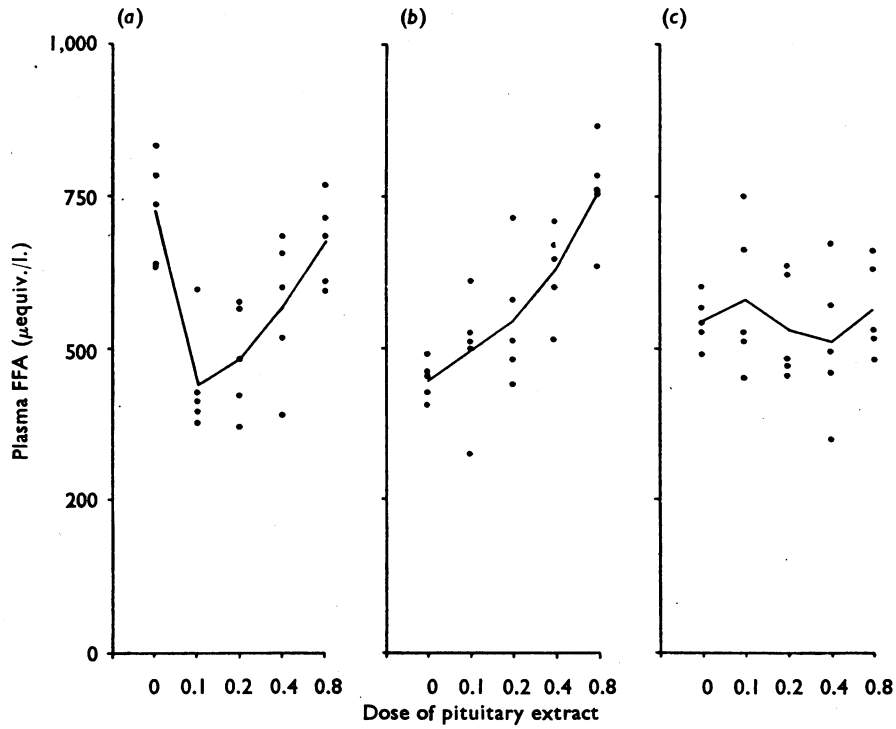

Fig. 1. The effect on male rats of intraperitoneal injection of pituitary extracts (dose expressed in terms equivalent to the fraction of the gland per 100g of body weight) upon plasma free fatty acid (FFA) concentration. Each point represents an individual value for one animal. The solid lines connect the means of the dose-groups. (*a*) Each dose-group bled in sequence: control group bled last. (*b*) Each dose-group bled in sequence: control group bled first. (*c*) One animal bled from each group in sequence but in random order within that group.

levels than animals housed in a "rat only" room. When rats were housed alone either at 21° C or at 27° C no significant effect of the environmental temperature upon plasma free fatty acids was observed. The number of rats per cage affected the levels of plasma free fatty acids. Individual variation was conspicuous in rats housed at twenty per cage compared with a much smaller range when five rats were housed in one cage. With one rat per cage no further reduction in individual variation was seen and it was found that the important factor was the number of drinking water points: most consistent results were obtained when there were not more than five rats per water bottle.

Effect of body weight. During the course of preliminary investigations it was noted that the free fatty acid concentrations in plasma of larger rats (over 200 g) tended to be lower than in smaller rats (less than 150 g). The mean plasma free fatty acid values for 158 rats grouped according to body weight are shown in Table 4. It can be seen that the highest values occurred in the smallest rats and that there was a gradual decrease with increase in body weight. When the individual values were submitted to linear regression analysis a highly significant regression was obtained. The values, however, suggested a levelling off at about 180 g body weight and from

TABLE 4

MEAN PLASMA FREE FATTY ACID (FFA) CONCENTRATIONS OF MALE RATS GROUPED ACCORDING TO BODY WEIGHT

Plasma free fatty acid concentrations are means with standard errors

Group	No. of rats	Weight range (g)	Plasma FFA concentration (μequiv./l.)
1	23	111–130	474±11
2	34	131–150	425±9
3	40	151–170	377±8
4	28	171–190	347±14
5	14	191–210	330±8
6	14	211–230	344±12
7	5	231–250	344±32

details of the analysis of variance for rats above and below 180 g it was evident that there was no significant regression for the former.

Diurnal variation. It is well known that the rat is a nocturnal animal and feeds mainly in the early hours of the morning. In view of the finding that a significant elevation of free fatty acid occurred after a 6 hr fast (see above) it seemed probable that there was a diurnal variation in the free fatty acid concentration of rat plasma. Groups of six rats, allowed free access to food and water and under conditions of minimal disturbance, were bled at various intervals throughout the day. Great care was taken to avoid stimulation of any of the remaining animals at each sampling time. Mean free fatty acid levels for each group are shown in Fig. 2. Between 9.00 a.m. and 12.00 noon there was no variation in the free fatty acid level. However, at 2.00 p.m. the concentration was significantly higher ($P<0.05$) and it continued to rise throughout the day. When the final sample was taken at 9.0 a.m. on the

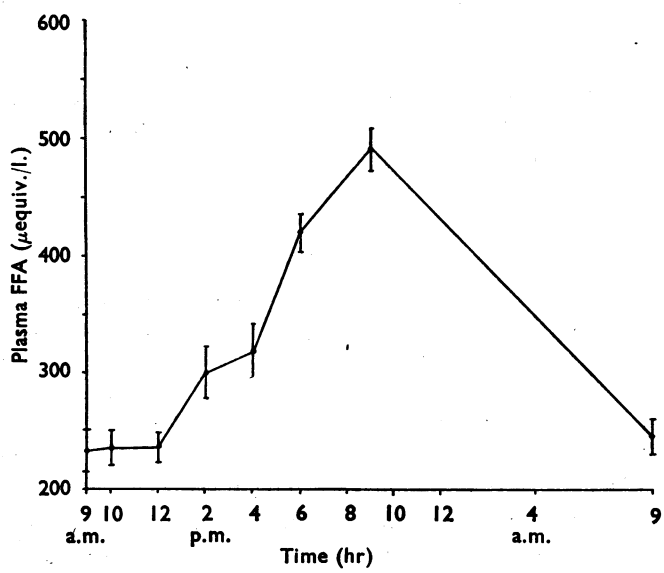

Fig. 2. Diurnal variation in plasma free fatty acids (FFA). Each point represents the mean from a group of six male rats. The vertical lines show the standard errors of the means.

following morning the free fatty acid level was not significantly different from that found for the previous morning.

Seasonal variation. The monthly mean free fatty acid values for all control rats weighing more than 180 g bled during 1962, are shown in Fig. 3. A dicyclic

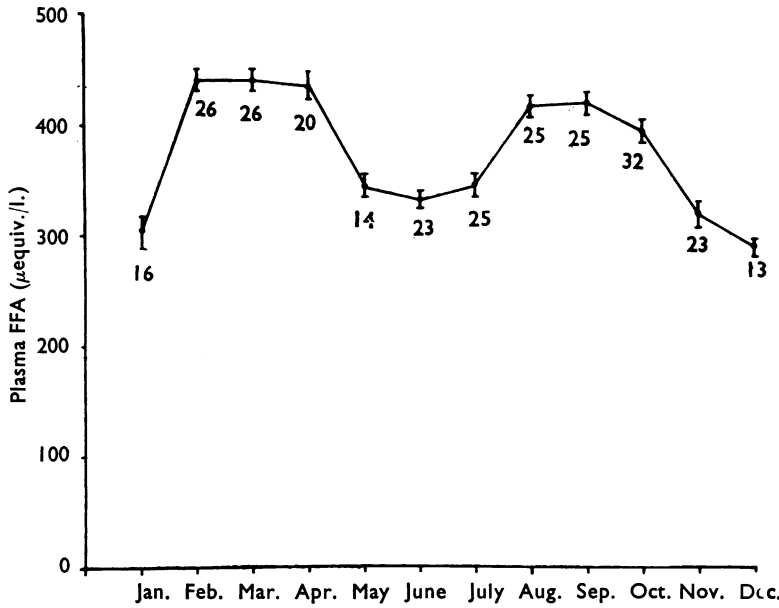

Fig. 3. Seasonal variation in free fatty acid (FFA) concentration in the plasma of male rats. Each point represents the mean value for the number of animals shown immediately below it. The vertical lines indicate the standard errors of the means.

seasonal variation was found with high levels occurring in February/April and August/September. The lowest values occurred in June and December. The peak values were some 30% higher than the low figures, the differences being very highly significant ($P<0.001$).

DISCUSSION

Excessive mobilization of free fatty acids from body fat depots, along with a fall in adrenal ascorbic acid and an increase in plasma corticosterone, is part of the classical response to stress (Westermann, Maickel & Brodie, 1962). The important role of the sympathetic nervous system in the control of free fatty acid liberation is also recognized (Havel & Goldfien, 1959). It is not surprising, therefore, that, as the results described here show, the plasma level of free fatty acids is extremely sensitive to a wide variety of nonspecific stimuli.

Careful control of environmental conditions makes it possible to obtain reproducible resting values. The free fatty acids of the plasma are raised when other species (rabbit, mouse) are kept in the animal house and by noise. Similar findings have been reported for the levels of corticosterone in the plasma of rats

(Barrett & Stockham, 1963). In man, a gradual and sustained rise in plasma free fatty acids has been observed in conscious volunteers during a 3 hr period of saline infusion (Bogdonoff, Weissler & Merritt, 1960). In another study, the basal level of free fatty acids of patients kept in bed overnight and until after blood sampling was 412 μequiv/l. (range 363 to 487) in contrast to a second group who were allowed up for "morning toilet" before sampling when the mean was 640 μequiv/l. (range 271 to 1,182) (Harlan, Laszlo, Bogdonoff & Estes, 1963).

In an attempt to reduce individual variation due to diet the majority of experimental studies concerning plasma free fatty acids in man and dog have been carried out in the fasting state. In the fasting rat, however, the individual variation was considerably greater than that found in the animal allowed food at all times and, for this reason, it would seem preferable to carry out experimental studies in the fed animal, in this species. The observation of a significant increase in the level of plasma free fatty acids only 6 hr after withdrawal of food prompted the inquiry into diurnal variation. In undisturbed quiescent rats the morning plateau is followed by a steep rise in free fatty acids during the afternoon and evening. This increase is almost certainly due to lack of feeding activity during the day for, in contrast, in an animal house in which discontinuous human activity occurred the rats tended to feed for several minutes after each disturbance. In such animals the levels of free fatty acids are higher than the morning values of undisturbed rats and the afternoon rise is obscured. Diurnal variation between 8.00 a.m. and 3.00 p.m. was not observed by Barreto & Recant (1960).

To facilitate comparison of results obtained in different laboratories some knowledge of three other factors is desirable, namely sex of animal, body weight and time of year. The higher concentration of free fatty acids found in the plasma of adult female rats compared with that of adult males confirms the findings of Zucker & Zucker (1962). The response to fasting is similar in both sexes but the individual variation amongst the females is greater. The present results demonstrate an inverse relationship between plasma free fatty acids and body weight up to about 180 g of body weight, at which weight the rats are considered to have attained sexual maturity. In accordance with these findings isolated adipose tissue obtained from immature animals and subsequently incubated *in vitro* releases significantly higher amounts of free fatty acids under control conditions and is more sensitive to fatty acid releasing agents when compared with tissue excised from mature rats (Barrett, unpublished). Other workers have found that the glucose uptake of both adipose tissue and diaphragm muscle obtained from rats weighing less than 180 g was greater than that observed in tissues obtained from rats weighing more than 220 g. Not only was the control glucose uptake increased in tissues from smaller rats but the sensitivity to added insulin was also markedly greater (Beigelman, 1962 ; Martin, Tranquada, Beigelman & Jones, 1962). The dicyclic seasonal variation in plasma free fatty acids corresponds to a similar pattern seen in serum cholesterol levels (Thorp & Waring, 1962 ; Edgren, 1963). Similar seasonal variations in adrenocortical and thyroid function have been considered by Thorp (1963) to reaffirm the interdependence of the animal with its environment and of its metabolism with its endocrine system.

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