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ACID LEVELS IN THE RAT

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It has been suggested that emotional stress is etiologically linked with the development of atherosclerosis and coronary artery disease (Russek, 1959; Wolf, 1958; Friedman and Rosenman, 1959). One mechanism proposed as the basis of this relationship is that stress, by inducing a rise in blood lipid concentrations, promotes a tendency for lipids to deposit in arterial walls. Evidence exists that various forms of stress do increase the levels of certain lipids in the blood. Thus, it has been reported that rats placed on an atherogenic diet, and brought into a state of anxiety periodically, showed higher serum total lipid and cholesterol levels, as well as a greater incidence of coronary artery atherosclerosis, after a period of 10 months, than did non-frightened control animals on the same diet (Uhley and Friedman, 1959). Periods of stress, or driving, pressing, behavioral patterns, have been found to be strongly correlated with high serum cholesterol levels in humans (Friedman and Rosenman, 1959; Hammarsten *et al.*, 1957). Recent investigations have shown that the free fatty acids (FFA) of plasma respond even more quickly than do cholesterol levels to changes in physiological and emotional states. It has been reported, for example, that fear, anxiety, or hostility, induced in human subjects, is rapidly followed by marked increases in plasma FFA levels (Cardon and Gordon, 1959; Bogdonoff *et al.*, 1959a). Trauma induced by femur fracture in the rat has been shown to be followed by significant rises in plasma FFA (Wadström, 1958).

Considerable evidence has now accumulated that these alterations in plasma FFA concentrations occur as a result of altered metabolic responses of the fat depots to varying impulses from the autonomic nervous system, and hence to changes of stimuli from the central nervous system itself (Bogdonoff, 1960; Havel and Goldfien, 1959). If high plasma FFA levels are in

any way related to atherogenesis, it would be of importance to be able to inhibit increases in blood FFA under certain circumstances. It has been shown that it is possible to inhibit the increased output of FFA from the fat depots resulting from increase in autonomic nervous stimulation, or the administration of the neurohumors epinephrine and norepinephrine, by employing autonomic ganglionic or peripheral adrenergic blocking agents. Thus, hexamethonium has been found to lower plasma FFA levels by interfering with the rate of release of FFA into plasma, and dibenzchloroethylamine (Dibenamine) by blocking the epinephrine- and norepinephrine-induced increases of plasma FFA levels in dogs (Havel and Goldfien, 1959). The ganglionic blocking agent trimethaphan camphor sulfonate (Arfonad) has been reported not only to reduce normal plasma FFA values in human subjects, but to reduce the FFA response to stressful experiences as well (Bogdonoff *et al.*, 1959b). The adrenergic agent phentolamine methane sulfonate (Regitine) has also been reported to be effective in inhibiting the plasma FFA rise in humans subjected to an orthostatic stress (Hickler and Hoskins, 1960).

The purposes of the experiments reported here were threefold: a) to determine the effect of an anxiety provoking stress on the plasma FFA levels of a laboratory animal; b) to determine the relationship between plasma FFA levels and duration of stress; and c) to examine the possibility of inhibiting any FFA response to stress at the central nervous system level by reducing the reaction to or perception of stress, by means of tranquilizing agents. Two different types of tranquilizers, differing in certain peripheral effects, were chosen.

METHODS. Female white rats of the Sprague-Dawley strain, 200 to 250 g in weight, were put into plastic cages containing metal grid floors,

and were subjected to electric shocks 1 second in duration at repeated irregular intervals of 1.25, 1.25, 2.5, 2.5, and 2.5 minutes. Each grid was connected to the output of its own variable voltage transformer, the transformers being connected in parallel *via* a common push button to the 110 V AC outlet. In the first series of experiments, the voltage across each cage was adjusted at the start, so that each rat, at the induction of the shock, exhibited a similarly severe response (subjectively determined) as indicated by jumping, squealing, defecation and urination. The voltage required to produce this response varied from 20 to 60, although in the majority of cases 40 V were required. Shocking was continued for periods of from $\frac{1}{2}$ to 7 hours. During these intervals of time neither food nor water was given. Control, non-shocked rats of the same strain, weight and sex were placed in cages in the same room, without food or water for the same intervals of time.

In the second group of experiments, rats were similarly shocked for periods of 4 hours only. Some of the shocked rats as well as non-shocked controls received, 5 minutes or 1 hour prior to the beginning of shocking, subcutaneous injections of 4 mg/kg or 8 mg/kg of chlorpromazine hydrochloride as a 0.4% or a 0.8% solution in saline, respectively, or injections of physiological saline in equal volumes. Other rats were given 200 mg/kg of meprobamate by stomach tube 1 hour prior to the shocking. The meprobamate was administered as a 4% suspension in a 5% solution of gum ghatti. In this series of experiments, the voltage across each grid was arbitrarily kept at 40, since the rats that received the tranquilizing agents reacted far less or not at all to the electric shocks.

At the end of the period of shocking, both control and shocked rats were removed from their cages and anesthetized by the intraperitoneal administration of sodium pentobarbital. Their abdomens were opened, and 5 to 6 ml of blood were withdrawn from the inferior vena cavae below the level of the renal veins and placed into heparinized centrifuge tubes. The cells were then immediately spun down in a refrigerated centrifuge at 0°C. Two 1-ml samples of plasma were taken from each tube for duplicate analyses for free fatty acid concentration by the method of Dole (1956). In a number of instances the more elaborate method of Schotz *et al.* (1959) was also used. Both methods gave similar results.

RESULTS. *Behavior of rats not receiving drugs.* Rats subjected to the electric shocks responded by squealing, jumping, defecating and urinating. After 20 minutes or so they remained immobile between shocks, generally in a corner of the

TABLE 1

Effect of periodic electric shocking on plasma free fatty acid levels of rats

Period of Shocking	No. of Rats		Plasma Free Fatty Acids		Statistical Significance
	Control rats	Shocked rats	Control rats	Shocked rats	
hr			mEq/l		
0.5	5	11	0.36 $\pm$ 0.009*	0.36 $\pm$ 0.009	P > 0.1
2	17	22	0.35 $\pm$ 0.005	0.47 $\pm$ 0.028	P < 0.01
4	9	13	0.36 $\pm$ 0.008	0.55 $\pm$ 0.046	P < 0.01
7	6	9	0.34 $\pm$ 0.006	0.78 $\pm$ 0.072	P < 0.01

* Results given as mean $\pm$ standard error of the mean.

cage, with arched backs, and often with bristling hair and tails raised. Sometimes they remained standing rigidly on the hind legs with the front paws against the cage wall during the interval between shocks, and it appeared that in this position the electric stimuli caused less discomfort. Altogether, these rats gave the appearance of being frightened, uncertain and apprehensive. In contrast, control rats continued to move about freely and easily in their cages.

Effect of shocking on plasma free fatty acid levels. The results of these experiments are given in table 1. After $\frac{1}{2}$ hour of shocking, plasma FFA levels of shocked rats and non-shocked controls were similar. However, as the shocking was continued, the plasma FFA levels of the shocked rats became significantly higher than those of the controls, the longer the shocking, the higher the levels. Increases of FFA concentrations over control values after 2, 4 and 7 hours were 32, 51 and 126% respectively.

A number of experiments were performed with rats that had been fasted overnight prior to being subjected to the electric shock procedure. Both control and shocked rats had higher FFA levels than did the corresponding non-fasted rats, owing to the effect of fasting, but again values for the fasted experimental rats were significantly higher than those of the fasted controls.

Effect of tranquilizers on behavioral response to shock procedure. Animals receiving 4 mg/kg chlorpromazine reacted much more weakly to the electric shocks in terms of jumping, squealing, frequency of urination and defecation than did those animals not receiving tranquilizers. Those rats receiving 8 mg/kg of this drug reacted even less, some not at all, especially during the last 2

TABLE 2

Effect of chlorpromazine and meprobamate on plasma free fatty acid levels of electrically shocked rats

Group No.	Drug Administered	Experimental Condition	No. of Rats	Plasma Free Fatty Acids (mEq/l)	Statistical Significance P Value Comparison with	
					Group 2	Group 7
1	None	Shocked 4 hr	13*	0.55 $\pm$ 0.046†	>0.1	<0.01
2	None	Shocked 4 hr	11	0.50 $\pm$ 0.013		<0.01
3	Chlorpromazine, 4 mg/kg	Shocked 4 hr	9	0.43 $\pm$ 0.035	=0.07	=0.08
4	Chlorpromazine, 8 mg/kg	Shocked 4 hr	12	0.41 $\pm$ 0.023	<0.01	=0.06
5	Chlorpromazine, 8 mg/kg‡	Shocked 4 hr	9	0.38 $\pm$ 0.013	<0.01	>0.10
6	Meprobamate, 200 mg/kg‡	Shocked 4 hr	15	0.36 $\pm$ 0.008	<0.01	>0.10
7	None	Not shocked	9	0.36 $\pm$ 0.008		
8	Physiological saline	Not shocked	7	0.33 $\pm$ 0.008		
9	Chlorpromazine, 4 mg/kg	Not shocked	7	0.35 $\pm$ 0.015		
10	Chlorpromazine, 8 mg/kg	Not shocked	8	0.34 $\pm$ 0.010		
11	Chlorpromazine, 8 mg/kg‡	Not shocked	8	0.33 $\pm$ 0.016		
12	Meprobamate, 200 mg/kg‡	Not shocked	13	0.35 $\pm$ 0.010		

Chlorpromazine and saline were administered subcutaneously; meprobamate was administered orally.

* In this group, voltage was adjusted for each rat so as to obtain severe response to shocks; in all other cases 40 V were applied across grid.

† Results are expressed as mean $\pm$ standard error of the mean.

‡ In these groups drug was administered 1 hour before shocking was begun.

of the 4 hours of the experimental procedure. These animals appeared relaxed and sleepy, but were not unconscious or anesthetized, and could be awakened readily by being touched. When 8 mg/kg of chlorpromazine were administered 1 hour before shocking was begun, the animals appeared sleepy and relaxed throughout the 4-hour period of shocking, and again reacted only slightly or not at all to the electric shocks. Defecation and urination rarely occurred. It has been reported that the inhibitory effect exerted by the phenothiazines on emotional defecation may be regarded as a manifestation of a central action relieving the state of tension and anxiety (Taeschler *et al.*, 1960). The same reduction in reaction to shocking, and a similar apparent relaxation of tension between shocks occurred in rats receiving meprobamate. In general one could say that while the tranquilized animals felt the shocks, they reacted much less to these unpleasant experiences than did control rats, and did not appear rigid, anxious and apprehensive during the longer periods between shocks.

Effect of tranquilizers on plasma FFA responses to electric shock stress. The results of these experiments are given in table 2. Three groups of non-stressed controls were employed: a) rats

receiving no drug; b) rats receiving physiological saline; and c) rats receiving a tranquilizer. The controls receiving either tranquilizer or saline manifested the same normal FFA levels as did the untreated controls. Thus the drug did not affect plasma FFA concentrations under normal conditions.

The rats receiving no drug, but shocked for 4 hours, with the potential across each cage at 40 V, showed the same significant rise in plasma FFA as did the animals shocked for 4 hours in the previous experiments that were exposed to a subjectively determined "maximal" stress. The rats given chlorpromazine in a dose of 4 mg/kg 5 minutes before stressing was begun, showed lower plasma FFA levels after the 4-hour period than did the shocked rats receiving no drugs, although the levels in the former animals were still significantly higher than those of non-stressed controls. When the dose of this drug was increased to 8 mg/kg, plasma FFA levels of the shocked rats were further reduced after the period of stress, being significantly lower than those of shocked rats receiving no drug. Finally, when the same dose of chlorpromazine was administered 1 hour prior to the beginning of the stressful procedure, no increase in plasma FFA levels above those of

non-stressed untreated controls occurred at all. When meprobamate was administered 1 hour prior to shocking, there was again no rise in plasma FFA levels after 4 hours of shocking, above those of non-shocked controls.

DISCUSSION. It was observed that irregular, periodic electric shocking of rats not only produced strong reactions to the disagreeable stimuli themselves, at the moment of shock, but also induced behavioral characteristics resembling fright, anxiety and apprehension during the longer intervals between the shocks, in untreated animals. The entire stressful procedure was accompanied by a rise in plasma FFA, and the longer the stress was continued, the higher was the ultimate level of plasma FFA. Since there was no rise in plasma FFA during the first half-hour of shocking, but only after the animals had become rigid and tense in appearance, it is possible that the rise in FFA might not have occurred as a result of the pain caused by the stimuli *per se*, but as a result of the tension and apprehension engendered by the recurrence of the stimuli after irregular and unexpected intervals of time. Within any group of animals (receiving drugs or controls) there was a fairly wide variation in intensity of response to the individual shocks, but no correlation was found between the intensity of the response and the final FFA value. This would be understandable if the tension generated by the irregular shocking rather than the responses at the moments of shock were related to the FFA increases. It is possible, however, that the apparent lack of even a small rise of plasma FFA during the first half-hour of stimulation of rats receiving no drugs may be due to a) a suppressing effect on FFA output of a hyperglycemia induced by the release of epinephrine from the adrenal medulla, or b) an increased utilization of released FFA as a result of the physical activity of the rats (Carlson and Pernow, 1959).

The possibility was considered that excess lactic acid, formed as a result of the jumping and movement in response to each shock, particularly among the rats not receiving tranquilizers, might be included in our FFA values. The results obtained with the Dole method were therefore compared with those obtained with the analytical method of Schotz *et al.* (1959) on the same samples of plasma. The latter method includes extra washing procedures for the elimination of lactic acid from the determination. The results

were the same with either method. In addition, it has been reported that exercise results in decreased rather than increased plasma FFA concentrations (Carlson and Pernow, 1959).

The results obtained with the tranquilized animals indicate that these drugs may, depending on the dose and time of administration, reduce or completely inhibit the FFA response as well as the behavioral response to stressful situations. Presumably, the effects are mediated *via* the central nervous system. Since chlorpromazine also possesses some peripheral anti-adrenergic effects, meprobamate was also used. The effectiveness of the latter suggests that the tranquilizers do not inhibit the FFA response to stress by peripheral adrenolytic actions only. Meprobamate also possesses muscle relaxant properties which chlorpromazine does not. Part of the relaxation observed in meprobamate-treated shocked rats was undoubtedly due to these properties. However, the inhibitory effect on FFA rise was probably not due to this muscle effect, since chlorpromazine affected the FFA rise in the same manner.

The doses of chlorpromazine and meprobamate used were several times as high as the therapeutic doses of these drugs generally administered to humans. However, our aim was to produce a maximum effect on FFA, and similar doses of these tranquilizers have been used by other investigators to study various other effects of these drugs in laboratory animals (Hunt, 1957; Berger *et al.*, 1957). Furthermore, it was found in preliminary experiments in our laboratory, that doses of chlorpromazine as high as those used here were necessary to suppress the conditioned avoidance response in rats for 4 hours or more. It would be of interest to determine whether the usual therapeutic doses produce a similar inhibition of the plasma FFA response to stress in human subjects.

SUMMARY

Female albino rats were subjected to an anxiety provoking stress induced by irregular unavoidable electric shocking for periods of $\frac{1}{2}$ to 7 hours. Plasma free fatty acid (FFA) concentrations rose to levels significantly above normal, the longer the period of stress, the greater being the rise. Rats receiving the tranquilizer chlorpromazine or meprobamate, and subjected to the stress for 4 hours, manifested either smaller or no rises in plasma FFA, depending upon the dose and time

of drug administration, in contrast to control animals not receiving a drug. It is concluded that it is possible to inhibit the FFA response to stress by affecting the central nervous system with tranquilizing drugs as well as by inhibiting peripheral autonomic stimulation of fatty depots.

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