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Morphological Changes Induced in Rats Following Prolonged Exposure to Stress[1]

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BASSETT, J. R. AND K. D. CAIRNCROSS. *Morphological changes induced in rats following prolonged exposure to stress.* PHARMAC. BIOCHEM. BEHAV. 3(3) 411–420, 1975. – Prolonged exposure of male C. S. F. rats to irregular signalled footshock from which they could escape for up to 71 days produced profound morphological changes. Retardation in growth, adrenal hypertrophy associated with an increase in the zona fasciculata and zona reticularis, and changes in the microcirculation of the heart were observed. There was a significant degree of congestion and dilatation of the microcirculation which was most marked in large venules, collecting venules and veins. An increase in PAS +ve material marginated in the venous endothelium was observed also, together with a suggested increase in mast cells and presence of vacuoles in the intima-media of the coronary arterioles. No pathological changes were observed in the renal cortex and medulla or the gastric lining. The changes in the microcirculation of the heart are discussed in terms of an oedematous reaction and a histamine type leakage of the endothelial lining.

Prolonged stress Weight gain Adrenal hypertrophy Myocardial changes Oedematous reaction Endothelial leakage Corticosteroid response

EMOTIONAL, environmental and sensory stress have been implicated in the etiology of many degenerative disease processes and especially in the pathogenesis of cardiovascular disease. Gastric ulceration [18, 30, 32, 39, 48, 49, 50], hypertension [7, 16, 41, 45], arterio - and atherosclerosis [37, 43, 44, 46], thrombosis [13, 17, 43], and myocardial ischaemia, necrosis and fibrosis [8, 26, 33, 34, 35] have all been linked with stress in which the psychological parameters of anxiety or fear play a major role.

Bassett *et al.* [2] proposed that irregular signalled footshock, with the possibility of escape, was a stressor having a large psychological component which produced an extreme plasma corticosteroid elevation. In view of the fact that glucocorticoids are strongly implicated in stress induced pathogenesis of the cardiovascular system [33,34], prolonged exposure to such a stressor could be expected to induce pathological changes. To examine such a premise, animals were subjected to irregular-signalled escape stress for prolonged periods, at the end of which time the cardiovascular system, stomach and kidney were examined for gross morphological and histological changes.

Decreased gain in body weight, adrenal hypertrophy and cardiac hypertrophy have been reported frequently to occur in association with many of the experimentally induced pathogenic states indicated [1, 23, 30, 31, 32, 48]. Retardation in growth and adrenal hypertrophy are also regarded as good physiological indices of stress [5, 6, 9, 12, 30]. In the previous study Bassett *et al.* [2] utilized only one physiological measure of stress, namely elevation of plasma corticosterone. In the present study changes in body, adrenal and cardiac weight, as well as plasma corticosteroid elevation were examined.

METHOD

Animals

Male CSF rats 87–93 days old were used in all experiments. The animals were housed in groups of 3 under conditions of constant temperature and humidity (21 ± 0.5°C, 46% humidity) and subjected to a 12 hr night–day regimen (light 8 a.m. – 8 p.m.) beginning at least 14 days prior to commencement of experimentation and continuing until its conclusion. Food (Fidelity Feeds, Murrumburrah, N.S.W.) and water were provided ad lib. Both control and stressed rats were housed under identical conditions.

Apparatus

The stress procedure was the same as that described in detail by Bassett *et al.* [2]. Animals were placed in auto-

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mated 1-way avoidance boxes (Lafayette Model No. 85200). An escape platform was made available to the animal by an automated movable partition. A light conditioned stimulus (CS) of 2 W was located on the wall of the grid chamber opposite to the escape platform. The unconditioned stimulus (UCS) was delivered by a generator-scrambler through the grids as a 2 mA, 50 pulses/sec square wave. Each rat was placed on the escape platform at the commencement of the treatment session. On each trial the CS onset 4 sec before the animal was pushed by the movable partition from the platform onto the grid which was simultaneous with the onset of the UCS. At this point in time the movable partition immediately retracted and the animal was able to jump from the grid to the re-exposed platform with a minimum latency of 0.3 sec. The UCS was terminated by the return of the animal to the platform. The time taken for the animal to return was the escape latency. The total escape latency for a stress session was recorded and used as a measure of escape performance.

Procedure

Seventy-one day stress. The stress procedure was that described by Bassett *et al.* [2]. The stress treatment consisted of 7CS-UCS exposures randomly placed in a 35 min stress session. Each stress session was repeated once daily for an arbitrary period of 71 days. Stress treatments were carried out between the hours 9 a.m. – 12 noon. The stress and control groups each consisted of 9 animals.

Twenty-five day stress. The stress procedure consisted of 14CS-UCS exposures randomly placed in a 60 min stress session. Each stress session was repeated twice daily (once in the morning and once in the afternoon), for an arbitrary period of 25 days. The stress and control groups each consisted of 6 animals.

In both the 71 day and 25 day stressed groups experimental animals were killed immediately following the completion of the last morning stress period by cervical dislocation and exsanguinated. The blood was collected in heparinized tubes and centrifuged in order to obtain cell free plasma which was then frozen. Corticosterone levels in plasma were determined subsequently by the fluorimetric method of Mattingley [25] which is specific for free 11-hydroxy-corticosteroids. The heart, both kidneys and both adrenals were removed for histological examination. The heart and adrenal glands were weighed before fixation. The stomach was removed and the gastric lining examined for gross morphological lesions.

Changes in body weight. Animals were weighed for 2 days prior to commencement of the experiment and throughout the experimental period at daily intervals before stressing. The average weight gain for individual animals over the 2 day period before the commencement of the experiment, and over 3 day intervals after the commencement of experiment were pooled for both stressed and control animals. The means (± S.E.) were plotted against time.

Escape performance. Escape performance was measured only in the 71 day stress group. The average daily total escape latencies for individual animals over 3 day periods were pooled, and the mean total escape latency (± S.E.) plotted against time.

Histology. Specimens were fixed in 10% formal saline for 7 days then paraffin-embedded. Serial sections (7 μ thick) were cut through the heart, adrenal glands and kidney. In the case of the heart, sections were cut transversely commencing at the apex. Adrenal and kidney sections were stained with haematoxylin (Harris) and eosin, (H & E). Heart sections were stained either with H & E, or were diastase treated to remove glycogen and stained with periodic acid leucofuchin and counterstained with Weigert's iron haematoxylin, (PAS); alternating 6 sections of each stain.

RESULTS

Changes in Body Weight

The effect of the 71 day stress procedure on body weight is shown in Fig. 1. In both the control and stressed groups body weight increased with the maturity of the animal. The critical values for a significant difference between any two means in the control group, using the Tukey test, were 33.4 at the 5 percent level and 38.0 at the 1 percent level of confidence (MS_{error} = 339.4, df = 168). For the stressed group the critical values were 32.0 at the 5 percent level and 36.3 at the 1 percent level (MS_{error} = 310.6, df = 168). While there was no significant difference in body weight or weight gain between the control and stressed animals at the start of the experimental period (t test, $p>0.8$ in both cases), the onset of stress resulted in a marked reduction in the subsequent rate of weight gain. This retardation became statistically significant ($p<0.05$) in the period from 9 to 12 days and remained significant throughout the remaining experimental period.

The effect of the 25 day stress procedure is shown in Fig. 2. Whereas the control group continuously increased body weight over the duration of the experimental period, the stressed animals initially lost weight, gradually recovering by Day 15–18 and then gained weight. The critical values for a significant difference between means in the control group (Tukey test) were 11.5 at the 5 percent level and 13.8 at the 1 percent level of confidence (MS_{error} = 39.1, df = 40). For the stress group the critical values were 15.3 at the 5 percent level and 18.3 at the 1 percent level (MS_{error} = 69.2, df = 40). Body weight and weight gain were not significantly different between the two groups at the start of experiment (t test, $p>0.8$ wt. gain, $p>0.6$ body wt). However following the commencement of the stress procedure there were significant differences in the average weight gain between the control and stressed groups ($p<0.001$).

Escape Performance

Variation in total escape latency over the period of experimentation (71 day stress) is shown in Fig. 3. There was a significant improvement in performance (represented by a fall in total escape latency) over the first 3 days of stress but performance then plateaued to a constant level and no further significant alteration was observed. The critical values for a significant difference between any two means (Tukey test) were 5.2 at the 5 percent and 5.8 at the 1 percent level of confidence (MS_{error} = 8.12, df = 168).

Plasma Corticosteroid Response

The mean plasma 11-hydroxy-corticosteroid levels (in μg/100 ml plasma ± S.E.) for the 71 day experiment were 34.5 ± 3.1 for the stressed animals and 13.4 ± 1.8 for the control group. This difference was found to be significant

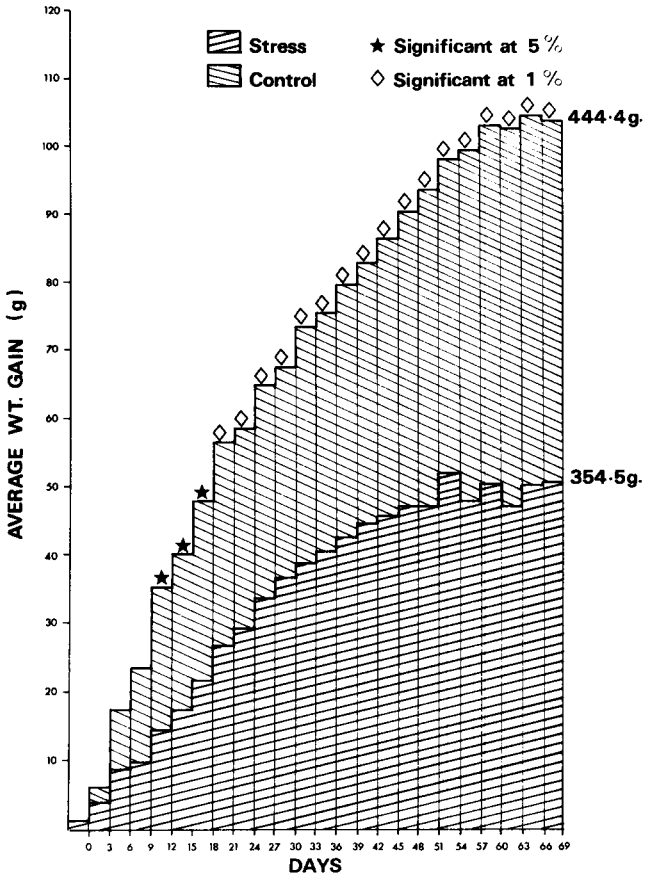

FIG. 1. Effect of 71 day stress procedure on body weight. Mean body weight for both control and stressed group at completion of the experimental period is shown on the right hand side of the histogram.

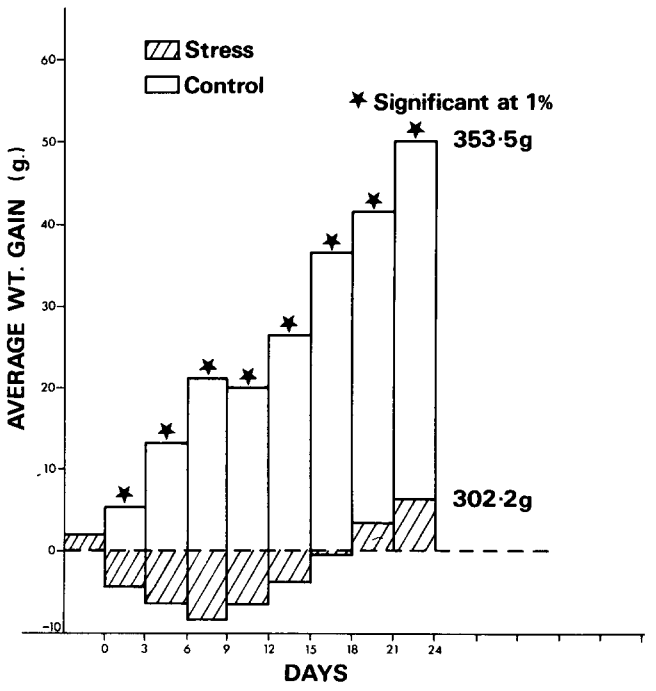

FIG. 2. Effect of 25 day stress procedure on body weight. Mean body weight for both the control and stressed group at completion of the experimental period is shown on the right hand side of the histogram.

using a t test ($p<0.001$). In the case of the 25 day experiment the mean plasma steroid levels were found to be 40.7 ± 2.8 for the stressed animals and 14.3 ± 3.6 for the control group. The difference was significant ($p<0.001$). A t test comparing the plasma steroid levels obtained in both the stressed groups (71 day and 25 day) showed no significant difference between the two steroid responses ($p>0.10$).

Changes in Adrenal Gland

Total adrenal weight was measured and expressed as both actual weight and weight relative to body weight (adrenal weight in mg/100g body weight). The effect of prolonged stress on the adrenal weight is shown in Table 1. No significant increase in the actual adrenal weight occurred in the 25 day stressed animals when compared with their control group, however, adrenal weight/body weight was significantly increased. In the 71 day stressed animals there was a significant increase in both actual adrenal weight and adrenal weight/body weight compared to the corresponding control group.

In order to demonstrate which area of the adrenal gland was responsible for the observed hypertrophy, the width of the adrenal cortex was measured together with the width of its three composite zones, zona glomerulosa, zona fasciculata and zona reticularis. Measurements were made in mm from histological sections taken through the centre of the gland. As far as was feasible sections were cut with the same orientation and measurements made from identical regions. The results from such a study are shown in Table 2. Since there was no significant variation in either the actual adrenal weights between the 25 day and 71 day control groups (see Table 1) or the width of the cortex or its zones, the results from both control groups were pooled in Table 2.

It can be seen from Table 2 that in the 25 day stressed animals no significant change occurred either in the width of the cortex or in the proportion of the 3 cortical zones. In the 71 day stress animals however, there was a significant increase in the diameter of the total cortex, zona fasciculata and zona reticularis, while the zona glomerulosa remained unchanged.

Cardiovascular Changes

The effect of prolonged stress on cardiac weight is shown in Table 3. No significant change in actual cardiac weight occurred in either the 25 or 71 day stressed groups when compared with their corresponding controls. However, a measure of cardiac weight relative to body weight (cardiac weight in mg/100g body weight) showed the hearts of the 71 day stressed group to be significantly larger than their control group. No such significant change was observed with the 25 day stressed animals.

Histological examinations of hearts taken from 71 day stressed animals showed a number of pathological changes. There was a significant degree of congestion and dilatation

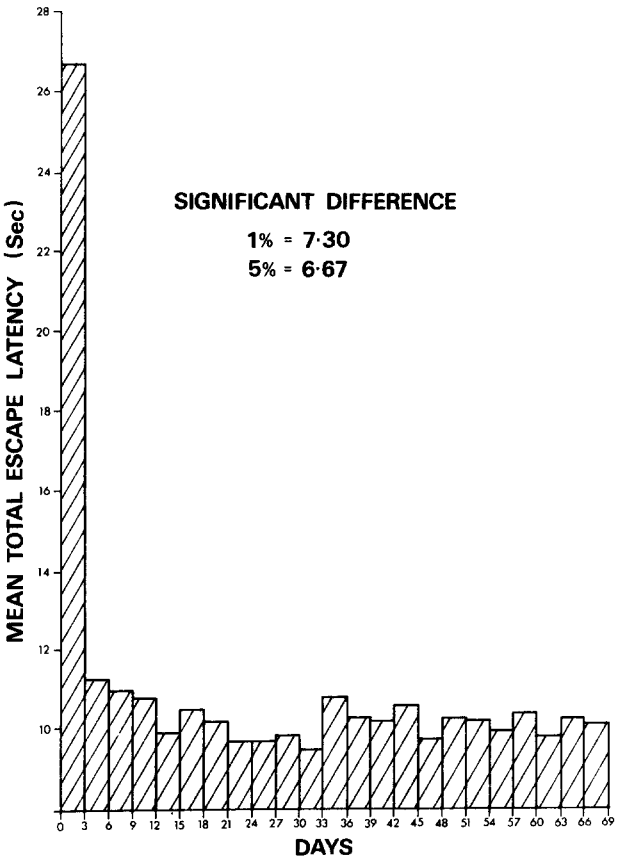

FIG. 3. Variation in escape performance over a 71 day stress period. Escape performance is measured as total escape latency (in seconds). Each lustogram represents the mean of the total escape latencies for individual animals averaged over 3 day periods.

of the microcirculation, which was most marked in large venules, collecting venules and veins (Fig. 4a,b). This venous dilatation, present in all 71 day stressed hearts, was observed in the apex and walls of both left and right ventricles, being most pronounced in the thicker left ventricular wall. The septum was the zone least involved in the venous dilatation. No such venous dilatation was observed in the hearts from any of the control animals. An increase in PAS +ve material marginated in the venous endothelium was observed, especially in the dilated vessels (Fig. 4a). This may represent sludged protein and platelet aggregation with or without fibrin. The absence of inflammatory infiltration, and negative results with Acid Fuchsin stains in areas of the myocardium other than those concerned with the coronary vascular system, excluded any suggestion of myocardial necrosis.

Some coronary arterioles in the 71 day stressed animals showed mild oedema and the presence of vacuoles in the intima-media (Fig. 5a,b). While all stressed hearts showed the presence of vacuoles in arterioles, not all arterioles within a heart demonstrated such an effect, i.e. vacuoles occurred in 42–60 percent of the arterioles examined in individual hearts. When present the number of vacuoles varied from one up to 8 visible in a transverse section of the arteriole. Vacuoles were not observed to be present in the arterioles of any of the control hearts.

Mast cells, commonly seen singly in the perivascular sheaths of arterioles but not elsewhere in the control hearts were found in the stressed animals occurring in groups of 2 or more and associated with capillaries as well as perivascular sheaths of large vessels (Figs. 5b and 6). On the basis of these observations it would appear that the number of mast cells in the cardiac tissue increased in the 71 day stressed animals, although no actual counts were performed.

TABLE 1

THE EFFECT OF PROLONGED STRESS ON ADRENAL GLAND WEIGHT

Experiment	Mean Adrenal Weight mg ± S.E.	Adrenal Weight/Body Weight mg/100g ± S.E.
25 DAY		
Control	49.3 ± 1.6	14.0 ± 0.7
Stressed	50.4 ± 0.7	16.7 ± 0.5
t test ($p<$)*	>0.50	0.01
71 DAY		
Control	49.2 ± 1.5	10.9 ± 0.4
Stressed	56.6 ± 1.5	16.0 ± 0.5
t test ($p<$)*	0.005	0.001

*$p<0.05$ is significant

TABLE 2

THE EFFECT OF PROLONGED STRESS ON ZONES OF THE ADRENAL CORTEX

Zone	Mean Width (mm) ± S.E.				
	Control	25 Day	p*	71 Day	p*
Total Cortex	0.75 ± 0.03	0.80 ± 0.03	>0.3	0.95 ± 0.05	<0.01
Glomerulosa	0.078 ± 0.003	0.074 ± 0.003	>0.3	0.080 ± 0.003	>0.6
Fasciculata	0.45 ± 0.02	0.50 ± 0.02	>0.05	0.57 ± 0.03	<0.01
Reticularis	0.23 ± 0.01	0.22 ± 0.01	>0.8	0.30 ± 0.03	<0.02

*t test, pooled control V's 25 or 71 day stressed group; $p<0.05$ is significant

TABLE 3

THE EFFECT OF PROLONGED STRESS ON CARDIAC WEIGHT

Experiment	Mean Cardiac Weight mg ± S.E.	Cardiac Weight/Body Weight mg/100g ± S.E.
25 DAY		
Control	1139.3 ± 36.9	323.9 ± 13.8
Stressed	1042.2 ± 42.5	345.2 ± 13.6
t test (p)*	>0.20	>0.30
71 DAY		
Control	1218.7 ± 33.3	269.7 ± 4.9
Stressed	1121.6 ± 32.7	316.9 ± 9.1
t test (p)*	>0.05	<0.001

*$p<0.05$ is significant

The histological differences between 25 day stressed animals and controls were not nearly as positive as those reported above. While the hearts from 25 day stressed animals tended to show some venular-venous dilatation such a phenomenon was only marginally present. Vacuoles were only occasionally observed in arterioles and there was no suggestion of an increase in the number of mast cells.

Changes in Stomach and Kidney

In both the 71 and 25 day stressed groups histological examination of both the renal cortex and medulla showed no apparent pathological changes compared with controls. Gross observation of the lining of the stomach showed no discernible lesions.

DISCUSSION

Both retardation in growth and adrenal hypertrophy are generally regarded as good physiological indices of stress, and in this study both reduced weight gain and adrenal hypertrophy were observed. However, it is apparent that changes in a single parameter cannot be assumed to reflect the severity of a stressor on the organism as a whole. In the case of retardation of body weight gain, the more intensive stress involved in the 25 day stressed group produced more marked effects than those obtained with the less intensive 71 day stress. If adrenal hypertrophy is the parameter measured the reverse occurs. The 71 day stress regime produced a significant adrenal hypertrophy, whereas with the 25 day stress procedure the effect was marginal.

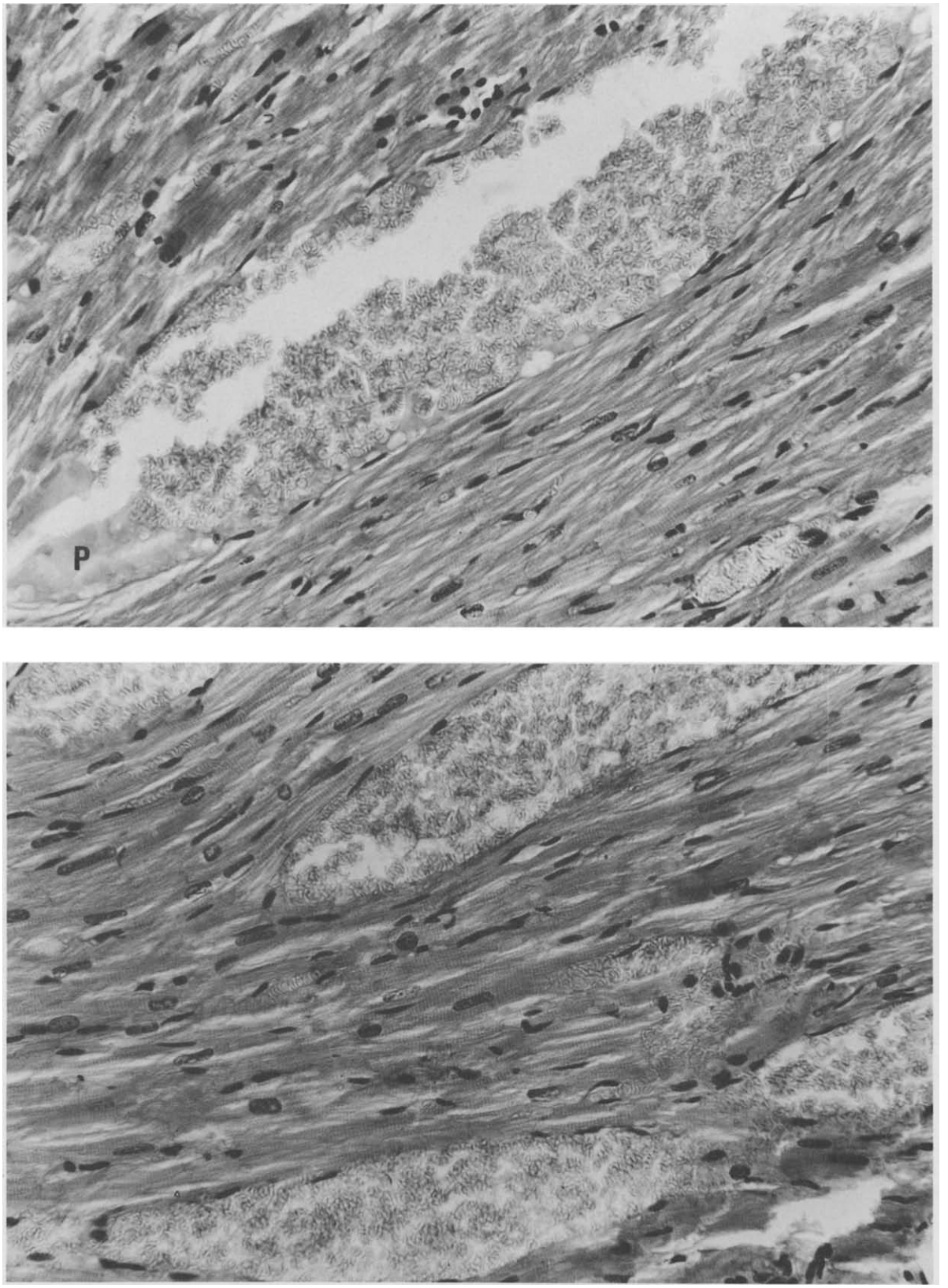

FIG. 4a and 4b. Extensive dilatation of venous radicles in hearts from 71 day stress animals. H & E (×80). P = deposition of material that will stain PAS +ve marginating the endothelium of venule.

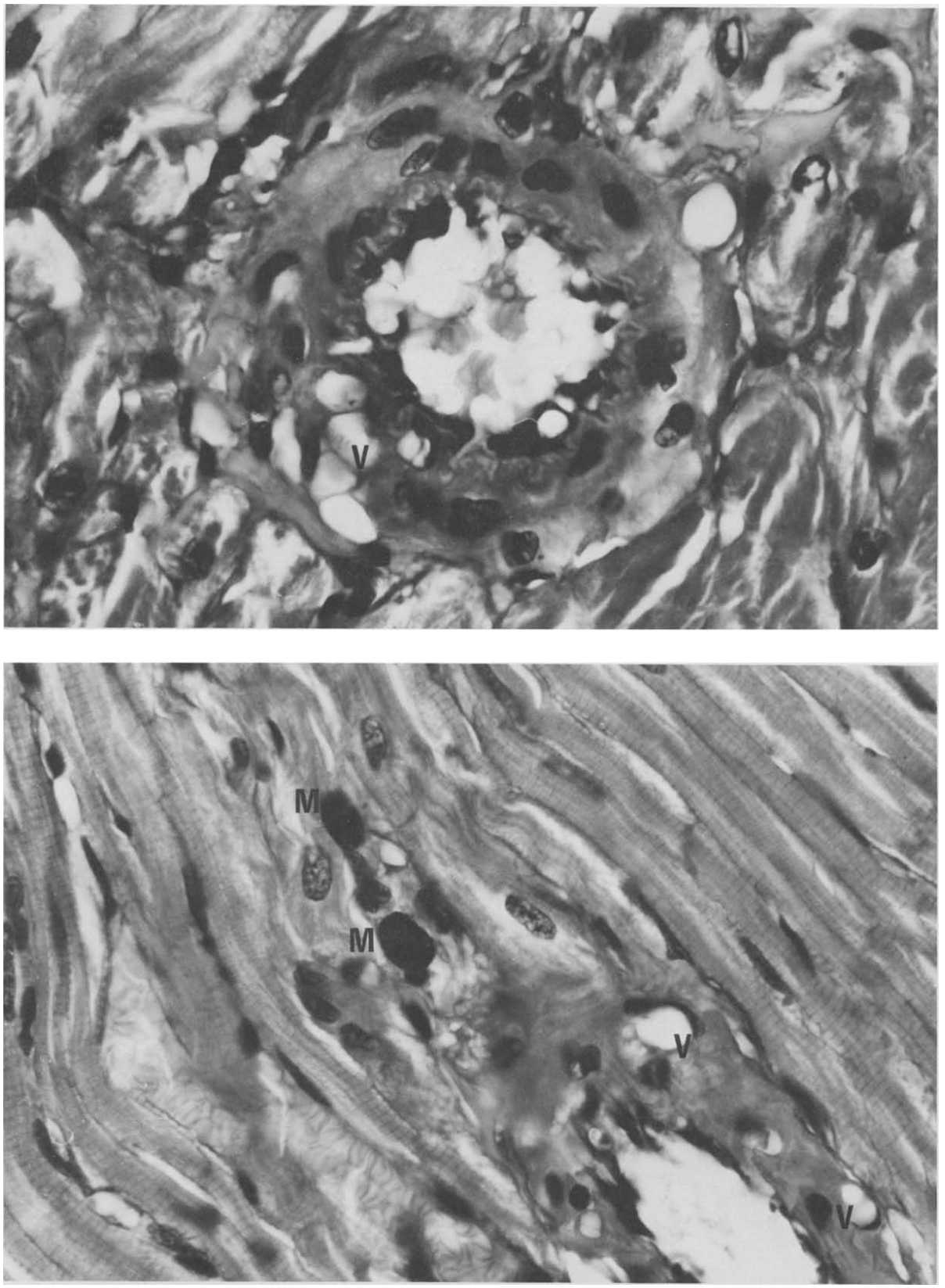

FIG. 5a and 5b. Coronary arterioles in hearts from 71 day stressed animals showing extensive vacuolization in the intima-media. H & E (×160). M = mast cells in perivascular sheath. V = vacuoles.

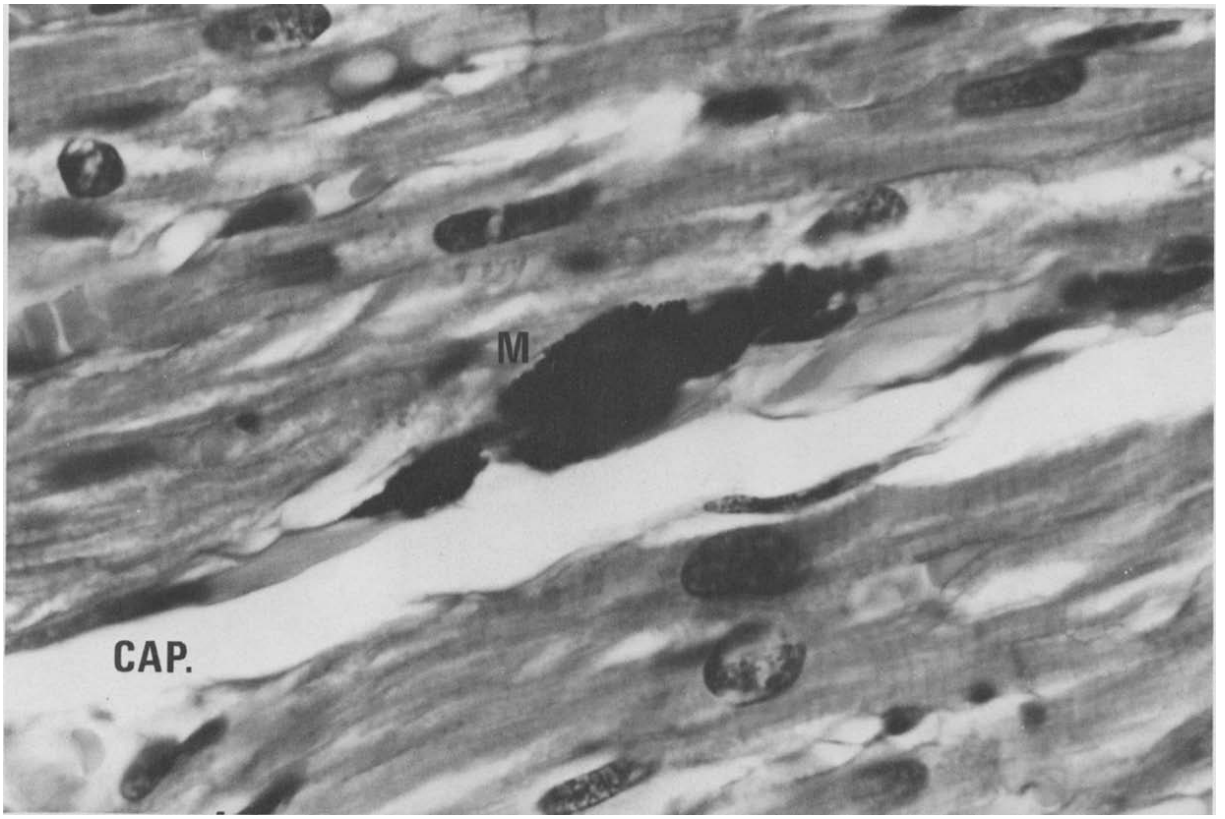

FIG. 6. Group of 5 mast cells associated with a small capillary in the left ventricular wall of 71 day stressed rat. H & E (×320).

In stress situations the increased levels of plasma catecholamines and glucocorticoids play an important role in mobilization and utilization of the body's energy stores. Accelerated protein catabolism which appears to characterise the response to many stressors whether psychological or physical, requires the presence of corticosteroid hormones [36]. Such responses would contribute to the general reduction in the rate of growth of young rats. Superimposed upon the catecholamine-glucocorticoid interaction in enhanced metabolism is the involvement of the thyroid gland. There is general agreement from animal experiments that the thyroid responds significantly to psychological stimuli, but the direction of the response is disputed. However, in view of the strong clinical association between emotional crises and onset of hyperthyroidism, it would seem that thyroid hormone levels increase in response to emotional stimuli, although the increases are often small in terms of percentage change [24]. Thyroxine increases the oxygen consumption of almost all metabolically active tissues and if food intake is not increased, endogenous protein and fat stores are catabolized and weight is lost [11]. The actions of thyroxine and the catecholamines are intimately interrelated. Thyroxine appears to potentiate the effects of the catecholamines and vice versa, but the basis of this interaction is not understood [11]. The decreased weight gain observed in this study can be explained on the basis of the metabolic disturbances induced by the elevated levels of the circulating hormones. In the absence of adaptation such metabolic changes should be most apparent in situations where the stressor is more prolonged or more frequent. This was the case with the 25 day stress procedure, a procedure involving hourly stress periods twice daily, where initially a fall in body weight was observed.

Adrenal hypertrophy in the form of a significant increase in actual adrenal weight, as well as a significant increase in adrenal weight relative to body weight, was only observed in the 71 day stressed animals. Corresponding to the increase in adrenal weight there was hypertrophy of the adrenal cortex mainly due to an increase in the zona fasciculata, the zone responsible for secretion of glucocorticoids, and to a lesser extent the zona reticularis. No significant increase in actual adrenal weight was found with the 25 day stressed animals. Only when adrenal weight was measured relative to body weight was a significant difference seen with this group. This significant difference, however, may not reflect hypertrophy of the gland but simply the dramatic decrease in body weight produced as the result of the metabolic changes discussed above. The fact that no significant alteration occurred in the width of the adrenal cortex or the proportions of its composite zones supports this hypothesis.

Adaptation of the pituitary-adrenal cortical axis, with a regression of the adrenal gland to its normal size and activity despite the continuing stress, is reported to occur following exposure to chronic stressors [36]. While no such

adaptation was apparent in the 71 day stressed animals with regard to adrenal size, adaptation in the form of a reduced 11-hydroxycorticosteroid response was observed with both the 71 day and 25 day stressed animals. Irregular-signalled escape stress would be expected to produce a plasma steroid response of approximately 90 μg steroid/100 ml plasma in naive animals or animals stressed for 4 days [2]. In the 71 day and 25 day stressed groups the same stressor could only produce a steroid elevation of 34.5 and 40.7 μg/100 ml respectively. A similar adaptation of the steroid response in rats following prolonged intermittent exposure to environmental stressors was reported by Smookler and Buckley [45]. In the Smookler study serum corticosterone levels in the stressed animals remained elevated for the first 4 weeks of exposure but by the end of week 5 declined dramatically to prestressed levels. The fact that there is no significant difference between the plasma steroid levels in the 25 and 71 day stressed groups would suggest that the plasma steroid response to irregular-signalled escape stress had established a new elevated level of response (35–40 μg/100 ml plasma), a result disimilar to the Smookler and Buckley study where the steroid response returned to prestressed levels.

It is apparent that traumatic stress can produce an oedematous reaction in the intima-media region of arteries and arterioles. This oedematous reaction takes the form of an enlargement of amorphous extracellular spaces due to the accumulation of serous substances including cholesterol [44]. Large molecules such as lipoproteins and cholesterol normally do not pass readily through the endothelial lining of the vascular system due to the overlapping nature of the cell junctions. However, endogenous inflammatory substances such as histamine, serotonin and bradykinin can induce swelling of the endothelial cells causing the separation of neighbouring cells from one another [19, 20, 21]. The opening up of such endothelial gaps would allow the passage of lipoproteins and cholesterol through the endothelial lining. In rat scrotum histamine, serotonin and bradykinin have all been shown to increase vascular leakage by the opening of endothelial gaps allowing the passage of large molecules into the extravascular spaces [21]. The suggested increase in mast cells reported in this study may be intimately concerned with such a histamine-type leakage. Mast cells are known to hold and release vasoactive substances [40], and in particular histamine and serotonin [3, 27, 28]. Their accumulation in the hearts of 71 day stressed animals may provide the source of the inflammatory substances necessary for the oedematous arterial reaction. Venoconstriction also plays a part in the histamine-type leakage. The endogenous inflammatory substances not only open endothelial gaps but also cause venoconstriction in a number of veins including the coronary veins [42,47]. In the stress situation catecholamine induced arteriolar dilatation together with venoconstriction, could raise the internal pressure within the coronary vessels and thus enhance the leakage of large particles through the endothelial gaps. Such a contention is supported by the observation that congestion and dilatation of vessels is observed in the 71 day stressed hearts, since these changes are indicative of an increased vascular perfusion with some defect of venous drainage. A similar congestion of coronary blood vessels resulting from traumatic stress has been reported by Prabhu [31]. The oedematous arterial reaction can be induced by elevated levels of adrenaline or oral administration of cholesterol, animal fats or saturated fatty acids possibly through an activation of bradykinin forming enzymes in the blood [44]. In stress situations the circulating adrenaline levels are markedly elevated as are serum cholesterol levels [12,46] and free fatty acid levels [4]. Further evidence in support of the hypothesis of an enhanced permeability of the endothelial lining of the coronary vessels, and the suggestion that the vacuoles seen are due to deposition of lipid material within the intima-media of coronary arterioles, comes from studies on the uptake of lipids into the heart. Following prolonged intermittent psychological stress the lipid content of the rat heart was found to be raised [23]. Noradrenaline infusion in dogs resulted in the accumulation of lipid droplets in areas of cardiac myolysis [29] and an enhanced triglyceride uptake by the myocardium [15,38].

Accompanying the oedematous arterial reaction induced by traumatic stress, adrenaline, cholesterol, or fatty acids, haematological changes occur which are indicative of activation of specific blood clotting factors, especially Hageman factor [22,44]. Intravascular aggregation of platelets similar to that found after noradrenaline infusion [14] has been demonstrated in rats subjected to stress [13] and clotting time is reduced under stressful conditions [46]. Activation of blood coagulation leading to thrombotic deposits lining the blood vessels, would explain the deposition of PAS +ve material marginated in the venous endothelium of the 71 day stressed animals. The formation of a platelet aggregate or thrombotic deposit at a site of prior narrowing of a venule or vein would further contribute to the observed congestion and dilatation of the microcirculation. The deposition of PAS +ve material in the 71 day stressed group was most apparent in the congested and dilated venules, collecting venules and veins.

There exists therefore, strong supportive evidence for the contention that the coronary vascular changes discussed reflect a progressive degeneration. In such circumstances it would be expected that continuing the stress period in excess of 71 days would induce myocardial pathogenesis and necrotic lesions associated with coronary occlusion. It could be expected also, that introducing a further stress factor of dietary or chemical origin would precipitate cardiac lesions. These possibilities are investigated.

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