Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 83 AUGUST-SEPTEMBER, 1953 No. 4

Eosinopenic Responses of Pyridoxine-Deficient Rats to Epinephrine, Adrenocorticotropic Hormone and Hypoxia.* (20448)

LILLIAN C. BUTLER† AND AGNES FAY MORGAN.

From the Department of Home Economics, University of California, Berkeley.

Few studies of the adrenal or pituitary function of the pyridoxine-deficient rat have been published. Histological studies of Dean and Shaw(1) showed at 3 weeks no more than a mild transitory stimulation of the zona fasciculata and reticularis of the pyridoxine-deficient rat. In 6 to 10 weeks this disappeared and the gland did not differ from that of the control. More recently, Stebbins(2) reported profound changes in the adrenal of the pyridoxine-deficient rat. At weekly intervals during a period of 5 weeks progressive depletion of the sudanophilic lipoid, birefringent material, cholesterol and ascorbic acid occurred in the fasciculata. These adrenal alterations were not present in the inanition and *ad libitum* controls. Impaired water diuresis which was restored to normal with desoxycorticosterone acetate (DOCA), lipo-adrenal extract or pyridoxine was also observed by Stebbins(3) in the pyridoxine-deficient rat. Ershoff and Parrott(4) found a normal lymphopenic response to a single dose of epinephrine in the pyridoxine-deficient rat. When epinephrine was injected daily for 10 successive days, however, an impaired lymphopenic response resulted. Whether this was caused by the deficiency itself or by the effects of inanition, these workers were not able to ascertain because caloric restricted rats were not studied.

The present study was restricted to one technic of determining the functional capacity of the pituitary and adrenal of the pyridoxine-deficient rat, that measured by eosinopenia. Both epinephrine and adrenocorticotropic hormone (ACTH) were used by Thorn(5) in eosinopenic tests for the diagnosis of hypopituitarism and adrenal insufficiency in humans. In addition, hypoxia has been well established as a stimulus of the pituitary adrenal system(6). Low barometric pressure causes specific metabolic changes in the intact rat(7) but not in the adrenalectomized rat(8).

Methods and materials. Diets and animals. Normal adult male Long-Evans rats with a mean weight of 376 g at a mean age of 103 days were placed in individual metabolism cages. Three groups were established; deficient, pair weighed, and those fed *ad libitum*. For 8 weeks all received a basal diet consisting of 24% casein (Labco, vitamin tested), 4% Hubbell *et al.*(9) salt mix and 62% sucrose. Vitamins‡ were fed 3 times weekly

* The authors wish to express their appreciation to Dr. William O. Reinhardt and Dr. Gerald F. Hungerford for suggestions and aid. We also wish to thank Dr. Choh Hao Li, University of California, Berkeley, for the ACTH preparation and Dr. Gladys A. Emerson of Merck and Co., Rahway, N. J. for the desoxypyridoxine.

† Public Health Service Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

in individual cups. The pyridoxine-deficient group received 30 mg of desoxypyridoxine per kg diet mixed directly into the ration, and pyridoxine was omitted from their vitamin supplements. The average weight loss in the deficient and pair weighed groups was 29%, while the *ad libitum* group gained 1%. In the deficient group mild acrodynia of the nose and fore paws in the 4th week progressed during the 7th and 8th weeks into severe involvement of the nose and all paws accompanied by alopecia.

Initial eosinophil counts were made immediately prior to the injections and 4 hours later. Thirty rats of *Series I* were injected during the 7th week on consecutive days with saline, 25 μg of epinephrine and 1 mg of ACTH. During the 8th week of the regimen these same animals were hypophysectomized. Twenty-four hours postoperatively 1 mg of ACTH was administered (Table I).

Series II comprising 48 rats was studied in the 4th and 7th weeks of the regimen for the purpose of determining any possible transitory eosinopenic response changes. Twenty-four rats received injections of 50 μg of epinephrine in these 2 periods and one injection of saline in the 4th week (Table I). In the 8th week of this dietary regimen the 24 rats of Series II were subjected to 24 hours of fasting and during the last 20 hours to hypoxia(10) equal to a decreased atmospheric pressure of 349 mm of mercury equivalent to 20,000 ft altitude. The animals were individually confined in 2-qt Mason jars which were attached to the low barometric pressure system. The animals had free access to water. The initial eosinophil counts used in this experiment were determined 3 days prior to subjecting the animals to hypoxia. The final eosinophil counts were completed immediately after the pressure was gradually increased to sea level. In like manner 24 rats of Series II in the 8th week of the regimen were fasted 24 hours and were placed into 2-qt jars during the last 20 hours of the 24-hour fasting period. These jars were also placed within the confines of the apparatus without connecting them to the hypoxia system. Eosinophil counts were similarly determined (Table I). The animals of Series III received saline injections followed by epinephrine injections 24 hours later during the 8th week of the regimen. This series consisted of 5 deficient and 5 pair weighed animals (Table I).

Blood technic and injections. Because diurnal variation(11) is an important factor in obtaining a constant eosinophil count from day to day all 4-hour tests began at 9:00 A.M. and were completed by 3:30 P.M. Insofar as possible environmental conditions were standardized(12). The animals were not disturbed 12 hours prior to the counts(13). Free flowing tail vein blood was obtained within 2 minutes after removing the animals from the cage and duplicate white blood pipettes were filled to the 0.5 mark. One chamber of a 0.2 mm Fuchs-Rosenthal hemocytometer was counted for each pipette. The diluent was prepared according to Speirs and Meyer(13). The per cent eosinophil change was statistically evaluated as a plus or minus value. The aqueous solution of 0.5 ml of 0.9% sodium chloride was administered intraperitoneally. Epinephrine hydrochloride (Adrenaline hydrochloride, Parke Davis & Co.) was diluted with 0.9% sodium chloride to contain either 50 μg or 25 μg per 0.5 ml. This solution was administered subcutaneously in 0.5 ml quantities. In the intact rat Hungerford(14) demonstrated graded eosinopenic responses to epinephrine in doses from 10 μg to 150 μg provided it was administered subcutaneously rather than intraperitoneally. A solution of 1 mg ACTH prepared from sheep pituitary glands(15) in 0.5 ml of 0.9% sodium chloride solution was administered intraperitoneally. A 0.5 ml syringe and a size 24 needle were used for all injections.

Results. Initial circulating eosinophil levels. The initial circulating eosinophil levels in Series I, II and III showed no differences

‡ The water soluble vitamins were dissolved in 20% aqueous ethanol solution and supplied each rat daily the following amounts in mg: thiamine hydrochloride, riboflavin, pyridoxine, and folic acid, .02 each, Ca pantothenate and para-aminobenzoic acid, .10 each, niacin amide, .07, inositol, 2.5, biotin, .002, and choline, 5.0. Vits. A and D and mixed tocopherols were dissolved in vegetable oil and were supplied separately in amounts of 1000 I. U., 100 I. U. and 3 mg/rat/week.

TABLE I. Eosinopenic Responses of the Pyridoxine-Deficient Rat Induced by Saline, Epinephrine, ACTH, Fasting and Hypoxia.

Series	Treatment	Week	No. of rats	Group	Eosinophils/mm — Initial	Eosinophils/mm — Final	% change†	P*
I. Intact	Saline .5 ml	7	10	D§	415 ± 60	275 ± 76	—41 ± 9	
			9	PW	369 ± 91	202 ± 79	—52 ± 10	
			10	AL	478 ± 81	313 ± 46	—40 ± 6	
	Epinephrine 25 μg	7	10	D	454 ± 89	108 ± 22	—79 ± 3	<.01
			10	PW	388 ± 38	167 ± 74	—62 ± 6	<.30
			10	AL	519 ± 69	213 ± 44	—59 ± 7	.07
	ACTH 1 mg	7	10	D	513 ± 60	167 ± 78	—73 ± 7	.02
			10	PW	382 ± 72	141 ± 46	—65 ± 8	<.20
			10	AL	514 ± 50	70 ± 62	—68 ± 6	<.01
Hypophysectomized	ACTH 1 mg	8	7	D	401 ± 111	152 ± 62	—66 ± 8	
			7	PW	559 ± 67	247 ± 78	—65 ± 11	
			7	AL	922 ± 225	388 ± 171	—60 ± 8	
II. Intact	Saline .5 ml	4	8	D	498 ± 144	426 ± 79	+ 2 ± 6	
			8	PW	621 ± 199	337 ± 70	—47 ± 8	
			8	AL	454 ± 92	385 ± 46	—19 ± 11	
	Epinephrine 50 μg	4	8	D	514 ± 136	93 ± 25	—76 ± 7	<.01
			8	PW	424 ± 128	134 ± 10	—83 ± 8	<.01
			8	AL	454 ± 38	83 ± 13	—80 ± 5	<.01
	Epinephrine 50 μg	7	8	D	374 ± 12	77 ± 24	—78 ± 6	<.01
			8	PW	297 ± 24	79 ± 24	—75 ± 8	<.01
			8	AL	529 ± 46	53 ± 8	—88 ± 3	<.01
	Sea level fasted	8	8	D	403 ± 29	170 ± 34	—55 ± 10	
			8	PW	364 ± 97	115 ± 32	—52 ± 13	
			8	AL	419 ± 35	284 ± 56	—31 ± 17	
	Hypoxia 20000 ft	8	8	D	370 ± 38	7 ± 4	—98 ± 1	<.01‡
			8	PW	297 ± 68	21 ± 9	—89 ± 6	.03
			8	AL	476 ± 120	25 ± 9	—93 ± 3	<.01
III. Intact	Saline .5 ml	8	5	D	514 ± 156	423 ± 145	—14 ± 14	
			5	PW	654 ± 17	250 ± 18	—60 ± 8	
	Epinephrine 50 μg	8	5	D	504 ± 145	114 ± 59	—86 ± 9	<.01
			5	PW	405 ± 90	46 ± 20	—91 ± 3	<.01

* Probability of significance of the % change of each group compared with its respective eosinopenia induced by saline injection.

† % change $= 100 \times \frac{\text{Initial} - \text{final}}{\text{Initial}}$.

‡ Probability of significance of the % change of each group compared with its respective % change at sea level.

§ D = deficient, PW = pair weighed, AL = *ad libitum*.

within each group from day to day or within the same week on the dietary regimen (Table I). The hypophysectomized animals showed no differences from their respective unoperated groups. However, in Series II during the 7th week and 8th week the initial circulating eosinophil levels of the deficient and pair weighed groups were significantly decreased when compared with their respective initial eosinophil levels during the 4th week.

Series I. Eosinopenias induced by saline, epinephrine and ACTH. During the 7th week the decrease in the eosinophils of all 3 groups following saline injection was —40% to —50% (Table I). This change was of the same magnitude in each group. Epinephrine injected at the 25 μg dose level gave a significant change in the deficient group when compared with its own saline-induced eosinopenia. Obviously, 25 μg of epinephrine was not large enough a dose to produce a significant response either in the pair weighed group or in the *ad libitum* group. ACTH caused a significant eosinopenia in the unoperated animals of the deficient group (—73%) and of the *ad libitum* group (—65%) as compared with

their respective saline responses. When these previously mentioned responses to ACTH are compared with each other, all 3 groups exhibited the same degree of eosinopenia. The 3 hypophysectomized groups showed equal eosinopenic responses to ACTH which did not differ from their corresponding ACTH-induced eosinopenias before the operation.

Series II. Eosinopenias induced by saline and epinephrine. Saline caused an eosinophilia of +2% during the 4th week in the deficient group. This value differed significantly from its corresponding deficient group (—41%) during the 7th week in Series I but not from the deficient group (—14%) in Series III in the 8th week. No differences in the saline-induced eosinopenia in the pair weighed (—47%) and *ad libitum* group (—19%) occurred. The degree of eosinopenia resulting from the administration of 50 μg of epinephrine during the 4th week and 7th week was comparable (—75% to —88%) in all 3 groups, and all were significantly below their respective saline eosinopenias.

Series II. Eosinopenias induced by fasting and hypoxia. The changes at sea level of the fasted animals reflected the stresses of handling, fasting 24 hours and of confinement in the hypoxia chamber for the last 20 hours. The eosinopenia in all 3 groups was of the same magnitude (—31% to —55%, Table I). In all 3 groups hypoxia caused comparable eosinopenias (—89% to —93%) which were significantly greater than the eosinopenic responses of their respective groups at sea level.

Series III. Eosinopenias induced by saline and epinephrine. During the 8th week a separate series of animals was studied for the purpose of confirming our previous results with epinephrine. The eosinopenia caused by saline was significantly larger in the pair weighed group (—60%) than in the deficient group (—14%). Administration of 50 μg of epinephrine again caused a significant eosinopenia in both the deficient and pair weighed groups (—86% and —91%, respectively) as compared with their saline-induced responses.

Discussion. The present experiment demonstrates that the pyridoxine-deficient rat was capable of responding to stress with a significant eosinopenia as well as did the pair weighed and *ad libitum* controls. It may be assumed therefore, that there was no interference in the elaboration or release of ACTH and adrenal 11-oxysteroids in the pyridoxine-deficient rat as determined by the epinephrine tests employed during the 4th week of mild deficiency signs and during the 7th and 8th weeks when severe acrodynia was present. It has generally been found that epinephrine acts directly upon the pituitary of rats causing the release of ACTH(16-19). Gemzell(19) in addition showed a fall of the pituitary ACTH content and a concomitant rise in the body fluid ACTH content in rats. In humans this action has not been demonstrated(20). When the pituitary adrenal axis was interrupted in rats by hypophysectomy or adrenalectomy, it has been established that no eosinopenic response followed the administration of a physiological dose of epinephrine(13,16,17). On the other hand, a synergistic action of epinephrine with cortisone has been demonstrated(21,22). When either substance alone is incapable of producing an eosinopenia at the dose levels used, evidence of their combined eosinopenic efficaciousness has been found both in adrenalectomized humans by Kark and Muehrcke(21) and adrenalectomized dogs by Henry *et al.*(22).

The use of ACTH-induced eosinopenia as a test for the adrenal function has been reported to be reliable for humans(21,23,24) and for rats and dogs(13,15). The data show that ACTH in the intact rat gave a significant eosinopenia in the pyridoxine-deficient and *ad libitum* groups but not in the pair weighed group. This was probably due to the greater saline-induced eosinopenia (by —11%) of this latter group. In order to obviate any activity mediated by the pituitary, hypophysectomized rats were used. The use of the hypophysectomized animal has the advantage of offering a more valid method of testing the adrenal function because endogenous ACTH is not present. All 3 of these hypophysectomized groups including the pyridoxine-deficient group gave eosinopenic responses greater than —50%. Our data made it evident that the adrenal cortex of the hypophysectomized pyridoxine-deficient rats is capable

of normal function as determined by the eosinopenia induced by ACTH administration.

Hypoxia was shown to cause eosinopenia in dogs by Furgeson and Smith(25) and in rats during recovery from hypoxia by King *et al.* (26). Our experiments demonstrate that the pyridoxine-deficient rat is capable of a normal eosinopenic response following 20 hours of exposure to an altitude of 20,000 ft including a 24-hour fast. Fasting of rats confined in the hypoxia chamber at sea level caused sufficient stress to produce a sharp drop in the circulating eosinophils. The latter finding suggests that eosinopenia is a more sensitive index of alterations in the pituitary and adrenal activity than are metabolic and adrenal changes (27) which fail to occur under these fasting conditions in the normal rat(28).

The differences between the adrenal histological findings of Stebbins(2) and Deane and Shaw(1) may be attributed to unequal susceptibility of dissimilar strains of rats to pyridoxine-deficiency(29); the former used Carworth male weanlings while the latter used Long-Evans young male rats. The current study of the adult pyridoxine-deficient male Long-Evans rat given desoxypyridoxine did not duplicate either of the previously mentioned experimental conditions. The eosinopenic responses in the adult pyridoxine-deficient male rat during the 4th, 7th, and 8th weeks indicated a normal response of the pituitary adrenal system to the stimuli used. Extra-adrenal factors may well be involved in eosinopenia but adrenal hormones appear to have at least an indirect influence.

Summary. Eosinopenic responses following, 1) hypoxia treatment, and 2) epinephrine injections, both in the intact rat, and 3) ACTH injections in the intact and in the hypophysectomized rat were of equal magnitude in the pyridoxine-deficient, pair weighed and normal animals. The significance of these findings is discussed.

1. Dean, H. W., and Shaw, J. H., *J. Nutr.*, 1947, v34, 1.

2. Stebbins, R. B., *Endocrinology,* 1950, v49, 25.

3. ———, *Am. J. Physiol.*, 1950, v106, 538.

4. Ershoff, B. H., and Parrott, F. D., *J. Clin. Nutr.*, 1953, v1, 124.

5. Thorn, G. W., *Diagnosis and Treatment of Adrenal Insufficiency,* pp. 53-61, 2nd Ed., 1951, Charles C. Thomas, Springfield.

6. Sayers, G., *Physiol. Rev.*, 1950, v30, 241.

7. Tepperman, J., Tepperman, H. M., Patton, B. W., and Nims, L. F., *Endocrinology,* 1947, v41, 356.

8. Lewis, R. A., Thorn, G. W., Koepf, G. F., and Dorrance, S. S., *J. Clin. Invest.*, 1942, v21, 33.

9. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, 1937, v14, 273.

10. Wickson, M. E., and Morgan, A. F., *J. Biol. Chem.*, 1946, v162, 209.

11. Halberg, F., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 846.

12. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood,* 1951, v6, 832.

13. Speirs, R. S., and Meyer, R. K., *Endocrinology,* 1949, v45, 403.

14. Hungerford, G. F., Unpublished data.

15. Li, Choh Hao, *J. Am. Chem. Soc.*, 1952, v74, 2124.

16. Recant, L., Hume, D. H., Forsham, P. H., and Thorn, G. W., *J. Clin. Endoc.*, 1950, v10, 187.

17. McDermott, W. V., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.*, 1950, v23, 52.

18. Gershberg, H., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.*, 1950, v23, 32.

19. Gemzell, C. A., *Endocrinology,* 1952, v50, 399.

20. Thorn, G. W., *Am. J. Med.*, 1953, v24, 139.

21. Kark, R. M., and Muehrcke, R. C., *Lancet,* 1952, v22, 1189.

22. Henry, W. L., Oliner, L., and Ramey, E. R., *Fed. Proc.*, 1953, v12, 66.

23. Thorn, G. W., Forsham, P. H., Prunty, F. T., and Hills, A. G., *J. Am. Med. Assn.*, 1948, v137, 1005.

24. Best, W. R., Muehrcke, R. C., and Kark, R. M., *J. Clin. Med.*, 1951, v38, 790.

25. Furgeson, F. P., and Smith, D. C., *Fed. Proc.*, 1953, v12, 42.

26. King, C. T. G., Mego, J. L., Williams, E. E., and Shaeffer, K. E., *Fed. Proc.*, 1953, v12, 76.

27. Gordon, A. S., and Padawer, J., *Fed. Proc.*, 1953, v12, 54.

28. Hurley, L. S., and Morgan, A. F., *J. Biol. Chem.*, 1952, v195, 583.

29. Cerecedo, L. R., and Renzo, E. C., *Arch. Biochem.*, 1948, v17, 397.

Received May 4, 1953. P.S.E.B.M., 1953, v83.