AAL-TDR-64-5

INFLUENCE OF CORTISONE ON GLYCONEOGENESIS, ENDOTOXIN LETHALITY AND TRYPTOPHAN PYRROLASE INDUCTION IN COLD-EXPOSED MICE

TECHNICAL DOCUMENTARY REPORT AAL-TDR-64-5

October 1964

ARCTIC AEROMEDICAL LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
FORT WAINWRIGHT, ALASKA

Project 8241, Task 824101

(Prepared under Contract AF 41(609)-1764 by
L. Joe Berry, Department of Biology,
Bryn Mawr College, Bryn Mawr, Pa.)

NOTICES

When US Government drawings, specifications, or other data are used for any purpose other than a definitely related government procurement operation, the government thereby incurs no responsibility nor any obligation whatsoever; and the fact that the government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

ABSTRACT

Mice singly housed and exposed to 5° C are almost as responsive as animals at 25° C in carrying out glyconeogenesis following a single injection of cortisone. An LD_{50} of endotoxin lowers liver glycogen about equally in mice housed at each of the two temperatures, but the dose for mice at 5° C is one one-hundredth the dose at 25° C. Cortisone given concurrently with the endotoxin prevents glycogen depletion in mice at 25° C but not in those housed at 5° C, except possibly at certain critical doses. Cortisone also fails to induce an increase in liver tryptophan pyrrolase activity in cold-exposed mice, in contrast to those maintained at room temperature, and this failure cannot be ascribed to any alteration in hematin level, the cofactor for the enzyme. These findings are considered as evidence for an impaired capacity for protein (enzyme) synthesis in normothermic animals during acute exposure to cold.

PUBLICATION REVIEW

HORACE F. DRURY
Director of Research

INFLUENCE OF CORTISONE ON GLYCONEOGENESIS, ENDOTOXIN LETHALITY AND TRYPTOPHAN PYRROLASE INDUCTION IN COLD-EXPOSED MICE *

SECTION 1. INTRODUCTION

Chedid (8) first observed that cortisone protects mice against the lethal effects of bacterial endotoxin. Despite numerous confirmatory publications in the intervening years, little is known about the way in which the hormone exerts this effect. Several publications from this laboratory (1, 4, 13) and elsewhere (11) have indicated that cortisone-treated animals are better able to maintain their carbohydrate reserves than untreated endointoxicated animals. More recently, the induction of enzymes that follows an injection of cortisone (or hydrocortisone) as reported by Knox and Mehler (16), Feigelson, Feigelson and Greengard (10) and Kenney and Flora (15) has been studied in mice injected with endotoxin (5). The enzyme used as the test system, tryptophan pyrrolase, becomes depleted in endotoxin poisoning but remains at a normal level of activity in animals given a concurrent injection of cortisone. Also of interest is the observation that certain metabolites (nicotinamide and diphosphopyridine nucleotide), substances that are formed from the product of tryptophan pyrrolase, have prophylactic potencies about equal to that of cortisone. This would be predicted if tryptophan pyrrolase promoted a reaction of survival value against endotoxin lethality.

In a different type of approach to the problem, Previte and Berry (20) reported that in mice singly housed at 5° C, cortisone protects against lethality of endotoxin only when a dose of critical size is given. This somewhat anomalous relationship serves as the basis for the present communication, in which two different phenomena have been studied. First, the ability of cortisone to augment carbohydrate reserves in cold-exposed mice has been determined and, second, the induction of tryptophan pyrrolase after cortisone administration has been measured. In each case, the behavior of the cold-exposed mice is different from that of animals maintained at room temperature.

---

* This research was conducted in accordance with the "Principles of Laboratory Animal Care" of the National Society for Medical Research.

SECTION 2. METHODS

Exposure to environmental temperature. Mice were housed singly, free of bedding, in Plexiglas compartments as previously described by Previte and Berry (20). Control animals were kept at 25° ± 2° C in an air-conditioned laboratory, while those in the cold were placed in a walk-in environmental room (Labline Modulab) maintained at 5° ± 0.5° C. Relative humidity was regulated in the cold room at 65%. No humidity control was possible at 25° C.

Carbohydrate determinations. Liver glycogen was determined by the method of Kemp and van Heijningen (14) as modified by Berry, Smythe and Young (1).

Liver tryptophan pyrrolase assays. The method of Knox and Auerbach (17) as adapted to mice by Berry and Smythe (5) was employed. Values are expressed as μmoles of kynurenine formed per hour per gram dry weight of liver tissue. In order to distinguish between the amounts of apoenzyme and holoenzyme present in the liver homogenate, hematin was added according to the procedure followed by Feigelson and Greengard (9). Hemin (Sigma Chemical Co., St. Louis) was dissolved in dilute NaOH. The alkalinity of the solution converts hemin to hematin, and by adding increasing amounts to a liver homogenate, it was found that 20 μg per flask gave maximum activity (all enzyme present as holoenzyme). Each flask contained a volume of 8 ml, hence the hematin was present as a 4.0 μM solution.

Endotoxin. Two types of endotoxin were used in all experiments. The material employed in all preliminary work was heat-killed Salmonella typhimurium, strain SR-11, suspended in nonpyrogenic saline (Baxter Laboratories). Large quantities, prepared at one time, were stored in the refrigerator and used as needed. The dry weight of this material was 6.0 mg per ml. Results obtained with this crude endotoxin have been confirmed with a more highly refined lipopolysaccaride derived from Serratia marcescens (Difco) by the Boivin procedure. All injections were given intraperitoneally in a volume of 0.5 ml of saline.

Cortisone. Cortisone acetate in stabilized suspension (United Research Labs) was injected subcutaneously in 5 mg amounts contained in 0.5 ml.

Protection experiments. Endotoxin and cortisone were each injected within a period of 10 to 15 minutes. Immediately thereafter, animals were placed at either 5° C or 25° C and were permitted to remain continuously at the given temperature for an observation period of 24 hours at 5° C and 48 hours at 25° C. No deaths occurred after these times. No experiments were done with cold "acclimatized" mice.

SECTION 2. METHODS

Exposure to environmental temperature. Mice were housed singly, free of bedding, in Plexiglas compartments as previously described by Previte and Berry (20). Control animals were kept at 25° ± 2° C in an air-conditioned laboratory, while those in the cold were placed in a walk-in environmental room (Labline Modulab) maintained at 5° ± 0.5° C. Relative humidity was regulated in the cold room at 65%. No humidity control was possible at 25° C.

Carbohydrate determinations. Liver glycogen was determined by the method of Kemp and van Heijningen (14) as modified by Berry, Smythe and Young (1).

Liver tryptophan pyrrolase assays. The method of Knox and Auerbach (17) as adapted to mice by Berry and Smythe (5) was employed. Values are expressed as μmoles of kynurenine formed per hour per gram dry weight of liver tissue. In order to distinguish between the amounts of apoenzyme and holoenzyme present in the liver homogenate, hematin was added according to the procedure followed by Feigelson and Greengard (9). Hemin (Sigma Chemical Co., St. Louis) was dissolved in dilute NaOH. The alkalinity of the solution converts hemin to hematin, and by adding increasing amounts to a liver homogenate, it was found that 20 μg per flask gave maximum activity (all enzyme present as holoenzyme). Each flask contained a volume of 8 ml, hence the hematin was present as a 4.0 μM solution.

Endotoxin. Two types of endotoxin were used in all experiments. The material employed in all preliminary work was heat-killed Salmonella typhimurium, strain SR-11, suspended in nonpyrogenic saline (Baxter Laboratories). Large quantities, prepared at one time, were stored in the refrigerator and used as needed. The dry weight of this material was 6.0 mg per ml. Results obtained with this crude endotoxin have been confirmed with a more highly refined lipopolysaccaride derived from Serratia marcescens (Difco) by the Boivin procedure. All injections were given intraperitoneally in a volume of 0.5 ml of saline.

Cortisone. Cortisone acetate in stabilized suspension (United Research Labs) was injected subcutaneously in 5 mg amounts contained in 0.5 ml.

Protection experiments. Endotoxin and cortisone were each injected within a period of 10 to 15 minutes. Immediately thereafter, animals were placed at either 5° C or 25° C and were permitted to remain continuously at the given temperature for an observation period of 24 hours at 5° C and 48 hours at 25° C. No deaths occurred after these times. No experiments were done with cold "acclimatized" mice.

TABLE I

PER CENT LIVER GLYCOGEN FIVE HOURS AFTER FASTING AND THE EXPERIMENTAL TREATMENTS INDICATED FOR MICE EXPOSED TO EITHER 25° OR 5° C

Experimental Treatment	Liver Glycogen in Mice (%)		Statistics
	Exposed to 25° C (A)	Exposed to 5° C (B)	
1. Control	3.36 ± 0.93 (8)	3.65 ± 1.15 (6)	1A vs 1B = N.S. *
2. 5 mg cortisone	7.24 ± 1.60 (10)	6.10 ± 2.26 (13)	1A vs 2A = 0.001 1B vs 2B = 0.05
3. 1000 µg endotoxin	1.86 ± 0.78 (9)		1A vs 3A <0.01
4. 1000 µg endotoxin + 5 mg cortisone	3.87 ± 1.48 (7)		3A vs 4A <0.01
5. 10 µg endotoxin	3.45 ± 1.14 (7)	1.51 ± 0.66 (16)	1A vs 5A = N.S. 1B vs 5B = 0.001
6. 10 µg endotoxin + 5 mg cortisone		2.19 ± 0.85 (9)	5B vs 6B = N.S.
7. 20 µg endotoxin		1.31 ± 0.62 (8)	1B vs 7B <0.01 6B vs 7B = N.S.
8. 20 µg endotoxin + 5 mg cortisone		2.03 ± 0.85 (8)	7B vs 8B = N.S.

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses. Mice were exposed to temperature (5° C) for the five-hour period only.

* N.S. = Not significantly different.

6B and is contrary to what is found in mice maintained at 25° C, as can be seen in lines 3A and 4A. When twice the amount of endotoxin, 20 μg, is given alone (line 7B) or concurrent with cortisone (line 8B), liver glycogen drops below the control level and fails to increase after the hormone in mice kept at 5° C.

Effect of cold on cortisone protection against endotoxin. Since cortisone at a dose level that maintains normal liver glycogen in mice given endotoxin at 25° C fails to have this effect in mice at 5° C, it was considered worthwhile to determine whether or not the hormone protects against the lethality of endotoxin in cold-exposed animals. The data of Table II summarize the results obtained at three dosages of endotoxin. All deaths occurred during the first 24 hours of exposure, and observations were terminated at that time.

Several points of importance emerge from these results. In the first place, cold exposure alone killed 5 of 40 mice. This is more than was found in earlier experiments (20, 21, 18, 19), but the strains of mice differ. In addition, cortisone failed to protect mice given concurrent injections of either 10 μg or 40 μg of endotoxin, while statistically significant protection is afforded animals given 20 μg of endotoxin. This last finding is primarily dependent on the results of 8 Nov. 63 and 16 Dec. 63, where only 1 of 10 untreated mice survived and 8 and 6, respectively, of the 10 animals injected with cortisone survived. On the remaining three dates, the combined results of 15/30 and 20/30 show no protective action of cortisone. It must be concluded, therefore, that cortisone in mice maintained at 5° C has, at best, only questionable protective ability, which may be dependent (21) in part on the dose of endotoxin or on the condition of the mice at the time of the experiment.

Effect of cold on cortisone induction of liver tryptophan pyrrolase. Both the lack of protection against endotoxin lethality afforded by a concurrent injection of cortisone and the failure of cortisone to maintain normal levels of liver glycogen in endointoxicated mice are clear indications that exposure to cold profoundly alters the metabolic competency of the mice. As direct evidence of this, the inducibility of liver tryptophan pyrrolase four hours after an injection of cortisone in mice maintained for the postinjection period at 5° C was determined. The results are presented in Table III. It is clear that the cortisone-induced increase in enzyme activity seen in mice housed at 25° C ($P < 0.001$ by rank order test of White, 23) fails to occur in mice similarly treated but maintained at 5° C. Moreover, the slightly but significantly higher level of enzyme activity observed in the cold-exposed mice not given cortisone, compared with the controls at 25° C (14.1 vs 10.4), is indicative of a limited enzyme induction, possibly in response to endogenous corticoids.

TABLE II

THE PROTECTIVE EFFECT OF CORTISONE AGAINST ENDOTOXIN LETHALITY IN MICE MAINTAINED AT 5° C

Date of Experiments	Number of Mice Surviving/Total Injected						
	No Injection (Controls)	10 µg Endotoxin	10 µg Endotoxin plus Cortisone	20 µg Endotoxin	20 µg Endotoxin plus Cortisone	40 µg Endotoxin	40 µg Endotoxin plus Cortisone
8 Nov. 63				1/10	8/10	2/10	1/10
18 Nov. 63	6/10	6/10	6/10	5/10	5/10		
26 Nov. 63	10/10	5/10	5/10	6/10	7/10	7/10	8/10
2 Dec. 63	10/10	7/10	8/10	4/10	8/10	4/10	1/10
16 Dec. 63	9/10			1/10	6/10	2/10	1/10
Totals	35/40	18/30	19/30	17/50	34/50	15/40	11/40

Each animal received 5 mg cortisone acetate subcutaneously and the indicated amount of crude S. typhimurium endotoxin by intraperitoneal injection.

TABLE III

CORTISONE INDUCTION OF LIVER TRYPTOPHAN PYRROLASE IN MICE EXPOSED TO EITHER 25° OR 5° C

Liver Tryptophan Pyrrolase Activity (μM kynurenine/hr/gm dry weight liver in mice)			
Maintained at 25° C		Maintained at 5° C	
Controls	5 mg Cortisone	Controls	5 mg Cortisone
10.4 ± 3.2 (17)	18.8 ± 4.3 (19)	14.1 ± 3.4 (25)	15.0 ± 2.9 (25)

Each animal either received 5 mg cortisone acetate subcutaneously or served as a control. Assays for tryptophan pyrrolase were begun four hours after the injection and four hours after the initial exposure to cold. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Tryptophan pyrrolase is known to require hematin as a cofactor (16), and in rats under normal conditions only about one-third of the total enzyme in liver is reported to be in the form of the holoenzyme (9). In order to determine whether or not the failure of cortisone to induce tryptophan pyrrolase in cold-exposed mice is attributable to changes in cofactor, the experiments summarized in Table IV were undertaken. First, the top line of this table agrees very well with the data of Table III, when the relative size of the samples and the fact that the values were obtained at different times of the year are taken into consideration. More important, however, are the changes resulting from the addition of hematin to the assay flasks. The increase in enzyme activity due to cortisone is clearly evident in mice at 25° C, but there is no significant increase due to cortisone in mice housed at 5° C, whether or not hematin is added. Thus 14.5 vs 15.9 and 27.3 vs 35.3 are not significantly different according to the rank order test of White (23). Inductive failure in mice at 5° C is apparently due, therefore, to lack of enzyme synthesis and not to lack of saturation of apoenzyme with cofactor. The data of Table IV also show that the increase in enzyme activity resulting from the addition of hematin to the assay flasks is the same under comparable conditions independent of the temperature at which the animals are housed. This is made evident by examining the hematin effect in control mice (21.2 - 94 = 11.8 vs 27.3 - 14.5 = 18.6 vs 35.3 - 15.9 = 19.6). Under both sets of conditions, the activity change is nearly identical.

SECTION 4. DISCUSSION

Greater lability in liver glycogen reserves following an injection of bacterial endotoxin, previously associated with mice exposed to hypoxia (3) or with tumors such as Sarcoma 37 or Krebs 2 carcinoma (13) has been extended to include animals exposed to cold. In the first of the above-cited publications, it was stated that the basis for such a lability may be due to an accelerated breakdown of glycogen, an impaired synthesis or a combination of the two. This remains equally true for the present results in light of our limited knowledge. Recent observations, however, have added new information which offers hope for greater understanding in the future. Greengard, Weber and Singhal (12) and Weber, Singhal and Stamm (22) have shown that in rats injected at the same time with cortisone and an inhibitor of protein synthesis (such as actinomycin D or puromycin) glycogen deposition fails to occur as does induction of selected liver enzymes. It is their contention that cortisone-mediated glyconeogenesis depends upon an increase in enzyme synthesis. Since their experiments are based upon a daily injection of hormone over a period of several days, the fact that cortisone, after a period of five hours, is able to result in an increase in liver glycogen almost as great in mice at 5° C as in those kept at 25° C is not necessarily inconsistent with

TABLE IV

EFFECT OF IN VITRO ADDITION OF HEMATIN ON LIVER TRYPTOPHAN PYRROLASE ACTIVITY FOLLOWING INDUCTION OF ENZYME BY CORTISONE IN MICE EXPOSED TO EITHER 25° OR 5° C

	Liver Tryptophan Pyrrolase Activity (μM kynurenine/hr/gm dry weight liver in mice)			
	Maintained at 25° C		Maintained at 5° C	
Assays Performed	Controls	5 mg Cortisone	Controls	5 mg Cortisone
Without hematin	9.4 ± 2.4 (7)	21.7 ± 5.0 (7)	14.5 ± 4.0 (7)	15.9 ± 2.6 (7)
Hematin added	21.2 ± 6.0 (7)	40.3 ± 11.5 (6)	27.3 ± 5.6 (7)	35.3 ± 6.2 (7)

Animals either received 5 mg cortisone acetate subcutaneously or served as controls. Assays for tryptophan pyrrolase were begun four hours after the injection and four hours after the initial exposure to cold. Assays were carried out either with no hematin added or with the addition of 20 μg per flask. Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

their findings. On the other hand, the failure of cortisone to induce tryptophan pyrrolase in livers of mice housed at 5° C versus the finding with animals at 25° C is in keeping with the failure of cortisone to protect mice against lethality of endotoxin. The importance of enzyme induction in the mouse's response to endotoxin has become increasingly evident from work already published (5), in press (6) or in preparation (7). Using tryptophan pyrrolase as a convenient test system and not necessarily as an enzyme of unique significance in endotoxin poisoning, it is apparent that cortisone fails to protect against lethality under each condition in which enzyme synthesis fails to occur. This includes delaying the injection of cortisone for an hour or two after the administration of the endotoxin or concurrently injecting an inhibitor of protein synthesis, such as actinomycin, ethionine, 8-azaguanine and 2-thiouracil.

Acute exposure to cold (5° C) of the type reported in this study must act, therefore, even in the absence of significant hypothermia (21) in some unexplained manner to block protein synthesis, at least as it is induced by adrenocortical hormones. The site of action remains to be determined, but with the rapidly expanding understanding of the various factors involved in protein synthesis, it should be possible to carry the analysis well beyond the present state of our knowledge.

REFERENCES

1. Berry, L. J., D. S. Smythe and L. G. Young. Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone. J. Exp. Med. 110:389-405, 1959.

2. Berry, L. J. and D. S. Smythe. Effects of bacterial endotoxin on metabolism. II. Protein-carbohydrate balance following cortisone. Inhibition of intestinal absorption and adrenal response to ACTH. J. Exp. Med. 110:407-418, 1959.

3. Berry, L. J. and D. S. Smythe. Effect of pure oxygen at reduced pressures on metabolic changes in mice. Amer. J. Physiol. 203:155-159, 1962.

4. Berry, L. J., D. S. Smythe and S. M. Kolbye. Effects of bacterial endotoxins on metabolism. V. The hyperreactivity of mice infected with mycobacterium tuberculosis, strain BCG. J. Exp. Med. 116:897-911, 1963.

5. Berry, L. J. and D. S. Smythe. Effects of bacterial endotoxins on metabolism. VI. The role of tryptophan pyrrolase in response of mice to endotoxin. J. Exp. Med. 118:587-603, 1963.

6. Berry, L. J. Effect of endotoxins on the level of selected enzymes and metabolites. In Bacterial Endotoxins, Braun, W. and M. Landy, Eds. New York, Academic Press, Inc., 1964.

7. Berry, L. J. and D. S. Smythe. Effects of bacterial endotoxins on metabolism. VII. Enzyme induction as a protective mechanism against endotoxin toxicity. To be submitted to J. Exp. Med.

8. Chedid, L. Hormones et infection, des effets opposes de la cortisone sur la resistance de l'hote au cours d'une salmonellase experimentale. Supplements, Bull. Biol. France Belg. 42:1, 1957.

9. Feigelson, P. and O. Greengard. A microsomal iron-porphyrin activator of rat liver tryptophan pyrrolase. J. Biol. Chem. 236:153-157, 1961.

10. Feigelson, P., M. Feigelson and O. Greengard. Comparison of the mechanisms of hormonal and substrate induction of rat liver tryptophan pyrrolase. Recent Progr. Hormone Res. 18:491, 1962.

11. Fukuda, T. On the mechanism of endotoxin intoxication in rabbits. Jap. J. Physiol. 13:155-168, 1963.

12. Greengard, O., G. Weber and R. L. Singhal. Glycogen deposition in the liver induced by cortisone : dependence on enzyme synthesis. Science 141:160-161, 1963.

13. Havas, H. F., L. J. Berry and D. S. Smythe. Effect of endotoxin on liver carbohydrate in mice bearing transplantable tumors. (In preparation.)

14. Kemp, A. and A. J. M. K. van Heijningen. A colorimetric micro-method for the determination of glycogen in tissues. Biochem. J. 56:646-648, 1954.

15. Kenney, F. T. and R. M. Flora. Induction of tyrosine-α-ketoglu-tarate transaminase in rat liver. J. Biol. Chem. 236:2699-2702, 1961.

16. Knox, W. E. and A. H. Mehler. The adaptive increase of the tryptophan peroxidase-oxidase system of liver. Science 113:237-238, 1951.

17. Knox, W. E. and V. H. Auerbach. The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem. 214:307, 1955.

18. Miraglia, G. J. and L. J. Berry. Enhancement of salmonellosis and emergence of secondary infection in mice exposed to cold. J. Bact. 84:1173-1180, 1962.

19. Miraglia, G. J. and L. J. Berry. Possible source of secondary invading staphylococci in mice exposed to acute cold. J. Bact. 85:345-348, 1963.

20. Previte, J. J. and L. J. Berry. The effect of environmental temperature on the host-parasite relationship in mice. J. Infect. Dis. 110:201-209, 1962.

21. Previte, J. J. and L. J. Berry. Studies on the potentiation of endotoxin in mice by exposure to cold. J. Infect. Dis. 113:43-51, 1963.

22. Weber, G., R. L. Singhal and N. B. Stamm. Actinomycin: inhibition of cortisone-induced synthesis of hepatic gluconeogenic enzymes. Science 142:390-392, 1963.

23. White, C. The use of ranks in a test for significance for comparing two treatments. Biometrics 8:33, 1952.