1961, H. C. Heinrich, ed., Stuttgart: Ferdinand Enke, 1962, 673.

5. Killander, A., Schilling, R. F., J. Lab. and Clin. Med., 1961, v57, 553.

6. Heinrich, H. C., Gabbe, E. E., Klin. Wschr., 1961, v13, 689.

7. Bauriedel, W. R., Picken, J. C., Underkofler, L. A., Proc. Soc. Exp. Biol. and Med., 1956, v91, 377.

8. Chosy, J. J., Killander, A., Schilling, R. F., in: Vitamin B_{12} und Intrinsic Factor. 2. Europ. Symposion, Hamburg, 1961, H. C. Heinrich, ed., Stuttgart: Ferdinand Enke, 1962, 668.

9. Glass, G. B. J., Pharmakotherapia, 1964, v2, 21.

10. Gabbe, E. E., Heinrich, H. C., Bornholt, E., Heyde, K., Weist, D., in: Vitamin B_{12} und Intrinsic Factor. 2. Europ. Symposion, Hamburg, 1961, H. C. Heinrich, ed., Stuttgart: Ferdinand Enke, 1962, 116.

11. Rosenblum, C., ibid., p306.

12. Berlin, H., Berlin, R., Brante, G., Spöberg, S. F., ibid., p485.

13. Doscherholmen, A., Hagen, P. S., Liu, M., Olin, L., J. Clin. Invest., 1957, v36, 1551.

14. Weisberg, H., Glass, G. B. J., (a) Gastroenterology, 1965, v48, 857; (b) J. Lab. and Clin. Med., in press.

15. Gräsbeck, R., Scand. J. Clin. Lab. Invest., 1959, v2, 250.

16. Reizenstein, P. G., Acta med. Scand., 1959, Suppl. 347, 1.

17. Bozian, R. C., Ferguson, J. L., Heyssel, R. M., Meneely, G. R., Darby, W. J., Am. J. Clin. Nutr., 1963, v12, 117.

Received February 4, 1966. P.S.E.B.M., 1966, v122

Inhibitory Effect of ACTH and Related Peptides on Extinction of Conditioned Avoidance Behavior in Rats. (31042)

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ACTH administered during avoidance learning delays extinction of a shuttle-box avoidance response in intact as well as in adrenalectomized rats(1,2). The removal of the posterior and intermediate lobe of the pituitary (posterior lobectomy) in rats facilitates extinction of a similar avoidance response, and the rapid rate of extinction can be inhibited by the treatment with ACTH, α-MSH and pitressin(3). These results indicate that ACTH and related peptides play an important role in maintenance of avoidance conditioned behavior.

Since synthetic ACTH as well as fragments of this peptide hormone have become available it was deemed of interest to study the effect of these peptides on the extinction of an avoidance response in intact rats in order to obtain information about the active part of the ACTH molecule responsible for this behavioral effect.

Materials and methods. Male white rats from an inbred strain weighing 140-180 g were used. Avoidance conditioning was studied in two different situations.

a. *Shuttle-box experiment.* Conditioning was performed in a 2-compartment box, divided into 2 equal compartments by a 5 cm barrier. The conditioned stimulus (CS) was the sound of a buzzer presented for 5 seconds prior to the unconditioned stimulus (US) of shock, delivered through the feet of the rat (40 V; 1.8 mA). If the animal crossed the barrier within 5 seconds, the CS was terminated and the rat avoided shock. Ten conditioning trials were given each day with a fixed intertrial interval averaging 60 seconds presented in a predetermined random sequence (4). Conditioning trials lasted till the rat had achieved criterion, *i.e.*, 80% or more avoidances during 3 consecutive days. Those rats which did not reach the criterion within 14 days were dropped from further participation in the experiment. The day after the criterion was reached extinction trials were run with the schedule and procedure as in conditioning, except that the US was never presented, and the CS, terminated after 5 seconds, if a barrier crossing had not occurred. Extinction was studied for 14 days.

b. *Pole jumping experiment.* Conditioning was performed in a square box of 29.5 cm length and width and 25 cm high, equipped with a grid floor through which the US of shock (25 V; 5 mA) could be delivered. The CS was a light produced by a 60 Watt bulb placed on top of the box presented for 5 seconds and followed by the US if the rat had not escaped by jumping into a pole of 1.5 cm diameter fixed in the middle of the box from top to bottom.

Ten conditioning trials were given each day for 3 days with a fixed intertrial interval, averaging 60 seconds in a predetermined random sequence. Rats which made more than 10 positive responses during these 3 days were used for extinction trials, run with the same schedule and procedure as in learning for the next 3 consecutive days.

The total number of conditioned responses (CR's) of every rat scored during acquisition or during extinction served as an index of avoidance behavior of the animal. Significant differences in CR's between groups were determined using Wilcoxon's 2-sample test(5).

Following substances were used: ACTH (A_1 peptide (Organon Comp.); synthetic ACTH β 1-24 (Ciba); purified β-MSH (Dr. Roger Guillemin); synthetic α-MSH (Ciba); ACTH fragments: ACTH 1-10, ACTH 5-10, ACTH 11-24 (Organon Comp.); protamine zinc insulin (PZI) 40 IU/ml (Leo).

All substances were administered as long acting preparations by addition of zinc and phosphate. The zinc phosphate complex was prepared as follows: 200 μg of the respective peptide was dissolved in 6 ml 0.01 N HCl. A mixture containing 0.3 ml $ZnCl_2$ (104 mg $ZnCl_2$ per ml), 1 ml Na_2HPO_4 (6.3 mg Na_2HPO_4 2 aq per ml + 35.0 mg NaCl per ml) and 0.5 ml 0.1 N HCl was added to the peptide solution under stirring. The pH of the final mixture was then brought to 7.8-8.0 with 0.5 N NaOH and the total volume of the complex was subsequently adjusted to 10 ml with distilled water. The freshly prepared long acting complexes were not used longer than 1 week in order to avoid loss of potency. Zinc phosphate complex without peptide material served as the placebo. Substances were injected subcutaneously, starting immediately after the last conditioning trial of the day the conditioning criterion was reached. In the shuttle-box experiment rats were treated every other day for 14 days. In the pole jumping experiment treatment was given only once.

TABLE I. Effect of ACTH and Related Peptides Administered During Extinction on the Rate of Extinction of the Avoidance Response in the Shuttle-Box Experiment.

Treatment	No. of conditioned avoidance responses (CR's) Learning	Extinction
ACTH (A_1 peptide), 3 IU every 2 days	42 ± 3.3*	135 ± 1.5 (8)
Placebo, 0.1 ml every 2 days	39 ± 4.3	61 ± 2.8 (7)
Purified β-MSH, 6 μg every 2 days	50 ± 2.5	133 ± 3.3 (10)
Placebo, 0.3 ml every 2 days	51 ± 2.3	53 ± 3.0 (10)
Synthetic α-MSH, 6 μg every 2 days	54 ± 2.2	111 ± 6.9 (12)
Placebo, 0.3 ml every 2 days	55 ± 1.7	41 ± 4.8 (11)
Protamine zinc insulin 2 IU every 2 days	43 ± 2.4	68 ± 5.0 (12)
Placebo, 0.05 ml every 2 days	41 ± 2.8	58 ± 2.5 (11)

* Mean ± standard error of the mean.
( ) No. of animals used.

Results. a. *Shuttle-box experiment.* Results with ACTH (A_1 peptide), β- and α-MSH, and PZI on extinction of the avoidance response are summarized in Table I.

The conditioned response in non-treated animals was generally extinguished within 10 days. Mean CR's during extinction of all placebo treated rats varied between 41 ± 4.8 and 61 ± 2.8. However, the number of positive responses in animals treated with ACTH (A_1 peptide), β- and α-MSH was significantly greater than that of controls, indicating that these 3 peptides delayed extinction of the avoidance response. Treatment with PZI did not materially affect the rate of extinction of the avoidance response.

b. *Pole jumping experiment.* The same peptides as in Experiment a were studied in the pole jumping situation. Table II shows that ACTH, β- and α-MSH exhibited similar effects on extinction under these conditions as in the shuttle-box experiment. In treated

TABLE II. Effect of ACTH and Related Peptides Administered During Extinction on the Rate of Extinction of the Avoidance Response in the Pole Jumping Experiment.

Treatment	No. of conditioned avoidance responses (CR's) Learning	Extinction	
ACTH (A_1 peptide), 3 IU	12 ± 0.7*	23 ± 0.8	(8)
Purified β-MSH, 6 μg	14 ± 0.7	20 ± 1.3	(9)
Synthetic α-MSH, 6 μg	13 ± 0.6	19 ± 0.8	(10)
Protamine zinc insulin 2 IU	15 ± 0.9	7 ± 1.4	(9)
Placebo, 0.3 ml	13 ± 0.2	9 ± 0.8	(9)

* Mean ± standard error of the mean.
( ) No. of animals used.

rats significantly more CR's were scored during extinction. PZI again failed to affect the number of conditioned responses.

In a subsequent experiment, the influence of the vehicle on the effect of ACTH (A_1 peptide) on extinction was studied. For this purpose the hormone was administered in partially hydrolyzed gelatine (3 parts of gelatine, and 1 part of ACTH solution in acid saline). It appeared that approximately 15 times as much ACTH had to be administered under these conditions to obtain a significant effect (Table III).

Table IV summarizes results with synthetic ACTH β 1-24 and several ACTH fragments. The synthetic ACTH was highly effective in delaying extinction of the avoidance response. The peptide ACTH 1-10 was nearly as active as the 24 amino acid molecule. In contrast, ACTH 11-24 only slightly affected the number of CR's; the difference between peptide

TABLE III. Effect of ACTH (A_1 Peptide) Administered in Partially Hydrolyzed Gelatine During Extinction, on the Rate of Extinction of the Avoidance Response in the Pole Jumping Experiment.

Treatment	Conditioned avoidance responses (CR's) Learning	Extinction	
ACTH (A_1 peptide), 15 IU every day during 3 days	14 ± 0.8*	16 ± 0.9	(8)
Placebo gelatine, 0.4 ml every day during 3 days	14 ± 0.5	8 ± 0.9	(7)

* Mean ± standard error of the mean.
( ) No. of animals used.

treated and placebo treated rats, however, was not significant. The administration of ACTH 5-10 peptide also induced a significantly greater score in the number of CR's. The effect was weaker than that of ACTH 1-10. Injection of the 5-10 peptide during 3 consecutive days did not further enhance its effect on extinction of the avoidance response.

Discussion. These results show that ACTH and related peptides, administered during extinction trials, delay the rate of extinction of an avoidance response in 2 different experimental situations. The smallest peptide used (ACTH 5-10) was still capable of delaying the rate of extinction of the avoidance response although its potency was less than that

TABLE IV. Effect of Synthetic ACTH and ACTH Fragments Administered During Extinction on the Rate of Extinction of the Avoidance Response in the Pole Jumping Experiment.†

Treatment	Conditioned avoidance responses (CR's) Learning	Extinction
ACTH β 1-24 10 μg	15 ± 0.5*	23 ± 0.5
Placebo 0.5 ml	15 ± 0.5	10 ± 1.2
ACTH 1-10 10 μg	14 ± 0.7	20 ± 1.0
ACTH 11-24 10 μg	13 ± 0.4	13 ± 1.4
ACTH 5-10 10 μg	14 ± 0.5	16 ± 1.6
Placebo 0.5 ml	14 ± 0.7	9 ± 1.8
ACTH 5-10 10 μg every day during 3 days	15 ± 0.7	16 ± 1.2
Placebo 0.5 ml every day during 3 days	14 ± 0.6	9 ± 1.2

* Mean ± standard error of the mean.
† Mean of 8 observations.

of ACTH 1-10. Since ACTH 1-10, α- and β-MSH were approximately as potent as ACTH 1-24, the active part of the ACTH molecule affecting avoidance behavior presumably is located within the first 10 amino acids. This is supported by the fact that ACTH 11-24 failed to affect the rate of extinction of the avoidance response. No other fragments of ACTH were available for this study so that the precise structural requirements for the behavioral effect could not be determined.

The behavioral effect of the peptides as shown by the present experiments could be obtained only by the use of long acting zinc phosphate preparations. Apparently, the chronic presence of the peptides is obligatory

to exert the effect. A long acting gelatine preparation which is known to possess less potent corticotrophic activities than the zinc phosphate complex, also was considerably less active on extinction.

In view of the fact that ACTH and related peptides exhibit corticotrophic, lipid mobilizing, and melanotrophic effects, one might expect a relationship between these intrinsic activities and the observed behavioral effect.

These activities all depend on the presence of the N-terminal part of the ACTH molecule. The corticotrophic-, melanotrophic-(6) respectively lipolytic activity(7) vary with the chain length of ACTH, ACTH 1-24 being stronger than ACTH 1-10, respectively ACTH 1-16. In contrast, the effect of ACTH 1-10 and ACTH 1-24 on behavior is approximately equal. Accordingly, the behavioral effect, although related to the other 3 biological activities, can be differentiated from these on a structural basis.

The site of action of the peptides studied in the present experiments presumably is located within the central nervous system. In this respect it is noteworthy that ACTH and α-MSH have been shown to possess central nervous activities(8).

The active part seems to be confined to a relatively small peptide common to ACTH and MSH. It has been suggested that such peptides may be released under a variety of conditions(9). The marked effect of these peptides on behavior strongly suggests a possible physiological role of similar and related peptides of pituitary or central origin in this respect in the central nervous system. This is supported by the observations that removal of the posterior and intermediate lobe of the rat facilitates extinction of the avoidance response(3).

In what way these peptides interfere with neural mechanisms is not clear. Whether they act *via* autonomic nervous transmission or by a direct action on the cells remains to be investigated. In this respect it is worth noting that Cook(10) showed that the administration of yeast RNA delayed extinction of an avoidance response in rats in a similar fashion as that found with ACTH and related peptides. Recent findings in which transfer of conditioned responses was demonstrated by RNA extracts obtained from the brain of trained rats(11,12) suggest a possible interference of learned behavior through RNA participation. Findings of Ungar and Oceguera-Navarro(13) and of Rosenblatt *et al*(14), however, are indicative that a polypeptide is the information bearing molecule. The molecular weight of this peptide was preliminarily estimated between 1000 and 5000(14). This is well within the range of that of ACTH and related peptides used in the present experiments.

Summary. ACTH, administered during extinction, delays extinction of an avoidance response in rats. To study structure activity relationships, the rate of extinction of an avoidance response performed in a shuttle-box and a pole jumping situation, was investigated in rats treated during the extinction period with ACTH (A_1 peptide), synthetic ACTH β 1-24, β-MSH, α-MSH, ACTH 1-10, ACTH 5-10, and ACTH 11-24, administered as long acting zinc phosphate preparations. Zinc phosphate complex and protamine zinc insulin (PZI) were used as placebo control substances. It appeared that ACTH and related peptides significantly delayed the rate of extinction of the avoidance response. Full activity was obtained with ACTH 1-10 peptide while ACTH 5-10 was less active. ACTH 11-24 and PZI were without effect. These results were interpreted to indicate that the active part of ACTH in this respect is located in the N-terminal part of the molecule, presumably in the first 10 amino acids.

The generous supply of the synthetic ACTH β 1-24 and the α-MSH by Ciba, the ACTH fragments by the Organon Company, and the purified β-MSH by Dr. Roger Guillemin is gratefully acknowledged.

1. Murphy, J. V., Miller, R. E., J. comp. physiol. Psychol., 1955, v48, 47.

2. Miller, R. E., Ogawa, N., ibid., 1962, v55, 211.

3. de Wied, D., Int. J. Neuropharmacol., 1965, v4, 157.

4. Murphy, J. V., Miller, R. E., J. Comp. Physiol. Psychol., 1956, v52, 77.

5. Wilcoxon, F., Biometrics, 1945, v1, 80.

6. Schwyzer, R., Ann. Rev. Biochem., 1964, v33, 259.

7. Lebovitz, H. E., Engel, F. E., Metabolism, 1964, v13, 1230.

8. Ferrari, W., Gessa, G. L., Vargiu, L., Ann. N. Y. Acad. Sci., 1963, v104, 330.

9. Li, C. H., Surv. Biol. Progress, 1962, v4, 93.

10. Cook, L., In: Animal Behavior and Drug Action, J. A. Churchill, Ltd., 1964, 91-119.

11. Fjerdingstad, E. J., Nissen, Th., Roigaards-Petersen, H. H., Scand. J. Psychol., 1965, v6, 1.

12. Babich, F., Jacobson, A. L., Bubash, S., Jacobson, A., Science, 1965, v149, 656.

13. Ungar, G., Oceguera-Navarro, C., Nature, 1965, v207, 301.

14. Rosenblatt, F., Farrow, J. T., Herblin, W. F., ibid., 1966, v209, 46.

Received February 4, 1966, P.S.E.B.M., 1966, v122.

Interferon Production by Human Leucocytes *in vitro*. Reduced Levels In Lymphatic Leukemia.* (31043)

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Humans with lymphatic leukemia, acute or chronic, have reduced resistance to infection by fungi and bacteria(1). They also seem to be peculiarly prone to develop serious infections by certain viruses which in normal subjects usually produce self-limited illnesses, *e.g.*, measles(2) chickenpox(3) and vaccinia (4). Other than these specific examples, however, there is no overall evidence of increased susceptibility to virus infection in leukemic patients.

The production of interferon by human leucocytes *in vitro* has been shown by several investigators(5,6,7). We have previously shown that both polymorphonuclear and mononuclear leucocytes share the capacity to produce interferon when stimulated by Sendai virus(6).

The present paper relates to similar studies of *in vitro* interferon production by suspension of leucocytes from patients with acute or chronic lymphatic leukemia.

Materials and methods. Viruses. Stock Sendai virus used to induce interferon formation in leucocyte cultures was grown in allantois of 9-11-day-old embryonated eggs and concentrated by high speed centrifugation as previously described(6). Sindbis virus was propagated and titrated in established human amnion cells (HA-FL)(6). For purposes of interferon assay, approximately 1,000 $TCID_{50}$ of the virus was used.

Tissue culture. Interferon assay was carried out using monolayer tube cultures of HA-FL cells as previously reported(6).

Preparation of leucocyte suspensions. The procedure for preparing leucocyte suspensions from normal donors and leukemic patients was as described previously(6). Dextran in a proportion of 1:6 was added to the heparinized blood sample to aid sedimentation of erythrocytes. The mixture was allowed to stand for 30 minutes or longer at 37°C. The leucocyte-rich plasma was removed and sedimented at 2,500 rpm for 15 minutes. The sedimented leucocytes were washed 3 times and resuspended in medium 199 containing 10% calf serum. A total cell count was then performed. Cytogenetic examination was also conducted in a number of normal control specimens and the karyogram shown to exhibit the normal number of 46 chromosomes.

Interferon production and assay. The production of interferon was carried out by the method already described(6). For comparative purposes, each blood sample obtained from leukemic patients was tested along with that of a normal control subject, during each experiment. Each cell suspension so prepared was divided into 2 aliquots. To one of these, stock Sendai virus at an input of 10-50 EID_{50}/cell was added, and to the other, the control, an equal volume of medium, free of

* The studies were supported by the Medical Research Council of Canada, Grant MA 1461.