There was, however, no significant difference in the virus titer of the spleens in control and test animals.

Among animals receiving the transplantable tumor, mitomycin C, TEM, 6MP and urethane, all significantly inhibited tumor growth, although the incidence of takes in test and control animals surviving to the end of the experiment was about the same. Estradiol had little effect on rate of tumor growth. The titer of virus in the tumors and spleens showed no significant differences between test and control animals. Mitomycin C proved toxic in the dosage given and the number of survivors was small; this accounted for our failure to titrate the tumors, and may also account for the low virus titer in the spleen. The spleens of tumor-bearing animals were smaller than those encountered in animals receiving cell-free virus preparations. However, the spleens in the former always yielded slightly more virus than the tumors, findings in agreement with those reported previously(5).

Discussion and summary. The results show that neither estradiol nor mitomycin C influence the replication of Friend virus as judged by virus titer in the spleen, although both inhibit development of the leukemia following inoculation of cell-free virus(1,2).

The results also show that mitomycin C, TEM, 6MP and urethane inhibited the growth of cellular implants of a transplantable reticulum cell sarcoma variant of Friend leukemia, although none of these substances affected the concentration of virus in either the spleen or tumor. Estradiol occupied an anomalous position in that it inhibited the development of splenomegaly in animals receiving both cell-free virus and tumor transplants, but did not inhibit the growth of the subcutaneous tumor.

While these drugs influence the response to Friend virus, it seems likely that their withdrawal, even after complete suppression of the original cellular response, would be followed by recrudescence of leukemia from new foci of neoplasia induced by the large amount of virus in the tissues. Evidence to support this concept has been reported for Maloney virus by Glynn *et al*(6). The fallacy of using only one parameter in assessing the results of chemotherapy is highlighted. In the case of virus-induced neoplasms, treatment should ideally inhibit the growth of both the neoplastic cells and the virus.

1. Mirand, E. A., Back, N., Prentice, T. C., Ambrus, J. L., Grace, J. T., Proc. Soc. Exp. Biol. and Med., 1961, v108, 360.

2. Sugiura, K., Cancer Res., 1962, v22, 93.

3. Fieldsteel, A. H., Dawson, P. J., Bostick, W. L., Proc. Soc. Exp. Biol. and Med., 1961, v108, 826.

4. Dawson, P. J., Fieldsteel, A. H., Bostick, W. L., Cancer Res., 1963, v23, 349.

5. Fieldsteel, A. H., Dawson, P. J., Bostick, W. L., ibid., 1963, v23, 355.

6. Glynn, J. P., Moloney, J. B., Chirigos, M. A., Humphreys, S. R., Goldin, A., ibid., 1963, v23, 269.

Received January 5, 1965. P.S.E.B.M., 1965, v119.

Effect of Hypothalamic Extracts on Release of Growth Hormone *in vitro*.* (30138)

ANDREW V. SCHALLY, SANFORD L. STEELMAN AND CYRIL Y. BOWERS

Endocrine and Polypeptide Laboratories, Veterans Administration Hospital and Department of Medicine, Tulane University School of Medicine, New Orleans, La. and Merck Institute for Therapeutic Research, Rahway, N. J.

Although the impairment of growth in rats and dogs with hypothalamic lesions and a significant fall in pituitary growth hormone content of lesioned rats have been demonstrated(1-4), direct evidence of hypothalamic regulation of growth hormone secretion has been lacking.

Recently Franz *et al*(5) claimed the pres-

* Supported in part by USPHS Grants AM 07547-01, AM 07467-01 and AM 08743-01.

ence of a somatotropin releasing factor in acetic acid extracts of hog hypothalamus while Deuben and Meites(6) showed that rat hypothalamic extracts increased the release of growth hormone in tissue cultures of rat anterior pituitaries.

The work reported here was undertaken to corroborate and extend the reports(5,6) concerning the presence of growth hormone releasing factor (GRF) in hypothalamic extracts, to find GRF activity in hypothalamic extracts of domestic animals suitable for purification work and to develop a test for detection of GRF activity.

Materials and methods. Fragments of rat, beef, sheep and pig hypothalamic tissue, containing mainly the pituitary stalk and the median eminence (SME) area, were collected on dry ice and lyophilized. The fragments were then defatted with acetone and petroleum ether and extracted with 2 N acetic acid at 8°C. The extracts were quickly heated to boiling, chilled on ice, and lyophilized. Details of extraction have been reported(7). Lyophilized hypothalamic extracts stored in high vacuo over pellets of NaOH were extracted with incubation medium. The suspension was spun at 15,000 rpm at 4°C, pH was checked with a glass electrode on a Metrohm pH meter E-300 and the supernatant was added to incubated pituitaries.

Incubations. Pituitary incubations were carried out by the method of Saffran and Schally(8). Male rats of Holtzman Sprague-Dawley strain, 150 ± 10 g were used as pituitary donors in the experiments. Each anterior pituitary was cut in half(8) and transferred to a 30 ml Pyrex beaker containing 7.5 ml Krebs-Ringer bicarbonate medium with 200 mg% glucose(9). In some experiments rats were starved overnight and glucose in the medium was omitted since it has been reported(10) that hypoglycemia is a potent stimulus for release of growth hormone. A total of 6-8 pituitary halves was placed in each of 2 flasks, one flask serving as control and the other with an equal number of anterior pituitary halves from the same rats, being incubated with hypothalamic extracts. After an incubation period of 6-9 hours, the medium was filtered through a fine plug of washed glass wool and kept frozen until assayed. The assay of growth hormone activity in the incubation media was performed by the tibia test method of Greenspan *et al* (11). Five to eight hypophysectomized female rats were used to assay each sample. USP reference preparation of growth hormone was used as the standard. The estimates of per cent stimulation of growth hormone release from pituitary incubates are approximate since they were computed for the ratio of stimulated *vs* unstimulated (control) incubates from bracketed (3 point) assays using tibia width response curve to growth hormone standards in the same experiment.

Results and discussion. Anterior pituitary tissue from young male rats, when incubated *in vitro* in KRBG medium for periods of 6-9 hours, released 0.15-0.5 μg growth hormone/mg pituitary tissue/hour.

Rat or sheep hypothalamic extracts added to incubation media did not accelerate the release of growth hormone. This might reflect weaker GRF activity rather than absence of such activity. Addition of SME extracts of porcine and bovine origin resulted in significant stimulation of the release of growth hormone *in vitro*. The results are seen in Table I. Porcine extracts caused a 200-700% stimulation of release of growth hormone into the medium. Bovine extracts enhanced *in vitro* secretion of growth hormone by 270-720%. In 9 tests the SME extracts when injected directly into hypophysectomized animals at the dose of 0.5-4 SME had no effect on the tibia width, indicating absence of contamination with growth hormone. This lack of growth hormone activity in SME extracts is in contrast to a considerable contamination of such extracts of ovine, bovine, porcine or canine origin with other tropic hormones such as ACTH(12-14), luteinizing hormone (7,15), thyrotropic hormone(16), and follicle stimulating hormone(17). It is likely that the heat and acid treatment(7) of these extracts contributed to a decrease in growth hormone activity. It is of interest that addition of 0.3-1.6 U arginine vasopressin to isolated rat pituitaries was without effect on the secretion of growth hormone *in vitro*.

TABLE I. Effect of SME Extracts on Release of Growth Hormone *in vitro*.

Sample	Addition before incubation	Response mean tibia width ($\mu \pm$ S.E.)	P*	% Stimulation
Media from control pituitaries	—	185 ± 11	—	
" " treated "	8.3 rat SME	191 ± 12	N.S.	
" " control "	—	177 ± 8	—	
" " treated "	.83 sheep SME	174 ± 8	N.S.	
" " control "	—	156 ± 9	—	
" " treated "	1.25 hog SME	214 ± 11	.01	>700
Extract alone	8.3 rat SME	139 ± 10		
" "	.83 sheep SME	132 ± 4		
" "	1.25 hog SME	143 ± 2		
Control saline	—	146 ± 7		
20 μg GH	—	165 ± 4		
80 μg GH	—	194 ± 11		
Media from control pituitaries	—	160 ± 7.9	—	—
" " treated "	2.5 hog SME	194 ± 15	.005	340
" " control "	—	146 ± 7.1	—	—
" " treated "	1.0 beef SME	172 ± 3.5	.01	257
Extract alone	2.5 hog SME	143 ± 7.3		
" "	1.0 beef SME	138 ± 3.9		
Control saline	—	149 ± 5.7		
20 μg GH	—	175 ± 7.2		
100 μg GH	—	220 ± 12.8		
Media from control pituitaries	—	139 ± 5		
" " treated "	300 mU arginine vasopressin	140 ± 6		
" " control " 11C	—	169 ± 5		
" " treated " 11E	1.6 U arginine vasopressin	171 ± 9		
" " control "	—	165 ± 9		
" " treated "	1.8 sheep SME	168 ± 7		
" " control "	—	165 ± 6		
" " treated "	2.0 sheep SME	159 ± 5		
" " control "	—	156 ± 4		
" " treated "	4.0 sheep SME	145 ± 7		
" " control "	—	155 ± 7		
" " treated "	2 sheep SME	169 ± 9		
" " control "	—	172 ± 3		
" " treated "	2 sheep SME	183 ± 7	.1–.2	
" " control "	—	154 ± 7.8		
" " treated "	1 beef SME	202 ± 8.5	.001	720
" " control "	—	142 ± 11		
" " treated "	2 pig SME	187 ± 17	.05	600
Saline	—	128 ± 7.2		
5 μg GH	—	179 ± 12.5		
40 μg GH	—	230 ± 13.9		
Saline	—	151 ± 2.8		
Extract alone	1 beef SME	138 ± 10		
" "	2 " "	139 ± 8.3		
" "	2 pig "	137 ± 7.5		
GH	40 μg	194 ± 12.8		
Media from control pituitaries	—	174 ± 8.7		
" " treated "	2 pig SME	208 ± 9.6	.05–.01	200†‡
" " control "	—	162 ± 8.4		
" " treated "	1 beef SME	203 ± 7.3	.01	270†
Saline control	—	140 ± 4.6		
5 μg GH	—	147 ± 4.0		
40 μg GH	—	237 ± 4.7		

TABLE I (continued).

Sample	Addition before incubation	Response mean tibia width ($\mu \pm$ S.E.)	P*	% Stimulation
Media from control pituitaries	—	177 ± 6.2		
" " treated "	1 pig SME	208 ± 3.9	.01–.001	
Saline control	—	156 ± 11.6		
Extract alone	2 pig SME	145 ± 3.5		
" "	1 beef SME	137 ± 7.9		
40 μg GH	—	238 ± 17		
100 μg GH	—	265 ± 14		

* Significance of stimulation *vs* corresponding control.
† Used KRBG containing only 40 mg % glucose.
‡ Pituitary donors starved overnight.

Although Bogdanove(1) and Reichlin(3,4) showed that hypothalamus may be involved in release of growth hormone, the evidence was indirect and thyroidal, gonadal and adrenocortical deficiencies caused by the hypothalamic lesions may have contributed to the impairment of growth. A possible non-specific damage to the primary plexus of hypophysial portal vessels, through which the rat passes most of the blood to the anterior pituitary, further complicates the interpretation of their results.

Direct evidence for hypothalamic regulation of growth hormone secretion was provided recently by Franz *et al*(5) and Deuben and Meites(6). Franz *et al*(5) used the method of Saffran and Schally(8) for detection of GRF activity but assayed the incubation media together with homogenates of the pituitaries stimulated by addition of hypothalamic extracts. Their data may reflect the increased synthesis of growth hormone in the pituitary tissue rather than release of growth hormone into the medium. Deuben and Meites(6) showed increased release of growth hormone after addition of rat hypothalamic extracts during a 6-day tissue culture period of rat anterior pituitaries. This may also reflect synthesis and a gradual release into the media rather than relatively rapid release as shown in our experiments. Pecile *et al*(18) in *in vivo* experiments demonstrated that rat hypothalamic extracts can indeed induce a marked depletion of pituitary growth hormone content.

A careful investigation of a variety of materials may be needed to clearly establish the specificity of the Saffran and Schally pituitary incubation method(8), we have modified in this paper, as a test for the detection of GRF activity. However, this method has been reevaluated and found specific as an assay for corticotropin releasing factor (CRF)(19). It has also been used successfully for the characterization of thyrotropic hormone releasing factor (TRF)(20,21), luteinizing hormone releasing factor (LRF)(22) and follicle stimulating hormone releasing (FSH-RF)(23). In all cases there was a good agreement with the data provided by the *in vivo* experiment. Franz *et al*(5) and Deuben and Meites(6) found that brain cortex extracts and synthetic posterior pituitary hormones had no effect on growth hormone release *in vivo* and *in vitro*. We found brain cortex extracts, selected hypothalamic fractions from Sephadex other than the GRF area, and lysine vasopressin inactive *in vitro*. Using the *in vivo* test developed by Pecile *et al*(18) we found that 100 mU lysine vasopressin and brain cortex extracts in contrast to SME extracts did not change the pituitary growth hormone content of normal male rats. In the same test pig and beef SME extracts effected a 75-80% depletion of pituitary growth hormone content as compared with rats injected with saline(24,25).

The data reported here suggest the presence of growth hormone releasing activity (GRF) in hypothalamic extracts of pigs and cattle. These may serve as suitable sources for purification of GRF activity. These studies also show that the pituitary incubation system may be used as a test for the detection of GRF activity.

Summary. The pituitary incubation tech-

nique of Saffran and Schally was utilized for detection of factors affecting the release of growth hormone *in vitro*. Pituitary stalk-median eminence extracts of porcine and bovine origin were shown to contain a growth hormone releasing factor (GRF).

We gratefully acknowledge the participation in this project of Dr. A. Kuroshima, Dr. Y. Ishida and Mr. T. W. Redding.

1. Bogdanove, E. M., Lipner, H. J., Proc. Soc. Exp. Biol. and Med., 1952, v81, 410.
2. Endronczi, E., Kovacs, S., Szalay, G., Endokrinologie, 1957, v34, 168.
3. Reichlin, S., Endocrinology, 1960, v67, 760.
4. ———, ibid., 1961, v69, 225.
5. Franz, J., Haselbach, C. H., Libert, O., Acta Endoc., 1962, v41, 336.
6. Deuben, R., Meites, J., Endocrinology, 1964, v74, 408.
7. Schally, A. V., Bowers, C. Y., ibid., 1964, v75, 608.
8. Saffran, M., Schally, A. V., Can. J. Biochem. Physiol., 1955, v33, 408.
9. Umbreit, W. A., Burris, R. H., Stauffer, J. F., Manometric techniques and tissue metabolism, Burgess, Minneapolis, 1951.
10. Roth, J., Glick, F. M., Yallow, R. S., Berson, S. A., Science, 1963, v140, 987.
11. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. M., Endocrinology, 1949, v45, 455.
12. Guillemin, R., Schally, A. V., Texas Rep. Biol. Med., 1963, v21, 541.
13. Guillemin, R., Schally, A. V., Lipscomb, H. S., Andersen, R. N., Long, J. M., Endocrinology, 1962, v70, 471.
14. Schally, A. V., Lipscomb, H. S., Long, J. M., Dear, W. E., Guillemin, R., ibid., 1962, v70, 478.
15. Guillemin, R., Jutisz, M., Sakiz, E., Compt. rend. Acad. Sci. (Paris), 1963, v256, 504.
16. Guillemin, R., Yamazaki, E., Jutisz, M., ibid., 1962, v255, 1018.
17. Courrier, R., Jutisz, M., Colonge, A., ibid., 1963, v257, 3773.
18. Pecile, A., Muller, E., Falconi, G., Martini, L., Endocrine Soc. Program, 1964 Meeting, San Francisco, 132.
19. Guillemin, R., Schally, A. V., Endocrinology, 1959, v65, 555.
20. Guillemin, R., Yamazaki, E., Gard, D., Jutisz, M., Sakiz, E., ibid., 1963 v73, 564.
21. Bowers, C. Y., Redding, T. W., Schally, A. V., ibid., in press.
22. Schally, A. V., Bowers, C. Y., ibid., 1964, v75, 312.
23. Kuroshima, A., Ishida, Y., Bowers, C. Y., Schally, A. V., ibid., 1965, v76, 614.
24. Kuroshima, A., Ishida, Y., Bowers, C. Y., Redding, T., Schally, A. V., Abstract, International Physiological Congress, Tokyo, 1965.
25. Schally, A. V., Bowers, C. Y., Kuroshima, A., Ishida, Y., Redding, T. W. and Kastin, A. J., Proceedings of the XXIII International Congress of Physiological Sciences, Tokyo, September 1965, p183-191, Excerpta medica.

Received January 8, 1965. P.S.E.B.M., 1965, v119.

Blood Coagulation and Hemostasis in Thoroughbred Horses.* (30139)

CHARLES F. ABILDGAARD AND ROGER P. LINK (Introduced by Irving Schulman)
Department of Pediatrics, University of Illinois College of Medicine, Chicago and Department of Physiology and Pharmacology, University of Illinois College of Veterinary Medicine, Urbana

Relatively few observations concerning the hemostatic mechanism of the horse are available and conflicting results have been reported(1-4). As part of a current investigation of epistaxis in race horses it was possible to evaluate coagulation factors, platelet number and adhesiveness and bleeding time in a group of thoroughbred horses. The effect of exercise on several coagulation factors was also measured.

* Supported in part by Research Grant H-07418 from Nat. Heart Inst. and an appropriation from Illinois State Legislature.

Materials and methods. The horses studied were all in active training during the 1964 racing season. Coagulation studies were done on 20 horses: 13 normal and 7 "bleeders." The latter had history of at least one episode of epistaxis during or after running, but were otherwise well. Their ages ranged from 2 to 7 years (mean 3.5 years). The group included 6 gelding, 6 mares and 8 stallions. Bleeding times were done on 9 additional normal thoroughbreds.

Blood was collected from the jugular vein through an 18 gauge needle into plastic