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ACTH and the Stress-Induced Changes of Lysine Incorporation into Brain and Liver Proteins

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DUNN, A. J., H. D. REES AND P. M. IUVONE. *ACTH and the stress-induced changes of lysine incorporation into brain and liver proteins.* PHARMAC. BIOCHEM. BEHAV. 8(4) 455–465, 1978. – When mice were subjected to footshock treatment and subsequently injected with [^{3}H]lysine, the cerebral uptake of [^{3}H]lysine, its incorporation into brain protein and the relative radioactivity (RR = protein radioactivity divided by amino acid radioactivity) were all increased. In the liver, footshocked mice showed decreased free lysine radioactivity, and increased protein radioactivity and relative radioactivity compared to quiet mice. The possibility that ACTH mediated these effects was investigated. The injection of saline had no effect in the brain but partially mimicked the footshock responses in the liver. Injections of ACTH 1–24 mimicked the effects of footshock in the brain, and further augmented the saline-induced effect on the RR in the liver. ACTH 4–10 increased the RR of brain protein, but produced no significant change in brain free lysine radioactivity or in any measure in the liver. Pretreatment of mice with the synthetic glucocorticoid, dexamethasone, did not enhance these effects and diminished the effect of ACTH 4–10 in the brain. ACTH treatment did not alter the profiles of brain polyribosomes. Lysine vasopressin, which is also released during stress, did not alter the incorporation of [^{3}H]lysine into brain or liver protein, except at high doses when it decreased plasma radioactivity. These results suggest that secretion of ACTH at least partially mediates the stress-induced changes of [^{3}H]lysine incorporation into brain and liver proteins, but that it is probably not the only factor involved.

ACTH ACTH 1–24 ACTH 4–10 Protein synthesis Footshock [^{3}H]lysine Lysine vasopressin Corticosterone Polyribosomes

PREVIOUSLY we have shown that avoidance training and other behavioral stressors increased the rate of brain and liver protein synthesis measured by the incorporation of [^{3}H]lysine into tissue proteins in the mouse [27] and rat [29]. The mechanism of these responses may involve one or another of the hormones secreted in stressful situations. The pituitary rather than the adrenal is probably involved because the biochemical responses were observed in adrenalectomized mice, and corticosterone injections did not result in biochemical responses [26]. ACTH has been implicated because dexamethasone, which blocks the release of ACTH from the pituitary, prevented the brain response and decreased the liver response [26]. In the present study, we tested the involvement of ACTH by administering ACTH 1–24 exogenously and observing the [^{3}H]lysine incorporation. To test the adrenal involvement we also used an ACTH analog, ACTH 4–10, reported to have no adrenocortical activity [7,32]. Since vasopressin is also released during stress [8] we also tested the biochemical responses to injected lysine vasopressin.

METHOD

Animals

C57B1/6J male mice about 6 weeks old were obtained from Jackson Laboratories, Bar Harbor, Maine. Experiments were performed on 6–10 week-old mice. Mice were group-housed until 3 days before an experiment, when they were individually caged. This is important since acute isolation itself causes increases in [^{3}H]lysine incorporation into mouse brain and liver protein [25].

Materials

ACTH 1–24 (Cosyntropin) and ACTH 4–10 (OI63) were generous gifts from Dr. Henck van Riezen, Organon International, Oss, the Netherlands. For the polyribosome experiments only, ACTH from Parke Davis, Detroit, Michigan was used. Lysine vasopressin (LVP) was obtained from Sigma Chemical Co., St. Louis, MO. Peptides were dissolved in physiological saline at concentrations of 5×10^{-5}M (ACTH 1–24), 7.5×10^{-5}M (ACTH 4–10) and $9 \times$

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10^{-5} M, 9×10^{-7} M or 9×10^{-9} M (LVP). Peptide solutions were made up fresh on the day of the experiments. [4,5-^{3}H]lysine (19 Ci/mmole) was obtained from Amersham Searle, Inc., Northbrook, Illinois.

Procedure

During the experiments mice were kept in a quiet room with as little disturbance as possible. Footshock was administered in the conditioned avoidance apparatus previously described [27] in a separate room. Twenty footshocks (0.3 mA, 1 sec) were administered randomly during 15 min and the mice returned to the quiet room for 20 min before [^{3}H]lysine injection. Mice were weighed just before their first injection. All injections were performed in the quiet room, injecting subcutaneously at the back of the neck. [^{3}H]lysine was injected at a dose of 1 μCi/g body weight. Except in the time course experiment the incorporation period was precisely 10 min. Immediately before sacrifice, mice were transferred in their cages to a second room where the biochemical procedures were performed. In some experiments trunk blood was collected in heparinized tubes and samples taken for radioactivity and corticosterone determination. After centrifugation plasma was aspirated off and frozen for subsequent fluorometric corticosterone determination [17].

The free amino acid contents of brain and liver were determined on extracts of whole brain or the right lobe of the liver. The tissue was homogenized in 5 volumes of 3% sulfosalicylic acid containing an internal standard of 0.5 mM norleucine. After centrifugation the supernatant was analyzed on a Durrum D-500 amino acid analyzer courtesy of Dr. Owen Rennert using the manufacturers recommendations. The values obtained were corrected using the internal standard.

[^{3}H]Lysine incorporation was measured as previously described [26,27]. The brain and a sample of the right lobe of the liver were homogenized as rapidly as possible in 5 ml of 0.05 M borate buffer (pH 9.2). Use of this high pH facilitates good homogenization, breaking up visible particles, prevents further protein synthesis, and discharges aminoacyl-tRNA [36]. Aliquots of the homogenates were then used to determine total tissue radioactivity (H), total tissue non-volatile radioactivity (D), and protein radioactivity (T) by trichloroacetic acid (TCA) precipitation, using the paper disc method of Mans and Novelli [23]. Samples were counted following digestion with Soluene 350 (Packard Instrument Co.).

Polyribosome analysis was performed on post-mitochondrial supernatants of brain tissue using 20–40% sucrose gradients as previously described [12].

To evaluate the effects of behavioral and hormonal treatments the data from all experiments have been combined. In this way we hoped to eliminate chance results that appeared in only one experiment. Statistical analysis was performed by two-factor (treatment x experimental replicate) analysis of variance using an SAS ANOVA program on an Amdahl 470 computer. Where only one experiment was involved, Dunnett's test for multiple comparisons was used.

Validation of Biochemical Procedures

Radioactivity lost on drying of the homogenate (H – D) was presumed to be in water (see also [4, 11, 34]). This ^3H_2O does not affect the results, since there is negligible incorporation of ^3H_2O into brain protein [18].

The radioactivity in the TCA-supernatant was analyzed by Dowex-50 chromatography [11]. The amino acids eluted from the Dowex were analyzed by paper chromatography using n-butanol-acetic acid-water (12:3:5, v/v/v) as solvent. Table 1 shows the results of this analysis which indicate that most of the TCA-soluble radioactivity in the brain was lysine in agreement with previous results [9, 21, 34]. This is also consistent with the slow catabolism of lysine in brain tissue [6,21]. Thus the difference between the dried homogenate and TCA-precipitable radioactivity (D – T) corresponds to the free lysine radioactivity in the tissue. It has been shown that under our conditions the blood content of excised C57Bl/6J mouse brains is 1.6% (by weight) and that for a 10 min pulse of [^{3}H]lysine correction for the blood decreases brain dpm values for free lysine by about 7% and for protein by less than 4% [13]. Thus changes in the cerebral content of blood could not account for the observed changes in whole brain radioactivity.

TABLE 1

INCORPORATION OF [^{3}H]LYSINE IN CHEMICAL FRACTIONS

	TCA-Soluble		TCA-Insoluble
Pulse Length Minutes	Percentage in Amines or Amino Acids*	Percentage in Lysine†	Percentage in Lysine†
10	96	88	98
60	96	85	96

Mice were injected subcutaneously with [^{3}H]lysine and fractions analyzed as described in the Methods section.

*Percentage of total dried TCA-supernatant bound to Dowex-50 and eluted by NH_4OH.

†^{3}H in lysine spot as percentage of ^{3}H recovered from chromatogram.

The results of the analysis of free amino acids in brain and liver following footshock, or saline or ACTH 1–24 injections are shown in Table 2. The only significant change observed was in liver histidine following footshock. Since the histidine levels were very low and the result was only just significant, we doubt this result is real, and would replicate. More important for the present analysis was that there were no significant changes in free lysine in the brain or the liver. This means that we may use the radioactivity in free lysine to correct for the protein labelling without having to measure the free lysine specific activity, since the specific radioactivity will be directly proportional to the radioactivity. This avoids the necessity for the tedious amino acid analysis in each tissue in each animal.

The radioactivity in the TCA-precipitable fraction was presumed to be protein since it was solubilized by pronase digestion [27]. Its chemical identity was established by hydrolysis of protein with 6 M HCl for 18 hr at 105°C and separation of the amino acids by chromatography as

TABLE 2

EFFECTS OF FOOTSHOCK OR ACTH ON TISSUE FREE AMINO ACIDS

	Brain				Liver			
Amino Acid	Quiet	Footshock	Saline	ACTH	Quiet	Footshock	Saline	ACTH
Taurine	658 ± 19	632 ± 15	629 ± 21	635 ± 17	920 ± 40	954 ± 31	901 ± 34	938 ± 76
Asparate	282 ± 4	287 ± 8	287 ± 3	277 ± 12	133 ± 10	137 ± 6	139 ± 8	142 ± 9
Threonine	31 ± 1	22 ± 2	21 ± 1	21 ± 1	15 ± 2	16 ± 2	14 ± 4	13 ± 2
Serine	212 ± 10	203 ± 13	205 ± 10	204 ± 12	137 ± 12	132 ± 12	135 ± 14	121 ± 14
Glutamate	740 ± 15	744 ± 29	761 ± 20	739 ± 29	148 ± 8	161 ± 14	172 ± 13	156 ± 12
Glycine	102 ± 5	106 ± 6	104 ± 4	101 ± 6	158 ± 13	148 ± 9	139 ± 9	133 ± 7
Alanine	30 ± 2	28 ± 3	32 ± 4	30 ± 4	155 ± 13	182 ± 13	162 ± 14	180 ± 23
Valine	–	–	–	–	27 ± 1	32 ± 4	32 ± 3	27 ± 3
Isoleucine	–	–	–	–	10 ± 1	12 ± 3	12 ± 1	9 ± 2
Leucine	–	–	–	–	16 ± 2	18 ± 3	17 ± 2	15 ± 2
Tyrosine	–	–	–	–	4.5 ± 0.5	4.5 ± 0.5	4.4 ± 0.5	4.7 ± 0.4
Phenylalanine	–	–	–	–	6.3 ± 0.5	7.2 ± 1.0	6.0 ± 0.6	6.8 ± 0.5
GABA	107 ± 4	111 ± 4	113 ± 5	109 ± 4	–	–	–	–
Histidine	9.5 ± 0.5	9.5 ± 0.5	10.3 ± 0.7	9.6 ± 1.0	39 ± 3	31 ± 2*	33 ± 1	30 ± 4
Ornithine	–	–	–	–	24 ± 4	34 ± 8	33 ± 1	24 ± 4
Lysine	18.7 ± 1.3	18.2 ± 1.2	18.2 ± 1.9	17.6 ± 1.6	29 ± 3	26 ± 7	29 ± 3	27 ± 4
Arginine	8.5 ± 0.5	9.0 ± 0.5	9.0 ± 1.0	8.4 ± 0.4	–	–	–	–

*Significantly different from Quiet, $p<0.05$ (Student's t-test)

The free amino acid content is expressed in μmoles per 100 g fresh tissue weight (mean ± SEM). Mice (six per group) were sacrificed 30 min following a period of footshock as described in the text, or 25 min after an injection of ACTH 1-24 or saline. Details of the procedures and analyses are described under Methods.

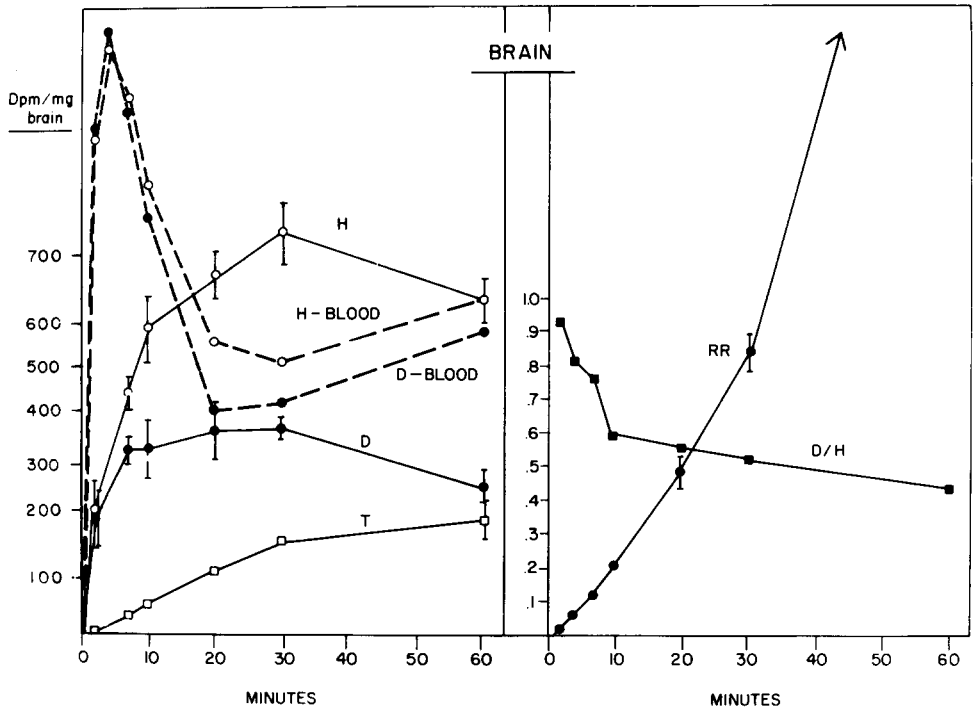

FIG. 1. Time course of labelling of blood and brain fractions with [4,5-^{3}H]lysine. [^{3}H]lysine was injected subcutaneously into C57B1/6J male mice (6 per group) and animals sacrificed at various later times. A. Solid lines brain tissue: open circles (H), total brain homogenate ^{3}H; solid circles (D), dried homogenate ^{3}H; squares (T), TCA-insoluble ^{3}H. Broken lines blood: open circles (H-BLOOD), total ^{3}H per 2.5 μl; solid circles (D-BLOOD), ^{3}H per 2.5 μl after drying. B. Circles (RR), brain relative radioactivity (=T/D-T); squares (D/H), proportion of homogenate ^{3}H not volatile. Bars represent the SEM; except for blood, their absence implies the SEM is smaller than the symbol.

described above. As Table 1 shows most of the radioactivity was recovered in the lysine spot confirming previous results [9,34]. Thus the TCA-insoluble radioactivity (T) represents protein-bound [^{3}H]lysine.

RESULTS

Figure 1 shows the time course of the incorporation of [^{3}H]lysine into the various brain fractions. The blood levels of ^{3}H reached a maximum approximately 4 min after injection, and then declined fairly rapidly. The amount of blood ^{3}H that was volatile (^3H_2O) increased with time. This probably reflects catabolism by peripheral tissues, because very little volatile ^{3}H is detected in brain following intracranial [^{3}H]lysine injections, (Dunn, unpublished observations). Total brain ^{3}H increased linearly for the first 10 min, after which the rate of uptake decreased, and total ^{3}H declined after 30 min. The proportion of ^3H_2O in the brain increased with time, especially noticeable in the ratio of dried to undried homogenate (Fig. 1B). Calculations correcting for volume showed that the ^3H_2O was equilibrated between blood and brain (and blood and liver). Following a short lag (2–3 min), the incorporation of ^{3}H into protein increased linearly for the first 30 min, with a slower rate of increase between 30 and 60 min. The relative radioactivity (RR = protein radioactivity/free lysine radioactivity) increased approximately linearly for the first 30 min and then the rate accelerated, principally because of a decline in the TCA-soluble ^{3}H (free lysine). The linearity with respect to time of the increases in brain RR suggests that it is a useful measure for estimating the rate of protein synthesis in the first 30 min, and that large fluctuations in pool radioactivity in cellular compartments do not occur.

Figure 2 shows the equivalent data for the liver. In this tissue the ^{3}H uptake reached a maximum earlier than in brain and there was a decline in all fractions between 30 and 60 min. This decline most probably reflects export of plasma proteins from the liver. The proportion of ^3H_2O in the liver was much lower than in the brain. Since the ^3H_2O was equilibrated between the blood and the liver, this reflects the greater ^{3}H uptake in terms of tissue weight (5 times that of brain). The liver RR was linear for the first 10 min, increased more rapidly up to 30 min, and then more slowly between 30 and 60 min. These data are in good agreement with those obtained using [^{14}C]lysine [21].

In all subsequent experiments we used a 10 min pulse of [^{3}H]lysine to estimate the rate of protein synthesis, since at this time the incorporation of ^{3}H into protein and the RR were still increasing linearly in both brain and liver. In the tables of data we have listed the mean values for the

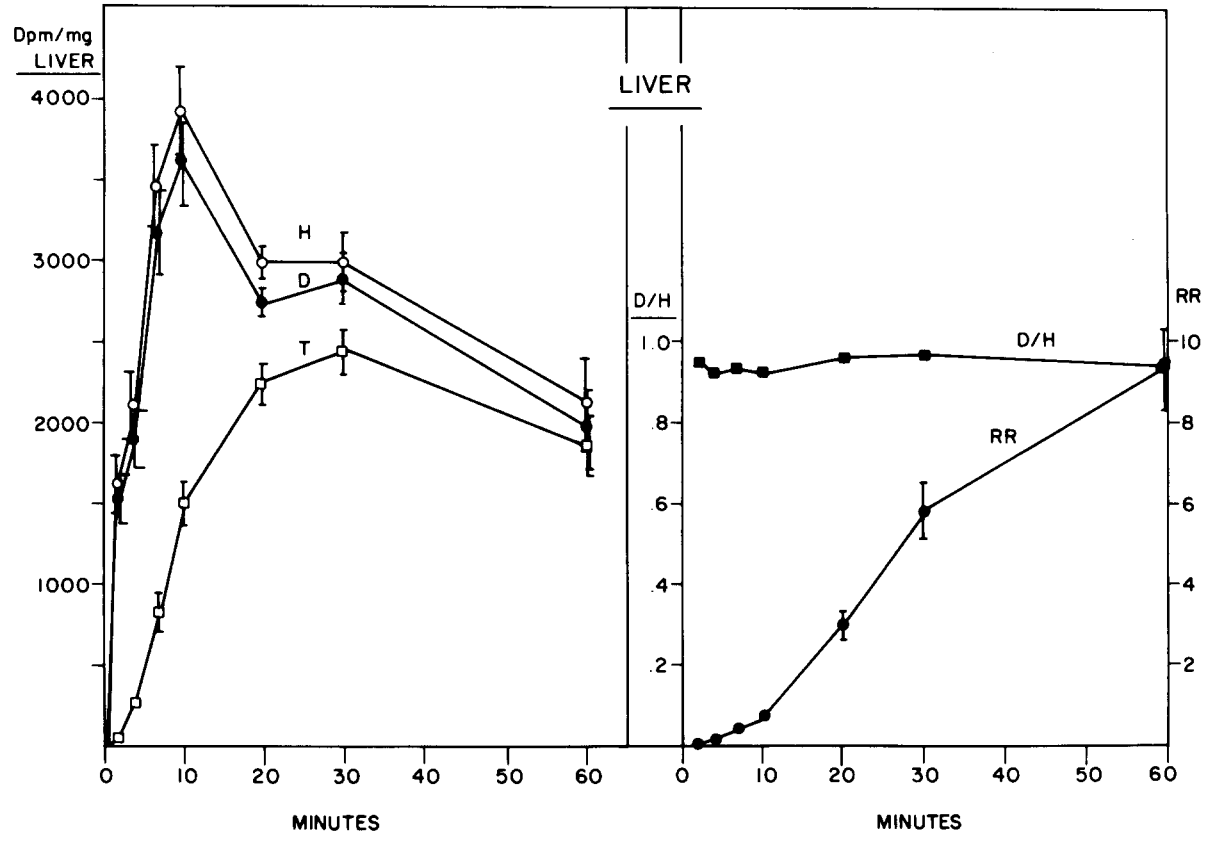

FIG. 2. Time course of labelling of liver fractions with [4,5-^{3}H]lysine. The same experiment as Fig. 1. A. Open circles (H), total liver homogenate ^{3}H per mg liver; solid circles (D) dried homogenate ^{3}H per mg liver; squares (T), TCA-insoluble ^{3}H per mg liver. B. Circles (RR), liver relative radioactivity (=T/D-T); squares (D/H), proportion of homogenate ^{3}H not volatile.

dpm in free lysine (D − T), for dpm in protein (T), and the relative radioactivity (RR). Each table (except Tables 7 and 8) contains the combined data from several separate experiments, including all experiments in which the particular experimental comparison was made. Thus a control group from one experiment may be represented in the data of more than one table.

Table 3 shows the combined data for the effect of footshock on [^{3}H]lysine incorporation into brain and liver for all experiments performed on C57Bl/6J mice in our present laboratory. As previously reported [26,27], footshock significantly increased the brain free lysine and protein radioactivity and the RR. In the liver, the free lysine radioactivity was decreased, whereas both the protein radioactivity and the RR were increased.

In the footshock experiments, the control mice were isolated and undisturbed until the [^{3}H]lysine injection. However, the most appropriate control for a hormone injection is a vehicle injection. But, the injection itself is stressful, as evidenced by the elevation of plasma corticosterone following a saline injection (Table 4 caption). Thus we first compared saline-injected with quiet mice (Table 4). The saline injection did not significantly alter the incorporation of [^{3}H]lysine into brain, but in the liver, the free [^{3}H]lysine was significantly decreased and the RR significantly increased. These changes in the liver resemble those caused by footshock (Table 3).

In Table 5 are shown the data from 5 separate experiments in which mice were injected with ACTH 1–24 (0.6 μg or 60 mU/g) or saline, 15 min before the [^{3}H]lysine pulse. ACTH 1–24 significantly elevated plasma corticosterone. In the brain there were significant increases in the free lysine and protein radioactivity and the RR just as in the footshocked mice. In the liver there were significant increases in the protein dpm and the RR. These changes were smaller than those observed following footshock, but to be comparable to the footshock data, these changes must be superimposed on the changes induced by saline (Table 4). However, ACTH 1–24 did not decrease the free lysine dpm as footshock did.

To test the involvement of the adrenal glands in the responses to ACTH, we injected ACTH 4–10 which has been reported to have no adrenocortical activity in the rat [7,32]. In our experiments with mice, ACTH 4–10 produced an elevation (27%) of plasma corticosterone that was significant (Table 6) but less pronounced than with ACTH 1–24 (83%). This result was observed in each of the 3 experiments in Table 6. Since this was an important point, we injected 2 further groups of 10 CD-1 mice each with ACTH 4–10 (0.33 μg/g) or saline and sacrificed them 15 min later. The plasma corticosterone values assayed were: Saline, 16.0 ± 1.1; ACTH 4–10, 21.8 ± 2.7 μg/100 ml. This 36% increase was statistically significant ($p<0.05$, Students t-test, one-tailed). When we performed a 2-factor

TABLE 3

THE EFFECT OF FOOTSHOCK ON [^{3}H]LYSINE INCORPORATION INTO MOUSE BRAIN AND LIVER

		Free Lysine dpm/mg		Protein dpm/mg		Relative Radioactivity	
	N	Mean ± SEM	% Δ	Mean ± SEM	% Δ	Mean ± SEM	% Δ
Brain							
Quiet	24	257 ± 8	[0]	41.0 ± 2.2	[0]	0.158 ± 0.004	[0]
Footshock	23	285 ± 9*	+11	50.5 ± 2.5‡	+24	0.176 ± 0.005†	+11
Liver							
Quiet	24	1434 ± 98	[0]	937 ± 69	[0]	0.673 ± 0.042	[0]
Footshock	23	1178 ± 60*	−18	1355 ± 74‡	+45	1.173 ± 0.054‡	+74

*Significantly different from quiet, $p<0.01$; †$p<0.001$; ‡$p<0.0001$ (ANOVA)

C57B1/6J mice were given 20 footshocks (1 sec, 0.3 mA) in 15 min or left quiet. Twenty min following the footshock session they were injected with [^{3}H]lysine and sacrificed 10 min later. The results of three separate experiments (Quiet n=10,7,7; Shock n=9,8,7 respectively) have been combined in this table. The changes in all measures occurred in all experiments. The increases in brain and liver protein radioactivity and the liver RR were significant in all experiments. The increase in brain RR was significant in 2 of the 3 experiments. Plasma corticosterone (2 experiments only): Quiet 13.7 ± 2.5; Footshock 21.8 ± 2.0 μg/100ml ($p<0.02$).

analysis of variance on all the experiments, the effect of ACTH 4–10 was statistically significant ($p<0.005$).

In contrast to ACTH 1–24, ACTH 4–10 had no effect on [^{3}H]lysine incorporation in the liver, but significantly increased the brain RR, with a nonsignificant increase in brain protein dpm and no change in free lysine dpm (Table 6).

In an attempt to accentuate the effects of ACTH, we suppressed endogenous ACTH secretion by pretreatment with dexamethasone. The dexamethasone treatment decreased plasma corticosterone levels in quiet mice from 13.7 ± 2.5 to 1.3 ± 0.5 μg/100 ml ($p<0.001$). In dexamethasone-treated mice, ACTH 1–24 significantly increased the plasma corticosterone, but ACTH 4–10 had no such effect (Table 7 caption). In the brain the results were equivocal; ACTH 1–24 still increased the free lysine dpm, protein dpm and RR, but none of these effects was statistically significant (Table 7). Likewise, ACTH 4–10 produced no significant changes in any parameter. In the liver, no significant changes were observed with either

TABLE 4

THE EFFECT OF A SALINE INJECTION ON [^{3}H]LYSINE INCORPORATION INTO MOUSE BRAIN AND LIVER

		Free Lysine dpm/mg		Protein dpm/mg		Relative Radioactivity	
	N	Mean ± SEM	% Δ	Mean ± SEM	% Δ	Mean ± SEM	% Δ
Brain							
Quiet	24	256 ± 14	[0]	39.5 ± 2.6	[0]	0.155 ± 0.005	[0]
Saline	25	235 ± 15	−8	35.6 ± 2.6	−10	0.151 ± 0.004	−3
Liver							
Quiet	24	1548 ± 82	[0]	978 ± 57	[0]	0.648 ± 0.036	[0]
Saline	25	1090 ± 74*	−30	1057 ± 81	+8	0.988 ± 0.058*	+52

*Significantly different from quiet, $p<0.001$ (ANOVA)

C57B1/6J mice were injected with 1 μl/g of physiological saline or left quiet. Fifteen min later they were injected with [^{3}H]lysine and sacrificed 10 min later. The results of four separate experiments (Quiet n=3,6,8,7; Saline n=6,5,7,7 respectively) have been combined in this table. The increase in liver RR was significant in each experiment. The decrease in liver free lysine was significant in 2 of the 4 experiments. Plasma corticosterone (1 experiment only): Quiet 12.9 ± 3.9; Saline 15.9 ± 3.5 μg/100ml.

TABLE 5

THE EFFECT OF ACTH 1-24 ON THE INCORPORATION OF [^{3}H]LYSINE INTO MOUSE BRAIN AND LIVER

		Free Lysine dpm/mg		Protein dpm/mg		Relative Radioactivity	
	N	Mean ± SEM	% Δ	Mean ± SEM	% Δ	Mean ± SEM	% Δ
Brain							
Saline	33	234 ± 13	[0]	37.0 ± 2.0	[0]	0.159 ± 0.004	[0]
ACTH 1-24	33	284 ± 17‡	+22	49.3 ± 2.9‡	+34	0.175 ± 0.005†	+10
Liver							
Saline	33	1204 ± 96	[0]	1157 ± 76	[0]	1.032 ± 0.061	[0]
ACTH 1-24	33	1317 ± 102	+9	1519 ± 111†	+31	1.195 ± 0.057*	+16

*Significantly different from control, $p<0.05$; †$p<0.01$; ‡$p<0.001$ (ANOVA)

C57B1/6J mice were injected with ACTH 1-24 (0.6 μg/g) or saline. Fifteen min later they were injected with [^{3}H]lysine and sacrificed after 10 min. The results of five separate experiments have been combined in this table (Saline n=6,5,7,5,10; ACTH 1-24 n=5,6,7,6,9 respectively). Values for brain and liver protein radioactivity and the relative radioactivity were increased in all five experiments; however, these increases were significant in only 2 of the 5 experiments in each case. Plasma corticosterone (2 experiments only): Saline 30.1 ± 3.0; ACTH 1-24 55.2 ± 2.7 μg/100ml ($p<0.001$).

ACTH analog. Nevertheless, dexamethasone itself approximately doubled the free lysine dpm in liver (compare Table 7 with Tables 3, 4, 5, 6, or 8, and see [26]).

Lysine vasopressin was tested at three doses (Table 8). At the 2 higher doses, the blood ^{3}H was significantly depressed by 15% ($p<0.01$) and 24% ($p<0.0001$), respectively, presumably reflecting the presser effect of these doses of LVP. At no dose of LVP was there any increase in free lysine or protein radioactivity or the RR in brain or liver. The highest dose significantly depressed the incorporation into protein and RR in both tissues, and depressed the free lysine dpm in brain.

To obtain an independent assessment of the effect of ACTH on brain protein synthesis, the state of aggregation of the ribosomes in polyribosomes was examined following ACTH injection (1 Unit per mouse SC). Two sample polyribosome profiles are shown in Fig. 3. Analysis of

TABLE 6

THE EFFECT OF ACTH 4-10 ON [^{3}H]LYSINE INCORPORATION INTO MOUSE BRAIN AND LIVER

		Free Lysine dpm/mg		Protein dpm/mg		Relative Radioactivity	
	N	Mean ± SEM	% Δ	Mean ± SEM	% Δ	Mean ± SEM	% Δ
Brain							
Saline	19	284 ± 12	[0]	42.1 ± 2.0	[0]	0.148 ± 0.003	[0]
ACTH 4-10	20	289 ± 15	+2	46.8 ± 2.2	+11	0.163 ± 0.004*	+10
Liver							
Saline	19	1298 ± 134	[0]	1206 ± 84	[0]	1.006 ± 0.071	[0]
ACTH 4-10	20	1493 ± 145	+15	1365 ± 79	+13	1.002 ± 0.063	0

*Significantly different from saline, $p<0.005$ (ANOVA)

C57B1/6J mice were injected with ACTH 4-10 (0.3 μg/g) or saline followed 15 min later by [^{3}H]lysine. The results of three separate experiments (Saline n = 7,5,7; ACTH 4-10 n=7,6,7 respectively) have been combined in this table. The increase of brain RR occurred in all experiments but was significant in only one. Plasma corticosterone: Saline 27.7 ± 2.5; ACTH 4-10 35.3 ± 2.6 μg/100ml ($p<0.05$).

TABLE 7

THE EFFECT OF ACTH ANALOGS ON DEXAMETHASONE PRETREATED MICE

	N	Free Lysine dpm/mg Mean ± SEM	% Δ	Protein dpm/mg Mean ± SEM	% Δ	Relative Radioactivity Mean ± SEM	% Δ
Brain							
Saline	7	289 ± 23	[0]	47.6 ± 5.8	[0]	0.163 ± 0.009	[0]
ACTH 1-24	7	331 ± 13	+14	59.8 ± 4.4	+26	0.180 ± 0.008	+10
ACTH 4-10	7	261 ± 22	−10	43.1 ± 3.7	−9	0.166 ± 0.007	+2
Liver							
Saline	7	2113 ± 192	[0]	1315 ± 79	[0]	0.651 ± 0.076	[0]
ACTH 1-24	7	2178 ± 152	+3	1478 ± 113	+12	0.715 ± 0.094	+10
ACTH 4-10	7	1938 ± 100	−8	1233 ± 79	−6	0.650 ± 0.055	0

C57B1/6J mice were treated with dexamethasone (300 ng/g) three hr prior to injection with ACTH 1-24, ACTH 4-10 or saline. [^{3}H]lysine was injected 15 min later. Plasma corticosterone: Saline 5.3 ± 1.4; ACTH 1-24 41.5 ± 3.3 ($p<0.001$); ACTH 4-10 3.9 ± 1.4 μg/100ml.

TABLE 8

THE EFFECT OF LYSINE VASOPRESSIN ON [^{3}H]LYSINE INCORPORATION INTO MOUSE BRAIN AND LIVER

	Free Lysine dpm/mg Mean ± SEM	% Δ	Protein dpm/mg Mean ± SEM	% Δ	Relative Radioactive Mean ± SEM	% Δ
Brain						
Quiet	248 ± 12	−10	37.4 ± 3.0	−11	0.150 ± 0.005	−1
Saline	277 ± 21	[0]	42.2 ± 4.1	[0]	0.151 ± 0.004	[0]
LVP 0.04 ng/g	255 ± 10	−8	38.7 ± 3.0	−10	0.151 ± 0.007	0
LVP 4 ng/g	232 ± 16	−16	34.8 ± 2.4	−18	0.151 ± 0.007	0
LVP 400 ng/g	175 ± 17†	−37	19.0 ± 1.7†	−55	0.110 ± 0.007†	−27
Liver						
Quiet	1410 ± 117†	+41	865 ± 42	−16	0.629 ± 0.052†	−39
Saline	1003 ± 28	[0]	1025 ± 90	[0]	1.025 ± 0.088	[0]
LVP 0.04 ng/g	1015 ± 81	+1	988 ± 157	−4	0.961 ± 0.085	−6
LVP 4 ng/g	903 ± 80	−10	945 ± 91	−8	1.058 ± 0.061	+3
LVP 400 ng/g	933 ± 94	−7	615 ± 72*	−40	0.659 ± 0.041†	−36

*Significantly different from saline, $p<0.05$, †$p<0.01$ (Dunnett's test, 2-tailed); N = 7 per group.

C57B1/6J mice treated with lysine vasopressin (LVP) or saline at the stated dose 15 min prior to a 10 min pulse of [^{3}H]lysine. Plasma corticosterone: Quiet 12.9 ± 3.9; Saline 15.9 ± 3.5; LVP 400 ng/g 25.4 ± 4.3 μg/100ml.

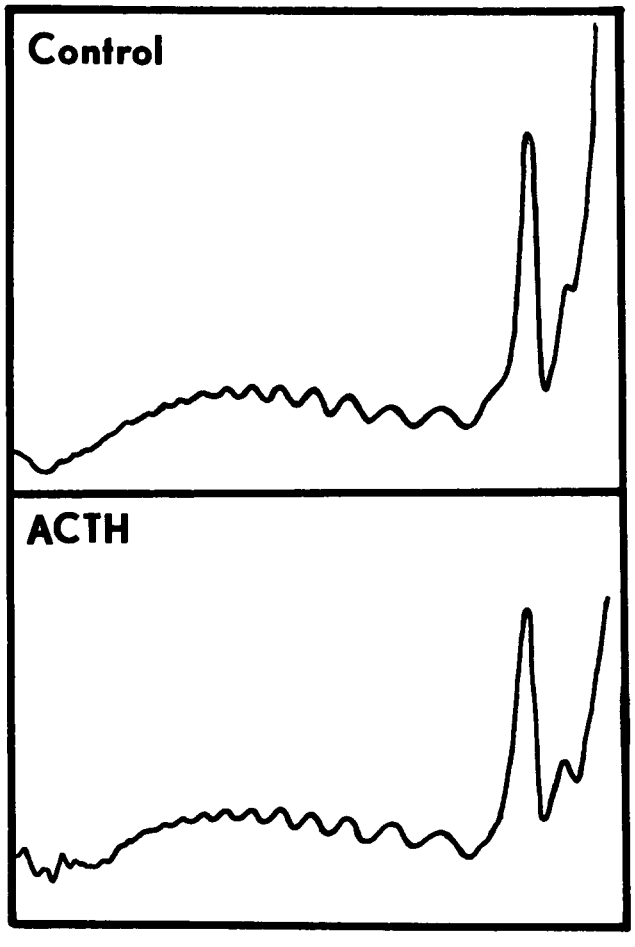

FIG. 3. Sucrose gradient analysis of brain polyribosomes of uninjected or ACTH-injected C57B1/6J mice. Postmitochondrial supernatants of brain homogenates were layered over 20–40% sucrose gradients and centrifuged for 4 hr at 63,000 × g (see [12]). The ordinate is ultraviolet absorbance (254 nm) in arbitrary units. Centrifugation direction is from right to left. The large peak near the top of the gradient is the 80S monomer peak, verified in control gradients following mild ribonuclease treatment. Analysis of these and other profiles, comparing the area under the monomer peak with that under the polyribosomal peaks or the total, did not reveal significant changes following ACTH treatment.

profiles from 6 pairs of mice did not reveal any difference in the state of aggregation of the ribosomes.

DISCUSSION

The major purpose of the present study was to assess the involvement of pituitary hormones in the previously observed biochemical responses to footshock [27]. We have shown previously that the responses were present in adrenalectomized mice and were not mimicked by corticosterone injection [26] so that adrenal corticosterone cannot be the effector. Dexamethasone, a potent inhibitor of pituitary ACTH secretion, almost completely suppressed the brain response, but only decreased the liver response [26]. This implicated ACTH in the brain response and possibly partly in the liver response.

The interpretation of the data following ACTH administration is complicated by the stress of the injection procedure, which causes ACTH and consequent corticosterone secretion. Nevertheless, injected ACTH 1–24 mimicked the effect of footshock in both the brain and liver. By contrast ACTH 4–10, an analog with low steroidogenic activity, increased the RR of brain but not of liver. ACTH 4–10 did not mimic footshock completely in the brain, since the free lysine radioactivity was not elevated.

In spite of the ability of ACTH 1–24 to mimic the effects of footshock on brain [^{3}H]lysine incorporation, ACTH 1–24 was less potent and consistent than footshock. Footshock treatment resulted in a significant increase of brain RR on all experiments, whereas significant increases were not observed in all experiments with ACTH 1–24. A possible explanation is that exogenous ACTH 1–24 does not readily penetrate brain tissue. Allen *et al.* [1] have suggested that ACTH may be secreted directly from the pituitary into the cerebral ventricles, and it is notable that intraventricular ACTH 1–24 increased [^{3}H]lysine incorporation into brain free lysine, brain protein and the brain RR [28].

Lysine vasopressin which is also released in response to stress, produced none of the changes seen with footshock. This resembles the lack of effect of intraventricular LVP [28]. Thus vasopressin apparently does not mediate the effects of footshock.

While ACTH seems likely to mediate the brain's response to footshock, a neural component cannot be excluded. This is most likely mediated via sympathetic stimulation which in the liver has previously been shown to increase the synthesis of specific proteins [5]. Jakoubek and Semiginovsky have shown that anticipation stress or restraint stress caused a decrease of [^{14}C]leucine incorporation into protein of rat cortex slices in vitro [19,39]. A similar but not identical effect was observed following in vivo treatment with ACTH [19]. ACTH treatment increased the in vivo incorporation of [U-^{14}C]leucine into mouse brain cortex [38]. The apparent discrepancy in the effects observed in vivo and in vitro has not been explained, but their data are consistent with ours showing similar effects of ACTH and stress on amino acid incorporation into proteins.

Previous studies have shown that ACTH increased the incorporation of amino acids into brain protein. In mice, the incorporation of [U-^{14}C]leucine [35,38] or [U-^{14}C]lysine, [U-^{14}C]tyrosine or [U-^{14}C]valine was increased [35]. In the latter study no change in the incorporation into liver or kidney was observed. The incorporation times used in these experiments were very long (1, 3, or 7 days), but we found ACTH increased [^{3}H]lysine incorporation into liver protein using a 24 hr pulse (Rees and Dunn, unpublished observation). In rats, ACTH 4–10 increased the [^{14}C]leucine incorporation into brain but not liver [24] in excellent agreement with our results. Furthermore, the diminished rate of incorporation of [^{3}H]leucine into brain protein caused by hypophysectomy could be reversed by ACTH 1–10 [37]. This effect of ACTH is most likely a direct one on the brain, since ACTH 1–24 is effective in producing the [^{3}H]lysine incorporation changes when administered into the cerebral ventricles [28] and ACTH analogs increase amino acid incorporation by brain slices in vitro [30]. It is also relevant that intracerebral implants of ACTH analogs are behaviorally [7] and electrophysiologically [40] active.

An important question is whether the effects of footshock or ACTH 1–24 truly reflect changes in brain protein synthesis. Insofar as the RR reflects protein synthesis rate, there may be a small change, but the small magnitude of the change has discouraged us from extending our analysis. Neither footshock nor ACTH 1–24 altered the free lysine content of brain (Table 2), the latter in agreement with previous results [35]. The changes in [^{3}H]lysine uptake suggest changes in brain lysine metabolism, so that it is possible that the biochemical responses do not reflect changes of the rate of protein synthesis, but changes in blood flow or other complex kinetic or compartmentation phenomena. No change in the aggregation of polyribosomes was observed, but this index is insensitive and it is doubtful whether it could reveal such a small change. (Following electroconvulsive shock there was more than a 50% inhibition of the incorporation of amino acids into protein, but the ratio of polyribosomes to monomeric ribosomes was decreased by only 20% [12]). However, there is other evidence that ACTH can affect protein synthesis. Hypophysectomy in rats produced a deficit in amino acid incorporation in vivo [37], which was also seen in brain slices [30] and in a cell-free system [10] where bloodflow and pool artifacts cannot occur. The in vivo deficit can be corrected by ACTH 1–10 [37] and the deficit in brain slices can be reversed by ACTH 1–10 in vitro [30].

It is undertermined to what extent previous reports of changes of amino acid incorporation in response to environmental changes may have been hormonally induced. The changes of [^{3}H]lysine incorporation into rat brain protein on first exposure to light [31] or of [^{3}H]leucine incorporation on exposure to light of dark-acclimatized rats [3] could have been due to ACTH, especially since most brain regions were affected in both cases. Likewise, the stimulation of [^{3}H]leucine incorporation into brain protein that occurred when rats were exercised [2], habituated like the footshock response and could have been due to ACTH.

It is interesting that while other investigators working with rats have not found any steroidogenic response to ACTH 4–10 [7,32], we observed a small increase in plasma corticosterone in mice with this peptide. We do not know whether this was a direct adrenocortical effect or some other effect of the peptide injection, perhaps causing stress. Since this effect was not observed in mice pretreated with dexamethasone, either the release of endogenous ACTH is essential to the ACTH 4–10-induced increase in plasma corticosterone, or dexamethasone can directly suppress adrenal corticosteroidogenesis caused by ACTH 4–10.

The work of de Wied's group has shown that ACTH or LVP can restore learning deficits in hypophysectomized rats and enhance acquisition and retard extinction of certain tasks in intact rats [7]. These activities of ACTH and LVP are distinct from their classical endocrine actions, since ACTH 4–10, which has little corticotrophic activity, and desglycinamide lysine vasopressin, which has little pressor or antidiuretic activity, are as effective behaviorally as their parent molecules. Thus it is pertinent to ask whether the [^{3}H]lysine incorporation changes caused by ACTH are related to its behavioral activity. It is interesting that treatments that decrease brain protein synthesis either specifically like cycloheximide or anisomycin, or nonspecifically like ECS, cause experimental amnesia in mice [12]; whereas arousal by footshock [15,22] or injection of ACTH [16] can enhance acquisition. Furthermore, ACTH can reverse amnesia due to CO_2 [33], ECS [20] or anisomycin ([14] and Dunn, unpublished observations). However, it is unlikely that protein synthesis is the common factor in these treatments, since LVP which is more potent than ACTH behaviorally has no detectable effect on protein synthesis. Perhaps agents that enhance cerebral performance incidentally stimulate protein synthesis, whereas those that impair cerebral activity inhibit protein synthesis.

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ADDENDUM

It is notable that Brain and Evans (*Pharmac. Biochem. Behav.* 7: 425–433, 1977) recently reported that ACTH 1–10 significantly elevated plasma corticosterone in mice.