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Corticotropin-Induced Changes in Protein Labelling: Lack of Molecular Specificity

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DUNN, A. J. AND N. B. GILDERSLEEVE. *Corticotropin-induced changes in protein labelling: Lack of molecular specificity.* PHARMAC. BIOCHEM. BEHAV. 13(6) 823–827, 1980.—Previous studies in this and other laboratories have indicated that corticotropin (ACTH) administered to intact or hypophysectomized rats or mice increased the labelling of cerebral proteins by radioactive amino acids, presumably indicative of increased protein synthesis. This study was designed to reveal whether this increased labelling was specific for particular proteins by studying labelling patterns in SDS polyacrylamide gels using a double isotope procedure. Two strains of mice, two different amino acid precursors, leucine and lysine, and both peripheral and central administration of ACTH and amino acids were used. In no case were treatment dependent changes in labelling observed in any region of the gel. This included regions containing S-100 or NSP, two brain specific proteins whose content had been reported to be increased by treatment of rats with an ACTH analog.

ACTH Brain protein synthesis Brain specific protein S-100 NSP

EXOGENOUS ACTH has been found to have a variety of behavioral effects in rodents [16]. Recently, we have shown that administered ACTH alters the rate of incorporation of amino acids into mouse brain proteins, and that this might account for a similar effect observed in stressed mice [5]. Thus the possibility exists that the ACTH released during the stress of training may act on the brain to increase the rate of protein synthesis and thereby influence behavior [1]. This study was designed to determine whether the effect of ACTH on brain protein synthesis is specific for any particular proteins.

Hypophysectomized animals are deficient in the synthesis of protein as estimated by amino acid incorporation into protein *in vivo* [15], *in vitro* in a slice preparation [13], by decreased polyribosomal aggregation [6], and by the activity of cell-free systems [4]. Some of these deficits are reversible by ACTH administration *in vivo* [15] and in the slice preparation *in vitro* [13]. We have shown that ACTH treatment of intact animals increases the incorporation of radioactive amino acids into protein whether the ACTH is administered subcutaneously [5] or intracerebroventricularly [12]. Although it seem slikely that this increased labelling of protein reflects an increased rate of cerebral protein synthesis, there are many problems associated with the interpretation of *in vivo* labelling data (see [2]). For this reason, we previously examined the polyribosome content of ACTH-treated animals but found that it was not significantly altered from controls [5]. However, it is doubtful if 10% changes in protein synthesis rate would be detectable by the analysis of polyribosome profiles. The present experiments were designed to determine whether the ACTH-induced changes of protein labelling were specific for any particular proteins. A double isotope procedure was used, to label proteins from ACTH-treated animals with ^{3}H and saline-treated animals with ^{14}C, or *vice versa*. Proteins from various subcellular fractions were then separated by electrophoresis on polyacrylamide slab gels containing sodium dodecyl sulphate (SDS). Because Zomzely-Neurath and Keller [17] had reported that ACTH treatment of intact rats increased the brain content of the proteins S-100 and NSP determined serologically, we paid particular attention to these two proteins.

METHOD

Animals used were male mice, either CD-1 from Charles River Laboratories (Wilmington, MA) or C57B1/6J from Jackson Laboratories (Bar Harbor, ME). [4,5-^{3}H]L-leucine and [U-^{14}C]L-leucine were obtained from Schwarz/Mann (Orangeburg, NY), [U-^{14}C]L-lysine was obtained from ICN Pharmaceuticals (Irvine, CA); [4,5-^{3}H]L-lysine, [U-^{14}C]L-lysine and [1-^{14}C]L-leucine were obtained from Amersham-Searle, Inc. (Arlington Heights, IL). $ACTH_{1-24}$ and [Met_4SO_2,D-Lys_8,Phe_9]$ACTH_{4-9}$ (ORG 2766) were a generous gift from Organon Internation B.V. (Oss, The Netherlands). Samples of S-100 and NSP were provided by Dr. Claire Zomzely-Neurath (Roche Institute, Nutley, NJ). Standard proteins were obtained from Sigma (St. Louis, MO). Mice were injected either subcutaneously (SC) or intracerebroventricularly (ICV) as previously described [12].

After one hour of protein labelling, mice were decapitated in pairs, one ^{3}H and ^{14}C, and the brains homogenized in 2 ml 0.32M sucrose. Subcellular fractionation into crude nuclear (P_1), crude mitochondrial (P_2), microsomal (P_3), and soluble fractions was performed as previously described [3].

Fractions were stored frozen until suspension in sample buffer (0.1M Tris-HCl, pH 6.8, 0.2% SDS, 0.5% 2-mercaptoethanol) for electrophoresis. The soluble fraction was either dialyzed against sample buffer or (in most experiments) passed over a small column (1×8 cm) of Sephadex G-10 equiligrated in sample buffer. Monitoring 1 μl aliquots

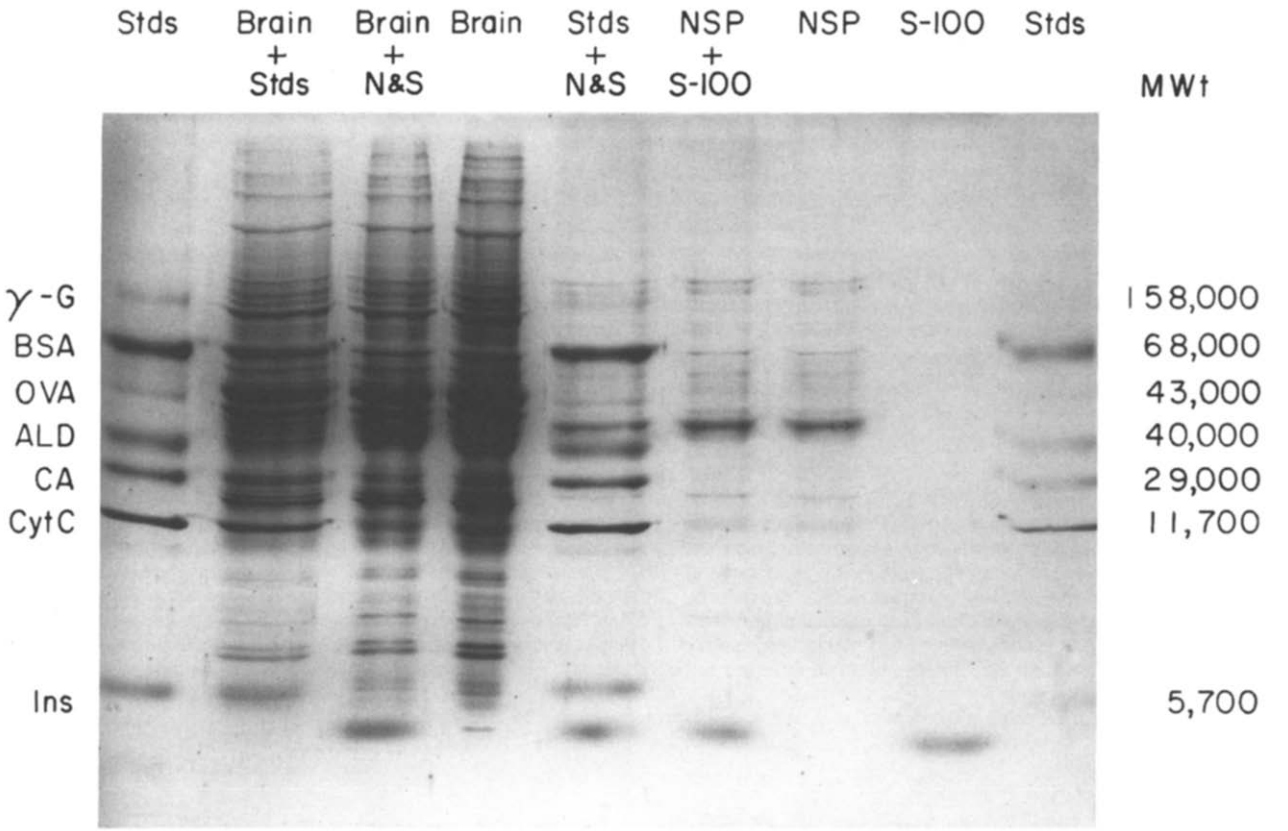

FIG. 1. Polyacrylamide gel electrophoretic separation of brain soluble proteins. SDS gradient gels (8–20%) NSP (N), neuron-specific protein; S-100 (S), brain-specific protein S-100; Stds, standards: γ-G, γ-globulin; BSA, bovine serum albumin; OVA, ovalbumin; ALD, adolase; CA, catalase; CytC, cytochrome C; Ins, insulin. The minor bands in the sample of NSP are impurities.

of the effluent indicated the positions of the protein and free amino acid peaks. This procedure not only removed free amino acids, but also other low molecular weight compounds that impaired the electrophoretic separation of this fraction. If necessary, samples were concentrated by addition of a small amount of Sephadex G-10 and centrifuging after equilibration for 1 hr.

Polyacrylamide gel electrophoresis was performed in the presence of SDS essentially according to the procedure of Laemmli [8]. Gradient gels (8 to 20%) were poured in a Bio-Rad slab gel apparatus using 3 mm spacers. Special combs were made to enable loading of up to 0.2 ml of sample, corresponding to approximately one half-brain. These combs made four slots 25 mm long, and one slot 9 mm long in the center of the gel for standard proteins. Combs were placed 5 mm above the gradient gel before pouring a 3% stacking gel. Samples were made 10% in glycerol, 5% in 2-mercaptoethanol, and 0.01% in bromphenol blue before loading. Current was applied at 20 mA until the marker dye entered the gradient gel (ca 45 min). Then electrophoresis was performed at 40 mA per slab until the marker dye reached the bottom of the slab (3–5 hr). Throughout electrophoresis the apparatus was cooled by water at 10°C. Proteins were fixed by soaking the gels in 7.5% trichloroacetic acid overnight. They were then washed in 10% acetic acid for 30 min and stained with Coomassie blue (G-250) (0.1% in 10% acetic acid and 50% methanol), for 2 hr, and destained in 10% acetic acid for 36 hr. Each gel was then photographed to obtain a permanent record. Gel slices (2 mm) were then cut using the procedure described by Luttge and Gray [10], and placed in 20 ml glass scintillation vials. Soluene 350 (0.5 ml, Packard Instrument Co., Downer's Grove, IL) was then added and the samples incubated for 16 hr at 50°C. After cooling, 10 ml of scintillation fluid (0.35% PPO, 0.01% POPOP in toluene) was added and the samples were equilibrated for at least 24 hr. Determination of radioactivity was performed in a Packard Model 2425 Liquid Scintillation Spectrometer using automatic external standardization to correct for quenching. All samples were counted to a standard deviation of 3% or less. Data are presented as corrected ^{3}H and ^{14}C disintegrations/min or as the appropriate ratio.

RESULTS

Figure 1 shows the separation of brain soluble proteins on the slab gels. Included are marker proteins of known molecular weight and samples of S-100 and NSP provided by Dr. Zomzely-Neurath. A number of different experiments was performed using different strains of mice (CD-1 and C56B1/6J), different precursors (lysine or leucine), different routes of injection of precursor (SC or ICV), different ACTH analogs ($ACTH_{1-24}$ or ORG 2766), and different routes of injection of ACTH (SC or ICV). The nature of these experiments is summarized in Table 1. All experiments included 6 or 8 pairs of mice in which ^{3}H or ^{14}C was randomly assigned (in equal numbers) to ACTH or saline groups. In all experi-

TABLE 1

COMBINATIONS OF EXPERIMENTAL PARAMETERS TESTED

Experiment no.	Mouse strain	ACTH form	Dose, route	Isotopes	Route	Time between ACTH and isotope
1	CD-1	ORG 2766	10 μg SC	[^{3}H]Lysine [U-^{14}C]Lysine	SC	15 min
2	C57Bl/6J	$ACTH_{1-24}$	0.5 μg/g SC	[^{3}H]Leucine [U-^{14}C]Leucine	SC	0
3	CD-1	$ACTH_{1-24}$	0.5 μg/g SC	[^{3}H]Lysine [U-^{14}C]Lysine	SC	0
4	CD-1	ORG 2766	1 μg ICV	[^{3}H]Lysine [U-^{14}C]Lysine	ICV	0
5	C57Bl/6J	ORG 2766	5 ng/g SC	[^{3}H]Leucine [1-^{14}C]Leucine	ICV	5 min
6	CD-1	$ACTH_{1-24}$	2 μg ICV	[^{3}H]Leucine [1-^{14}C]Leucine	ICV	0

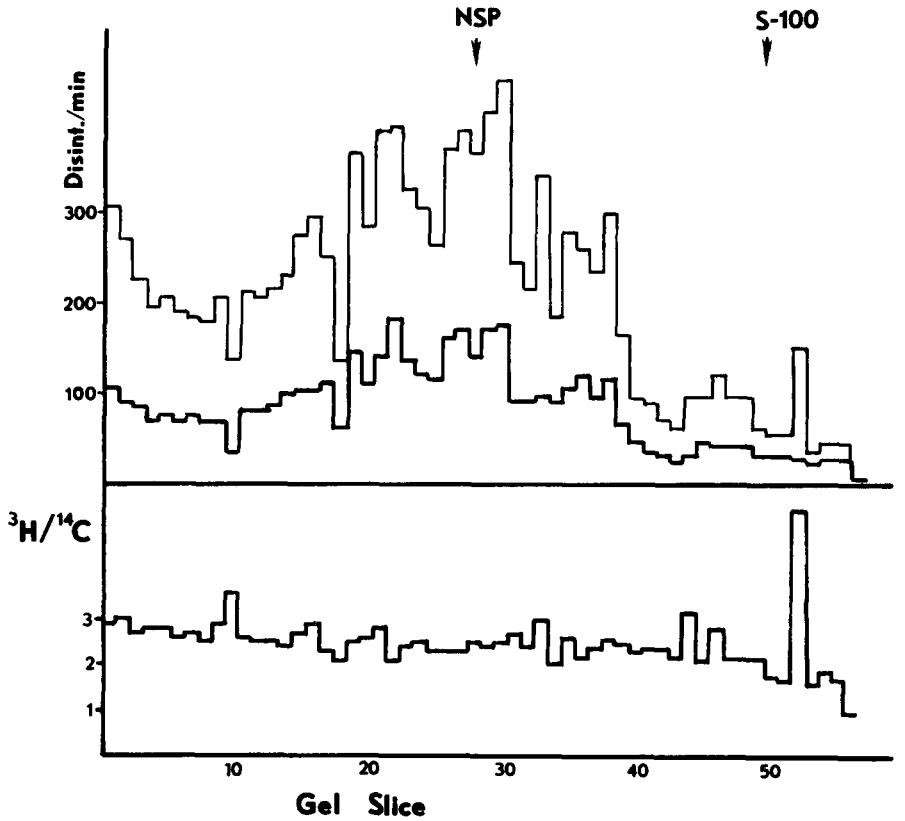

FIG. 2. Typical polyacrylamide gel electrophoresis profile of labelled soluble proteins. A pair of mice. C57B1/6J, was treated with $ACTH_{1-24}$ (0.5 μg/g, SC) or saline and then injected with SC [^{3}H]- or [^{14}C]leucine (Exp. 2). Upper profile indicates disint./min in each slice. Lower profile is $^3H/^{14}C$ ratio (^{14}C in $ACTH_{1-24}$-injected mouse). NSP and S-100 indicate the positions of these marker proteins.

ments the soluble (cytoplasmic) fraction was examined; in some of the experiments the other subcellular fractions were also run.

We concentrated our efforts on the soluble fraction for the following reasons: NSP and S-100 appear in this fraction; most newly synthesized cellular proteins appear first in this fraction—little newly synthesized protein is incorporated into membrane fractions at this time; labelled proteins in the nuclear, mitochondrial and synaptosomal fractions are mostly contaminants from the microsomal fraction; the

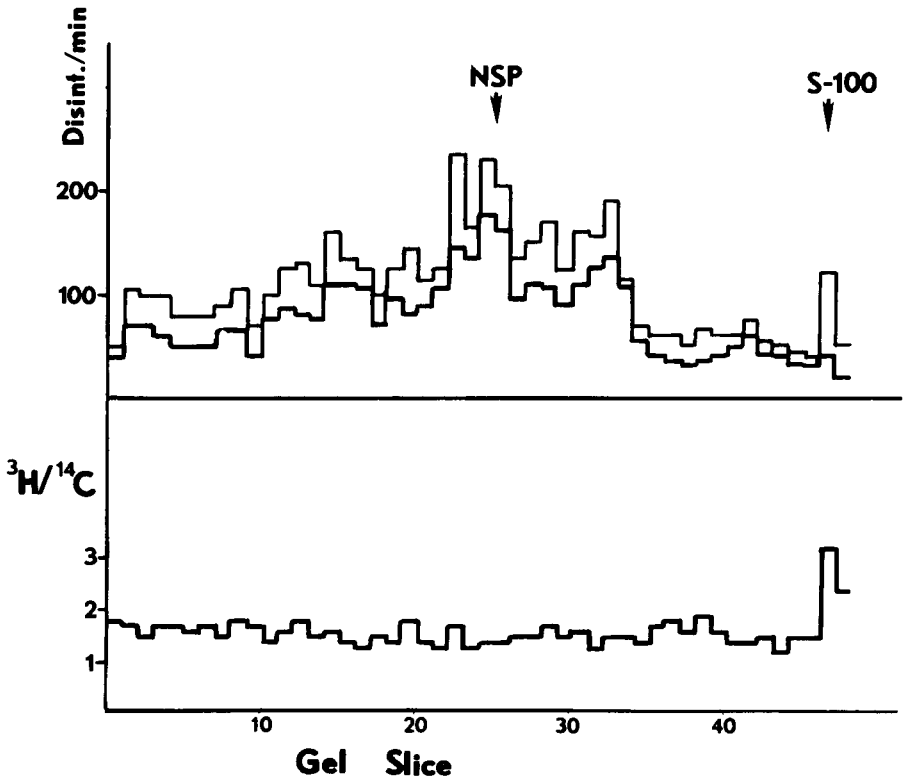

FIG. 3. Typical polyacrylamide gel electrophoresis profile of labelled soluble proteins. A pair of C57B1/6J mice was treated with ORG 2766 (5 ng/g, SC) or saline followed 5 min later by ICV [^{3}H]- or [^{14}C]leucine (Exp. 5). Otherwise as Fig. 2, but ^{3}H in ACTH-treated mouse.

labelling profile of the microsomal fraction is contaminated by the presence of incomplete highly labelled proteins from the ribosomes.

Typical results are shown in Figs. 2 and 3. In no case was any consistent difference seen in any part of the gels in any subcellular fraction. Particular attention was paid to parts of the gel containing NSP and S-100 as determined by the migration of these standards. Considerable deviation from normal isotope ratios was not uncommon in the region of the gel corresponding to S-100. However, the direction of the change was not consistently associated with ACTH or saline treatments. We can only assume that S-100 or some other labelled component that migrates in the same region was particularly susceptible to modification either *in vivo* or during the tissue preparation.

DISCUSSION

The results obtained and in part presented here provide no evidence for a selective effect of ACTH on the labelling of brain proteins. Although we examined a variety of different conditions including different peptides, different routes of injection, two strains of mice and two different amino acid precursors no consistent changes were observed. These results are similar to those reported by Reith *et al.* [14] who studied the labelling of proteins *in vivo* in hypophysectomized rats in response to $ACTH_{1-10}$ treatment, and Pavlík *et al.* [11] who studied proteins synthesized in brain cortex slices in response to ACTH. In particular, neither the latter study nor the present one found any evidence for a specific labelling of the brain proteins S-100 or NSP. Thus, these data do not provide support for the finding that treatment with ACTH increases the synthesis of these two proteins (S-100 and NSP) [17]. Either the increase in these proteins detected serologically does not represent increased protein synthesis, or both we and Reith *et al.* [14] failed to reproduce some crucial factor in Zomzely's experiments.

The finding of a lack of specificity of the effect of ACTH on cerebral protein synthesis can be interpreted in two ways: there is no specificity, and ACTH merely elevates the synthesis rates for all cellular proteins; or, the specificity is exerted not on synthesis, but on a subsequent processing or degradative step. Support for the latter arises from other studies on brain proteins. It is well established that the bulk of neuronal proteins is transported axoplasmically, to the terminals and that most, if not all, proteins found in the axon are transported at one rate or another [9]. Because it can take a very long time for a newly synthesized protein to arrive at the terminals, it may be that specificity is determined locally at the terminals by degrading unneeded proteins. This would explain why there is so little change in the type of proteins synthesized even after axotomy [7]. This apparently wasteful system may actually be more efficient. It has the advantage that the specificity of proteins functionally incorporated can be under direct local control. This possibility means the lack of specificity of ACTH exerted at the synthesis step, need not indicate a lack of functional specificity, which could be determined by local factors.

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