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Extracts from the brains of hibernating and alert ground squirrels: Effects on cells in culture

(brain peptides/cell culture bioassay/inhibitor of nucleoside incorporation)

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ABSTRACT **Aqueous extracts were prepared from pulverized, acetone-dehydrated brains of hibernating and alert ground squirrels. Addition of these extracts to Chinese hamster ovary cells in suspension culture resulted in a decrease in the amount of [^{3}H]thymidine incorporated into acid-precipitable material without affecting the transport or phosphorylation of the nucleoside. The inhibition was time- and dose-dependent and full recovery occurred about 2 hr after exposure of the cells to the active extract. The active factor is readily oxidized during storage at −70°C but full activity can be restored by treatment with 2-mercaptoethanol. The peptide nature of the active material is indicated by its susceptibility to proteases and by loss of activity after alkylation. Fasted or cold-acclimated rats also develop increased levels of active substance in their brains; however, brains of hibernating squirrels contain 10- to 50-fold more of the active substance than brains from either alert squirrels or rats. A time-dependent increase in activity of extracts from hibernating brain incubated with a homogenate from alert brain suggests that the peptide is activated or generated in the mixture.**

Attempts have been made to isolate and identify factors that induce or regulate the hibernating state. Dawe and co-workers (1–6) have obtained a fraction from the blood of hibernating woodchucks and ground squirrels ("trigger") that, when injected into alert ground squirrels, induces hibernation even in the summer. Other researchers have failed to obtain a significant induction of hibernation in squirrels (7, 8) or hamsters (9) using a similar protocol. Swan *et al.* (10–13) have prepared extracts of brains obtained from hibernating or aestivating animals that have been reported to cause a decline in both body temperature and oxygen consumption when injected intravenously into rats. The large quantities of brain extract required to elicit a response in rats precluded the use of this whole animal assay in isolating the factor responsible for lowering metabolic rates. In the present study we have developed a sensitive cell culture bioassay capable of distinguishing between extracts prepared from brains of hibernating and alert ground squirrels. Although the levels of the active substance in the extract correlate with entrance into hibernation, a similar activity can be detected in brain extracts of homeotherms where the levels of the active agent respond to altered environmental conditions. The nature of the active substance and the cellular response to it have been investigated.

MATERIALS AND METHODS

Materials. D-[^{14}C(U)]Glucose (300 mCi/mmol; 1 Ci = 3.7 × 10^{10} Bq), [^{3}H-methyl]thymidine (72 Ci/mmol), [5-^{3}H]uridine (63 Ci/mmol), Oxifluor-CO_2, and Protosol were purchased from New England Nuclear. Cholecystokinin (octapeptide fragment 26–33) and [Leu]enkephalin were obtained from Boehringer Mannheim. [Met]Enkephalin, β-endorphin, glycine, serotonin, γ-aminobutyric acid, spermine, spermidine, and putrescine were obtained from Sigma. Bombesin was a gift from J. Stewart (University of Colorado Medical School). Baker HPLC-analyzed grade solvents were used for HPLC. All other chemicals were reagent grade.

Source of Brain Tissue. Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were obtained from Zoological Center International (Hinsdale, IL); they were trapped in Wisconsin in late summer. They were individually housed in an environmentally controlled room at 8°C with 8 hr of light per day. Food, water, and nesting material were available *ad lib.*

Brains, obtained from animals sacrificed by decapitation, were removed immediately and frozen in either liquid nitrogen or acetone and dry ice. Cold-acclimated squirrels were maintained at 8°C and had been in hibernation previously but were not in hibernation on the day of sacrifice. Hibernating squirrels were also maintained at 8°C and had been in hibernation on the observation day previous to the day of sacrifice (2–3 days earlier). Squirrels that were entering hibernation were obtained by arousal of the animals and, 24 hr later, squirrels that appeared to be reentering hibernation (based on posture and respiratory rate) were sacrificed. Both thoracic and rectal body temperatures were recorded at the time of sacrifice. Squirrels that were exiting hibernation were sacrificed 20 min after arousal and removal from the cold room. Alert squirrels were maintained at room temperature (22°C) and were sacrificed prior to and following the hibernating season. Fasted rats had been deprived of food for 4 days prior to sacrifice. Cold-acclimated rats were housed at 8°C for 21 days prior to sacrifice. Brains from rats and mice were used as controls. Brain sections were obtained by surgical dissection prior to freezing.

Preparation of Brain Extracts. All steps were carried out at 4°C unless otherwise specified. The brains, frozen in acetone/dry ice, were thawed in acetone, washed twice in 3 vol of acetone, and homogenized in a VirTis homogenizer in acetone. The homogenate was centrifuged at 1,000 × *g* for 5 min; the acetone was discarded and the pellet was dried under reduced pressure and stored at −70°C. The acetone-powdered brain was homogenized in water (≈20 ml/g), centrifuged at 100,000 × *g* for 1 hr, and the supernatant was applied to an Amicon PM-10 ultrafiltration membrane. After concentration, the retained material was washed with a volume of H_2O equal to the initial volume. The total flow-through was collected and concentrated on an Amicon UM-2 membrane. The retained material was diluted to twice its volume and concentrated again. The materials re-

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Abbreviations: HSB, hibernating squirrel brain extract; ASB, alert squirrel brain extract; CHO, Chinese hamster ovary; HBSS, Hanks' balanced salt solution; ara-C, 1-β-D-arabinofuranosylcytosine.

tained by the PM-10, the UM-2, and the flow-through from the UM-2 [designated hibernating squirrel brain extract (HSB) >10, 1–10, and <1, respectively] were lyophilized and stored at −70°C. Extracts were also prepared from fresh frozen brain by using the HCl/acetone procedure of Li *et al.* (14).

Modification of Extract. Approximately 10 μg of lyophilized aqueous extract (fraction designated 1–10) of the acetone powder from brains of hibernating squirrels (HSB 1–10) was hydrated with 100 μl of 0.5 M ammonium bicarbonate at pH 8.0 and flushed with nitrogen; after 2-mercaptoethanol (10 μl) was added, the sample was sealed and incubated at 37°C for 1 hr. The solution was cooled to 4°C, 10 μl of iodoacetamide (1 mg/ml) was added, and the sample was resealed and placed in the dark at 4°C for 1 hr. The modified extract was desalted on an Amicon UM-2 ultrafiltration membrane by repetitive washing with water. The remaining material was lyophilized.

HSB 1–10 was incubated for periods up to 3 hr at 37°C with either Pronase P under conditions described by Vosbeck *et al.* (15) or with papain in 100 mM ammonium bicarbonate at pH 6.2 or with bromelain in 10 mM acetic acid at pH 4.5. The digestions were terminated by boiling and the digests were lyophilized.

The 100,000 × *g* supernatant obtained from an aqueous extraction of hibernating squirrel brain acetone powder was incubated at 37°C with an aqueous homogenate of fresh frozen alert brain for 0, 30, 60, 90, and 120 min. The mixture was clarified by centrifugation at 100,000 × *g* for 1 hr at 4°C. The supernatant was fractionated on the Amicon filters as described.

Fractionation of Brain Extracts. Gel filtration was carried out on a Sephadex G-25 column (2 × 60 cm) equilibrated with 50% acetic acid. Reverse-phase HPLC separations were obtained on a Bio-Sil C-18 column (Bio-Rad, 5 μm particle size; 0.4 cm × 15 cm) with 50 mM sodium phosphate at pH 7.0 and a gradient from 0% to 60% acetonitrile.

Cell Culture Bioassays. Chinese hamster ovary (CHO) cells were maintained in logarithmic phase as monolayer cultures grown in McCoy 5A medium supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were removed from the plates by trypsinization (16), diluted to 1×10^5 cells per ml, and maintained in suspension culture at densities of 1×10^5 to 4×10^5 cells per ml for at least 24 hr prior to the beginning of any experiment. Cells in suspension culture had doubling times of 18 hr or less.

For assays that measured thymidine (dThd) or uridine incorporation, 2 ml of CHO cells was dispensed into sterile culture tubes and incubated at 37°C in a water bath. For dose–response curves and routine activity assays, additions (100 μl) of either control or test substances were made at 0 time and the contents of the culture tubes were mixed. At 20 min, the ^{3}H-labeled nucleoside (2.0 μCi) was added, and the tubes were agitated. At 40 min, 1 ml of 20% trichloroacetic acid was added and the culture tubes were placed on ice. For the time-course experiments, the time after the addition of the test substance but prior to the addition of the labeled nucleoside was varied (the time for incorporation of label remained constant at 20 min).

Acid-precipitable material was collected on Whatman no. 3 filter paper (2.4 cm diameter) and washed four times with 3 ml of 5% cold trichloroacetic acid followed by washing with 2 ml of 95% ethanol. The filters were placed in scintillation vials with 0.3 ml of Protosol and incubated overnight. Scintillation cocktail (toluene base with 0.5% 2,5-diphenyloxazole/0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene) was added, the samples were mixed, and the amount of radioactivity was determined by liquid scintillation counting. Internal standards were used to determine uniformity in the degree of quenching.

Rate of dThd uptake was determined by preincubating 1 ml of cells with a test substance for 20 min in a Microfuge tube at 37°C, followed by addition of [^{3}H]dThd for varying periods of time up to 20 min. To terminate incorporation and uptake, the tubes were rapidly cooled on ice and centrifuged in a Beckman Microfuge B for 10 sec at 4°C, and the medium was removed. The cells were washed twice with 500 μl of ice-cold Hanks' balanced salt solution (HBSS). The cells were resuspended in 100 μl of H_2O and frozen and thawed; 100 μl of 20% trichloroacetic acid was added, and the tubes were centrifuged at 10,000 × *g* for 4 min at 4°C. Acid-soluble and insoluble radioactivity was determined by liquid scintillation counting. An aliquot of the thawed suspension was examined by light microscopy to determine that all cells had been lysed.

The effect of the HSB 1–10 on the extent of phosphorylation of dThd was determined. CHO cells (1 ml) were preincubated in a Microfuge tube with either HSB 1–10 or an equivalent volume of HBSS. At 20 min [^{3}H]dThd was added, and at 40 min cells were pelleted and washed as described above. After the second wash, the cells were resuspended in 100 μl of H_2O, frozen, and thawed in a boiling water bath. The precipitated material was removed by centrifugation. The supernatant was applied to a PEI-cellulose plate and chromatographed in 0.75 M KH_2PO_4 (17). Fluorography was performed as described (18). Standards were visualized under UV light. The radioactive regions were removed from the plate and eluted from the cellulose with greater than 90% recovery (unpublished data). The amount of radioactivity was determined by liquid scintillation counting.

The effect of HSB 1–10 on progression of CHO cells through the cell cycle was examined by making periodic additions (1-hr intervals for 10 hr) of the HSB 1–10 (0.03 brain equivalent per 2 ml of cells) to cells maintained in suspension culture. An equivalent amount of HBSS containing 0.1% 2-mercaptoethanol was added to a second control culture. Forty minutes after each addition three aliquots of cells were removed from each flask. One aliquot was incubated with [^{3}H]dThd for an additional 20 min to determine if continued inhibition of [^{3}H]dThd incorporation was occurring. A second aliquot was used to determine cell number by using a particle counter. A third aliquot was pelleted by centrifugation and resuspended in HBSS, and 95% ethanol was added to a final concentration of 75%. The fixed cells were stained with chromomycin (19) and their distribution through the cell cycle was analyzed on a flow cytometer (20).

Production of $^{14}CO_2$ from [^{14}C]glucose was measured by using 2 ml of a CHO cell suspension in a small glass vial containing a micro stirring bar. [^{14}C]glucose (20 μl) was added at 0 time, and the vial was stoppered with a silicone rubber stopper having an inlet and outlet port. A mixture of 5% CO_2/95% air was pumped (≈3 ml/min) through the vials while they were stirred at 37°C. The outflow gas bubbled through 4 ml of a solution of Oxifluor-CO_2/methanol, 1:1 (vol/vol), in a glass scintillation mini-vial. Scintillation vials were changed at 20 min intervals. The test or control substance was added to the cell suspension at 40 min.

RESULTS

Characterization of Cell Culture Bioassay. When HSB 1–10 was treated with 0.1% 2-mercaptoethanol and added to CHO cells in culture, a decrease was observed in the amount of [^{3}H]dThd incorporated into acid-precipitable material (Fig. 1*A*). Control cultures (those that received HBSS) incorporated [^{3}H]dThd at a level of about 12,000 dpm (1 dpm = 16.7 mBq) per 4×10^5 cells, whereas the HSB 1–10-treated cultures incorporated about 8,400 dpm per 4×10^5 cells. The other two fractions of brain extracts (HBS >10 and HBS <1) caused no significant change in [^{3}H]dThd incorporation up to the highest

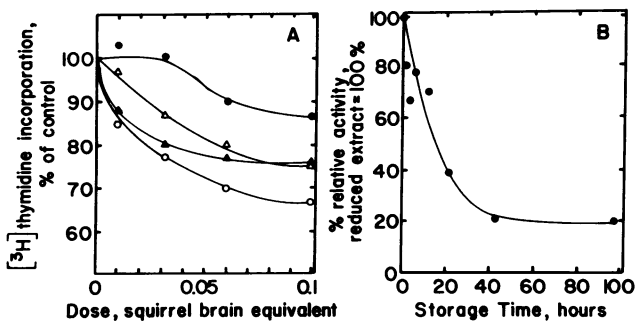

FIG. 1. Effects of storage on biological activity of HSB. (*A*) Dose–response curves of samples stored as lyophilized powders at −70°C. Storage times were 30 min (▲), 23 hr (△), and 1 yr (●). ○, Sample stored 1 yr and treated with 0.1% 2-mercaptoethanol immediately before assaying. (*B*) Relative activity as a function of storage time. The relative activity was calculated from the ID_{50} of the stored sample compared to that of a sample treated with 0.1% 2-mercaptoethanol. The $t_{1/2}$ of the biological activity of stored samples is about 16 hr.

levels tested (0.2 brain equivalent). The active agent in HSB 1–10 loses activity when stored as a lyophilized powder at −70°C in a sealed container (Fig. 1*A*) with a $t_{1/2}$ of about 16 hr (Fig. 1*B*). Activity can be fully restored by hydration with HBSS containing 0.1% 2-mercaptoethanol; consequently, all brain extracts were rehydrated with this solution before assaying.

The incorporation of [^{3}H]uridine (Urd) into acid-precipitable material by CHO cells in the presence of brain extracts was also measured. The dose–response curves in Fig. 2 compare the 1–10 fractions obtained from the brains of alert and hibernating ground squirrels. [^{3}H]Urd incorporation was inhibited to the same extent as was [^{3}H]dThd incorporation; the HSB 1–10 was about four times more active than the same fraction from brains of alert squirrels (ASB 1–10).

A single administration of HSB 1–10 (0.03 brain equivalent) resulted in a maximal inhibition of [^{3}H]dThd and [^{3}H]Urd incorporation in CHO cells at 40 min (Fig. 3). Complete recovery occurred by 120 min. A dose of 0.1 brain equivalent produced a response for [^{3}H]dThd incorporation identical in magnitude, rate of depression, duration of effect, and rate of recovery as observed for the dose of 0.03 brain equivalent (data not shown). However, a second addition of 0.03 brain equivalent at later times was capable of further inhibition. Recovery from multiple additions of HSB 1–10 took place at about the same rate as that observed after a single addition (Fig. 3 *A* and *B*).

The effects of HSB 1–10 on the rate of [^{3}H]dThd transport into CHO cells was compared to HBSS controls as well as to solutions containing 1-β-D-arabinofuranosylcytosine (ara-C) or colchicine. Colchicine inhibited the uptake of [^{3}H]dThd (21). The control cell cultures and those containing HSB 1–10 or

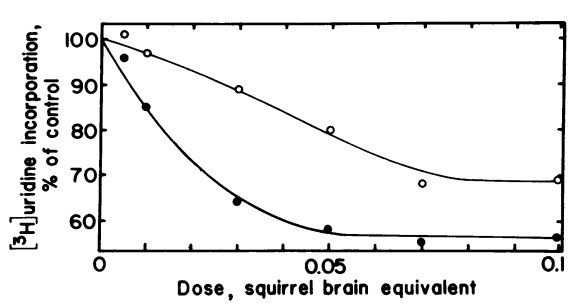

FIG. 2. Effect of brain extracts on [^{3}H]Urd incorporation into acid-precipitable material in CHO cells. Each point is the average of two separate experiments, each done in triplicate. Control samples contained HBSS and 2-mercaptoethanol. ○, ASB 1–10; ●, HSB 1–10.

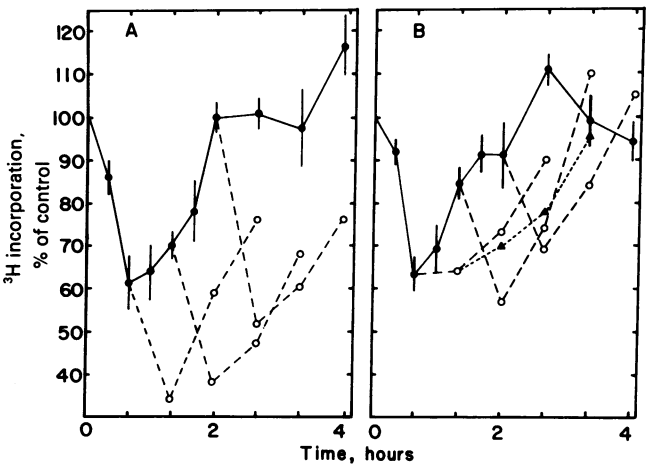

FIG. 3. Effects of single and multiple doses of HSB 1–10 on nucleoside incorporation by CHO cells. (*A*) [^{3}H]dThd incorporation (five experiments); (*B*) [^{3}H]Urd incorporation (three experiments). ●, Time course of a single addition of 0.03 brain equivalent. Bars represent SD for multiple experiments, each with triplicate determinations. ○, Values after second addition of 0.03 brain equivalent of HSB 1–10. ▲, Values after third addition of 0.03 brain equivalent of HSB 1–10.

ara-C showed similar increases in amount of radioactivity in the acid-soluble fraction; rates of increase over the first 15 min for HBSS-, HSB 1–10-, ara-C-, and colchicine-treated cultures were 1,170 dpm/min, 1,190 dpm/min, 1,260 dpm/min, and 970 dpm/min, respectively. The extent of phosphorylation of [^{3}H]dThd by cultured CHO cells treated with HSB 1–10 was identical to that of HBSS-treated controls. Distribution (± SD) of the [^{3}H]dThd into dThd, dTMP, and dTTP was 1.1 ± 1.5%, 79.9 ± 1.5%, and 19.9 ± 1.3%, respectively.

The periodic addition of HSB 1–10 to CHO cells in suspension culture resulted in a continuous 30–40% inhibition of [^{3}H]dThd incorporation over the 10 hr that these additions were made. [^{3}H]dThd incorporation returned to control levels within 2 hr of the last HSB 1–10 addition. The results of flow cytometry revealed no significant differences when compared to controls, indicating that there was no specific point in the cell cycle at which progression was arrested.

Fig. 4 shows the dose–response curves of [^{3}H]dThd incorporation obtained by using the 1–10 fractions prepared from rat, mouse, and squirrel brains. Identical curves were obtained from HSB 1–10 and extracts of hibernating squirrel brain prepared

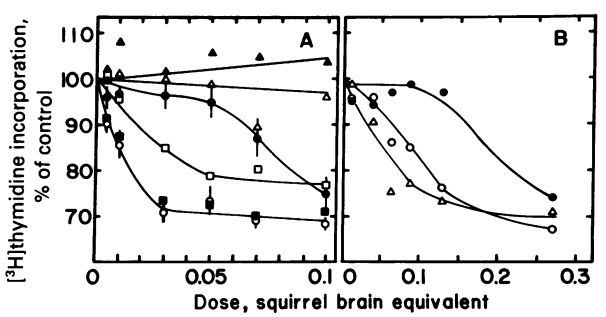

FIG. 4. Effects of brain extracts on [^{3}H]dThd incorporation into acid-precipitable material in CHO cells. Brain equivalents are based on the wet weight of the brains compared to the adult ground squirrel brain (2.30 g). (*A*) ○, HSB 1–10; ■, HSB prepared by HCl/acetone extraction; □, cold-acclimated ASB 1–10; ●, ASB 1–10; △, mouse brain 1–10; ▲, rat brain 1–10. Bars represent 95% confidence levels based on 13 and 9 experiments for HSB and ASB, respectively. (*B*) Comparison of brain extracts (fraction designated 1–10) from fasted rats (△), cold-acclimated rats (○), and control rats (●).

by using the HCl/acetone extraction scheme. Based on the difference in ID_{50} values obtained for HSB and ASB, hibernating animals have about 10 times more of the active factor in their brain than have alert animals (Fig. 4*A*). Lower levels of activity were found in rat brain; the level of the active agent increased in brain of rats subjected to either fasting or cold acclimation (Fig. 4*B*).

The levels of the active agent in the brains of squirrels entering and exiting from hibernation (expressed as the dose required in squirrel brain equivalents for half of the maximal response in inhibition of [^{3}H]dThd incorporation) are plotted as a function of body temperature at the time of sacrifice (Fig. 5). Maximal levels of the active agent are present in squirrels with body temperatures below 25°C. Cold-acclimated alert squirrels and squirrels exiting hibernation have somewhat lower levels of the active material but have more than is present in the brain of alert squirrels sacrificed during the summer. For comparison, the ID_{50} values obtained from the assay of rat brain extracts shown in Fig. 4*B* are included.

No inhibition of $^{14}CO_2$ production from [^{14}C]glucose was observed when CHO cells (5×10^5) were incubated with HBSS (control) or 0.03, 0.05, or 0.1 brain equivalent of HSB 1–10. In fact, production of $^{14}CO_2$ by the CHO cells was slightly stimulated by the HSB 1–10. Comparative values of the control and 0.1 brain equivalent of HSB 1–10, expressed as molecules of glucose converted to CO_2 per cell per 20 min, were 2.2×10^3 and 2.9×10^3, respectively, over the period of 20–80 min after addition. Control and experimental values of $^{14}CO_2$ production with the lower doses of extract were not significantly different.

Distribution of the Active Factor in Squirrel Brains. Aqueous extracts (fraction designated 1–10) from sections of surgically dissected brain obtained from hibernating squirrels were assayed for the active agent by use of the [^{3}H]dThd incorporation assay (Fig. 6*A*). The extract from the hemispheres showed activity comparable to whole brain extracts. The extracts of the hypothalamus and pituitary demonstrated the presence of the active agent at doses above 0.5 brain equivalent (data not shown). Therefore, the agent, though present, is not localized in the pituitary or hypothalamus.

Effects of Modified HSBs and Other Substances in the [^{3}H]dThd Incorporation Assay. Reduced and alkylated HSB 1–10 was tested for its ability to inhibit [^{3}H]dThd incorporation in CHO cells. HSB 1–10 that was either subjected to alkylation at pH 4.0 or that was treated with alkylating agent previously inactivated with an excess of 2-mercaptoethanol at pH 4.0 could not be assayed due to its extreme toxicity to the CHO cells even after readjustment to neutral pH. However, HSB 1–10 that was

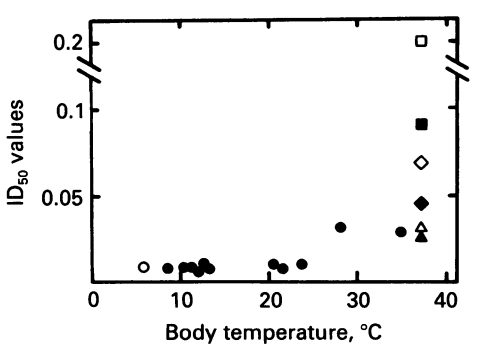

FIG. 5. ID_{50} values of brain extracts (fraction designated 1–10) on [^{3}H]dThd incorporation into acid-precipitable material in CHO cells. Source of brain: hibernating ground squirrel (○); ground squirrels entering hibernation (●); ground squirrels exiting hibernation (▲); cold-acclimated alert ground squirrels (△); fasted rats (◆); alert ground squirrels (◇); cold-acclimated rats (■); control rats (□).

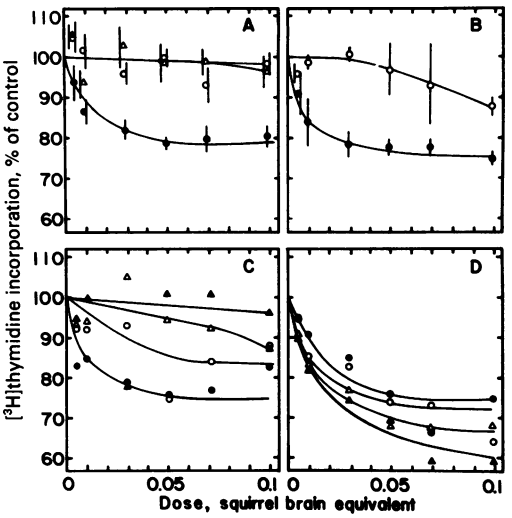

FIG. 6. Effects of brain extracts (fraction designated 1–10) on [^{3}H]dThd incorporation into acid-precipitable material in CHO cells. (*A*) Distribution of biologically active material in regions of the brain. ●, Hemispheres; ○, pituitary; △, hypothalamus. Bars represent SD. (*B*) Effects of alkylation at pH 8.0 on HSB 1–10. ○, Alkylated sample; ●, nonalkylated control. Bars represent SD. (*C*) Effect of bromelain treatment on HSB 1–10 activity. Incubation times at 37°C were 1 min (●), 30 min (○), 60 min (△), and 90 min (▲). Each point is the average of triplicate determinations. (*D*) Incubation of HSB with homogenate of alert squirrel brain. Activity of 1–10 fraction after incubation of the brain mixture for 0 min (●), 60 min (○), 90 min (△), and 120 min (▲). Each point is the average of triplicate determinations.

reduced and alkylated at pH 8.0 showed a loss of activity when compared to its nonalkylated control (Fig. 6*B*). These findings, in conjunction with the data showing a rapid loss in activity upon storage (Fig. 1*B*), strongly suggest the presence of an oxidizable, alkylatable group that must be in the reduced state for activity. The active agent was also destroyed by incubation with either papain or bromelain but was not destroyed by treatment with Pronase P. Use of papain resulted in nearly complete loss of activity with a 1-min incubation. Incubation with bromelain resulted in a slower time course of inactivation (Fig. 6*C*).

Incubation of the 100,000 × *g* supernatant from an aqueous HSB acetone powder with an aqueous homogenate of fresh frozen alert squirrel brain resulted in a level of active agent that increased upon longer incubation times (Fig. 6*D*). These findings suggest that the active factor is being produced, perhaps as a consequence of enzymatic activity.

Substances that have been suggested as having roles either in hibernation or in regulation of DNA synthesis include serotonin, bombesin, β-endorphin, [Leu]enkephalin, [Met]enkephalin, cholecystokinin, γ-aminobutyric acid, glycine, spermine, spermidine, and putrescine. These substances were tested for their effects on [^{3}H]dThd incorporation in CHO cells at doses between 1 and 30 μg [0.1 brain equivalent of HSB 1–10 = 10 μg of peptide based on $A_{210}^{0.1\%} = 21$ for peptides (22)]. None of these substances showed an inhibition of [^{3}H]dThd incorporation.

Partial Purification and Characterization of the Active Component in HSB 1–10. HSB 1–10, obtained from 50 brains, was fractionated on a column of Sephadex G-25. Fractions were lyophilized to remove the acetic acid and were assayed for their ability to inhibit [^{3}H]dThd incorporation. The active material eluted after the total volume, suggesting interaction with the resin. The concentrated fractions containing active substance were further fractionated by reverse-phase HPLC. The majority of the biologically active agent eluted very early with the

phosphate buffer. Use of gradients employing 0.1% trifluoroacetic acid resulted in the same early elution observed with phosphate buffers. A portion of the most active fraction was used to determine a dose–response curve. Another portion of this material was labeled with ^3H by using reductive methylation and the labeled peptides were separated by two-dimensional mapping on cellulose thin-layer plates (23). More than 10 spots were visualized by fluorography. Insufficient active material remained for further purification.

DISCUSSION

The results reported here demonstrate the existence of a molecule, presumably a peptide, that inhibits both [^3H]Urd and [^3H]dThd incorporation into acid-precipitable material in cultured CHO cells. This agent is not lethal to the cells because the inhibitory effect(s) is reversible with time. No single test dose inhibited nucleoside incorporation by >40%, but multiple additions of small doses at 40-min intervals resulted in additive inhibitory effects on [^3H]dThd incorporation, suggesting that a saturable receptor may be present.

The mechanism by which this agent affects nucleoside incorporation is not known. No inhibition of nucleoside transport or conversion to the nucleoside triphosphate was observed. Flow cytometry studies indicated that cells did not preferentially accumulate at a specific point in the cell cycle, suggesting that the cells simply traverse the cycle at a decreased rate. The inhibition of synthetic processes may result in a slower progression through the cell cycle. Decreased rates of synthesis could be expected to result in decreased utilization of ATP which, in turn, should slow down catabolic processes in the cell. However, the rate of $^{14}CO_2$ production from [^{14}C]glucose was not affected by the presence of the active agent. The use of a different energy source (i.e., fatty acid) might demonstrate a decreased rate of catabolism.

The incubation of the extract from hibernating brain with the homogenate of alert squirrel brain revealed two very interesting points. First, it suggested that the active agent is produced in the mixture. Several examples of the processing of neuroactive peptides from larger precursor proteins have been reported (24–26). Second, the brain, although it possesses the enzyme(s) necessary to generate the active agent, does not rapidly degrade it.

Though the inhibitory activity is not unique to extracts of brains from hibernating animals, the levels of the active agent are highest in squirrels whose body temperature has dropped below 25°C. Seasonal variation may be responsible for the increased levels of active substance in the brains of cold-acclimated squirrels in comparison to the alert animals sacrificed during the summer. Alternatively, the levels may be increased due to the physiological effects of cold, similar to the effects observed in rats, which would imply that the substance may be a result of, rather than a cause of, the altered metabolic state of hibernation.

Adelstein *et al.* (27) monitored the incorporation of [^3H]dThd into DNA of animal spleens and isolated spleen cells; they observed significantly decreased levels of incorporation in tissue obtained from alert hibernating species (including *S. tridecemlineatus*) compared to tissue from nonhibernating rodent species. The agent found in the brain extracts reported here may be the same as the factor responsible for these decreased rates of [^3H]dThd incorporation in spleen. Regardless of its role in hibernation, this agent may be valuable in regulating rates of DNA synthesis.

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