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Environmental Stimulation and Fucose Incorporation into Brain and Liver Glycoproteins[1]

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DAMSTRA-ENTINGH, T., D. ENTINGH, J. E. WILSON AND E. GLASSMAN. *Environmental stimulation and fucose incorporation into brain and liver glycoproteins.* PHARMAC. BIOCHEM. BEHAV. 2(1) 73–78, 1974. – Changes were observed in the amount of radioactive fucose incorporated into glycoproteins of brain and liver when mice were exposed to different environments. Mice were injected subcutaneously with ^{3}H-L-fucose, placed in a small chamber with an electrifiable grid floor for 15 min, and killed 1 min later. Exposure to the apparatus without shocks increased the levels of incorporation in both brain and liver compared to mice placed in individual cages after the injection. Increasing amounts of footshock reduced the level of incorporation. Five to 40 sec of footshock resulted in incorporation levels inversely proportional to the number of shocks.

Glycoproteins Environmental stimulation Neurochemistry Arousal Fear Brain Liver

MANY lines of evidence suggest that glycoprotein molecules on the surfaces of cells may participate in the establishment and maintenance of cell-to-cell contact [4,6]. While most of the evidence is based on studies of non-neuronal tissues, histochemical studies indicate that glycoproteins are localized on neuronal cell surfaces, and are a major component of the structures of the synaptic cleft [3, 17, 19]. It is thus tempting to speculate that changes in membrane glycoprotein structures might change the functional connectivity of neurons allowing new pathways or networks to be formed, and thus be intimately involved in neurobiological events that mediate the storage, consolidation, and retrieval of experiential information [1,2]. Since relatively rapid changes in the glycoside portions of these molecules seem to occur without major alterations in the protein backbone structure [11], synaptic connectivity might be altered without any gross disruption of membrane structure. Bogoch [2] has reported alterations in glycoprotein metabolism in pigeon brains following operant conditioning; however, Holian *et al.* [8] failed to detect changes in the amount of C^{14}-glucosamine incorporated into glycoproteins of control rats and rats trained on a one trial passive avoidance task.

Previous experiments (Entingh and Damstra-Entingh, in preparation) have suggested that foot-shock avoidance training alters the metabolism of uridine diphosphate sugars in mouse brain. Since nucleoside diphosphate sugars are involved as cofactors for glycoprotein synthesis, we have studied the effects of avoidance training on the incorporation of radioactive fucose into brain and liver glycoproteins. Fucose is a useful precursor for studying glycoprotein metabolism and synthesis, since this sugar is not a significant component of glycolipids or mucopolysaccharides [16,20], and is not readily metabolized to other sugars [5,10]. Also, since fucose occupies a terminal position on carbohydrate chains [7], it is possible that rapid, reversible modifications of glycoproteins as a result of behavioral stimulation may be detected.

METHOD

Animals

Male, C57BL/6J mice (Jackson Lab), seven to eight weeks old were used. They were housed six to a cage, 7–14 days before use, at 25°C with food and water available ad lib.

Apparatus

The apparatus was an automated version of a jumpup one-way active avoidance box [14]. The training side of the apparatus had a shelf on one wall to which the mouse could jump to escape or avoid footshock (0.3 ma). The yoked

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side had no shelf. A door buzzer (89 dB SPL) served as the conditional stimulus (CS).

Injections

Each mouse was immobilized by hand and injected subcutaneously in the back of the neck with 50 to 100 μC of L-fucose-1-H^3 (1.8-2.8 Ci/mmole, Amersham/Searle).

Behavioral Treatments

Quiet mice were injected, placed in individual cages and exposed to the ordinary laboratory environment for 20 min, and then decapitated.

Non-quiet mice were injected as above, subjected 4 min later to the behavioral treatments described below for 15 min, and decapitated 1 min later.

Trained mice were placed on the grid floor of the jump box for 5 sec at the start of the session. The CS was turned on, and 3 sec later the footshock was turned on. When the mouse jumped to the shelf, or after 20 sec of footshock, all stimuli were terminated and a 20 sec intertrial interval was begun. The mouse was pushed from the shelf by a piston at the start of each trial. Each mouse received about 33 training trials during the session.

Yoked mice were kept in the yoked compartment of the apparatus, while a naive mouse was being trained. The yoked mouse was exposed to the same stimuli as its trained partner, but no shelf was available for escaping footshock.

Classically conditioned mice were placed in the yoked side of the apparatus. For each trial, the CS was turned on and 3 sec later the mouse received 2 sec of inescapable footshock (0.3 ma). Fifteen trials at 53 sec intervals were given during the 15 min session.

Shocked mice received a preset number (5, 10, 20 or 40) of inescapable footshocks (0.3 ma, 1 sec duration). Delivery of shocks began 60 sec after the mouse was placed in the apparatus. Intervals between successive shocks varied randomly from 15–25 sec, with a mean interval of 20 sec.

Apparatus-exposed mice were placed in the yoked side of the same apparatus in which mice had just previously been shocked, and left undisturbed.

Urine-exposed mice were placed in a clean cage containing paper towels wetted with urine released by shocked animals.

Handled mice were transferred to a clean cage in the same room that housed the apparatus. After 15 min they were removed from this cage, placed in a new cage, and 1 min later decapitated.

General Biochemical Procedures

Each mouse brain, minus olfactory bulbs, was removed, rinsed in ice-cold 0.9% saline, and homogenized with 12 strokes in a Teflon-glass homogenizer in 4 or 5 ml ice cold 0.1 N NaOH or 0.05 M sodium borate, pH 9.0. The time from decapitation to homogenization was one minute. In some experiments 100–180 mg of the right lobe of the liver was removed from each mouse, weighed, and homogenized as above.

From each homogenate samples were prepared in triplicate as follows:

For estimates of total radioactivity (T-samples) in the brain and liver, 0.1 or 0.2 ml of homogenate was pipetted into a scintillation vial containing 10 ml of scintillation fluid: Triton X-100: water (200:100:21) and capped. The scintillation fluid contained 4.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4 methyl-5-phenyloxazolyl)]-benzene per liter of toluene. Note these aliquots are undried and contain tritiated water.

For estimates of total radioactivity exclusive of tritiated water (dried, unwashed, D samples), 0.1 or 0.2 ml of homogenate was pipetted into a scintillation vial, and dried for 2 hr at 100°C to remove tritiated water. 0.8 ml of Soluene (Packard, tissue solubilizer) was added and the vials capped.

For estimates of the total radioactivity in the washed TCA-insoluble (glycoprotein) fraction (W samples), 0.1 or 0.2 ml of homogenate was spotted onto a filter paper disc (Whatman, 3MM, 2.3 cm) mounted on a straight pin [13]. The discs were dried at room temperature for 1 hr and placed in ice-cold 5% trichloroacetic acid (TCA). Discs from brain and liver were washed separately. About 2 hours later, all discs, along with several blank discs, were placed in 500 to 1000 ml of 5% TCA for 30 minutes and then heated for 15 min at 90°C. They were then washed three times in ice cold 5% TCA for 20 min each, twice in 95% ethanol-ether (1:1) for 20 min at 40°C, and once in ether at room temperature for 20 min. The discs were placed in scintillation vials, 0.1 ml of Soluene was added and the vials capped.

To insure adequate solubilization of macromolecules, vials containing D or W samples were heated at 50°C for 8 hr and allowed to sit at room temperature for at least 36 hr. Ten ml of scintillation fluid was added to each vial. All samples were counted on a Packard 3375 Scintillation Spectrometer, and radioactivity counts were converted to disintegrations per minute (dpm) using external standards.

The results were expressed in terms of relative radioactivity where the radioactivity in the TCA-insoluble

$$RR = \frac{\text{dpm W}}{\text{dpm D} - \text{dpm W}}$$

material on the paper discs is divided by that in the TCA-soluble material in the unwashed D samples from the same tissue (see Discussion).

For comparisons of means, *t*-tests for unpaired samples were used.

RESULTS

In the first set of experiments, the amount of ^{3}H-fucose incorporated into acid-insoluble substances in brains and livers of quiet mice and mice trained on a jump-up active avoidance task was studied. As shown in Table 1, an increase in the levels of incorporation expressed as RR in trained mice occurred, but this increase was quite variable. An analysis of these results suggested that the seconds of shock the trained mice received might be affecting the amount of fucose incorporated into glycoproteins. This idea is supported by data showing a significantly decreased RR in the brains and livers of classically conditioned mice (Table 2). These results and those from the training experiments in Table 1 indicate that footshock reduces ^{3}H-fucose incorporation into brain glycoprotein.

Figure 1 shows the results of an experiment designed to measure directly the influence of the apparatus and the amount of footshock on the level of fucose incorporation. As predicted, increasing amounts of footshock resulted in decreased levels of incorporation. The data in Fig. 1 resolved most of the inconsistencies seen in the previous experiments with trained mice. When the curve of Fig. 1 was used to predict the percentage changes in RR on the basis of

TABLE 1

THE EFFECT OF AVOIDANCE TRAINING ON THE INCORPORATION OF ^{3}H–FUCOSE INTO BRAIN

Exp.	Treatment	N	Avg. Sec Shock	dpm D	dpm W	RR	% Change in RR Relative to Quiet
A	Trained	6	5.9	3,793 ± 502	537 ± 61	0.165 ± 0.014	+100*
	Quiet	6	–	2,929 ± 493	222 ± 31	0.082 ± 0.011	
B	Trained	6	10.2	6,175 ± 1,570	404 ± 56	0.070 ± 0.005	+ 35*
	Quiet	6	–	5,301 ± 982	262 ± 68	0.052 ± 0.006	
C	Trained	6	14.3	3,577 ± 455	265 ± 33	0.080 ± 0.012	+ 4
	Quiet	6	–	3,398 ± 443	243 ± 33	0.077 ± 0.011	

Results are the means ± standard deviations. DPM are for 0.1 ml aliquots of 4 ml total homogenate.
Mice were injected and treated as described in the Methods.
Radioactivity was determined in D and W as described in the Methods. $RR = \frac{dpm\ W}{dpm\ D - dpm\ W}$
*$p<0.05$, two-tailed

TABLE 2

THE EFFECT OF CLASSICAL CONDITIONING ON THE INCORPORATION OF ^{3}H–FUCOSE INTO BRAIN AND LIVER

Treatment	N	dpm T	dpm D	dpm W	$\frac{dpm\ D}{dpm\ T}$	RR	% Change in RR Relative to Quiet
				BRAIN			
Quiet	6	4,475 ± 641	3,142 ± 443	243 ± 33	0.702 ± 0.007	0.085 ± 0.013	–
Classically Conditioned (30 sec total shock)	6	4,682 ± 552	3,330 ± 390	229 ± 24	0.712 ± 0.016	0.072 ± 0.005	−15.0*
				LIVER			
Quiet	6		36,430 ± 11,360	2,688 ± 1,020		0.078 ± 0.007	–
Classically Conditioned (30 sec total shock)	6		40,420 ± 14,660	2,185 ± 307		0.056 ± 0.006	−28.0†

Results are the means ± standard deviations. DPM are for 0.1 ml aliquots of 4 ml total homogenate.
Mice were injected and treated as described in the Methods. Radioactivity was determined in D and W as described in the Methods.
$RR = \frac{dpm\ W}{dpm\ D - dpm\ W}$
*$p<0.05$, two-tailed
†$p<0.001$, two-tailed

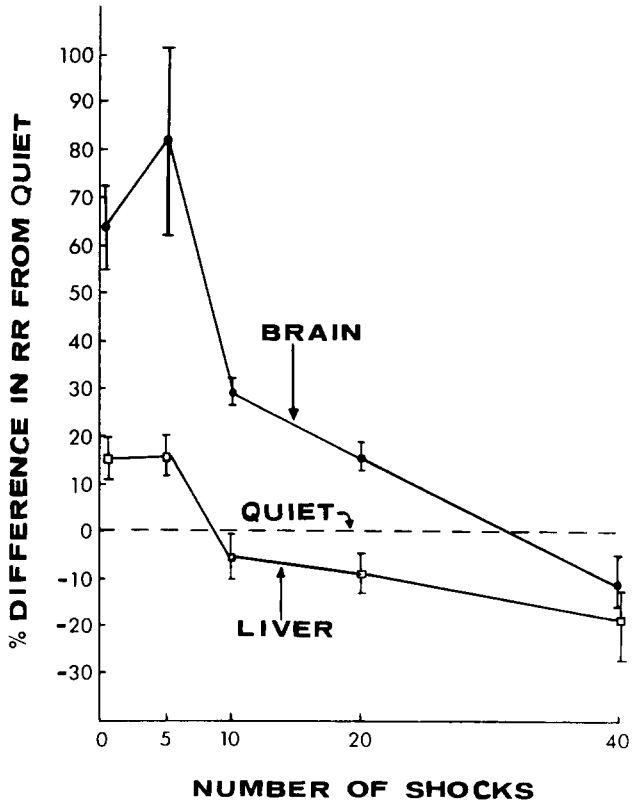

FIG. 1. The effects of exposure to the apparatus and subsequent footshock on RR of brain and liver. Results are means (N=5 or 6) ± standard error of the mean. Mice were injected, placed in the apparatus, and shocked as described in the Methods. Twenty minutes after the injection, they were sacrificed, and dpm W and dpm D determined as described in the Methods.

total seconds of footshock received on the average by trained mice, the linear correlation coefficient between the observed and predicted changes was 0.92.

Figure 1 also revealed a totally unexpected phenomenon. Significant increases in the incorporation of fucose into brain and liver glycoproteins occurred in mice that were exposed to the apparatus, but never given footshock. This phenomenon has been confirmed many times. It appears that the increased incorporation observed in these experiments may be due to the exposure of the animals to certain sensory stimuli present in the apparatus. It may also be due, in part, to the additional handling of these animals. Table 3 shows that a small nonsignificant increase occurs in the RR of brain and liver of handled mice. A significant increase is observed in the RR of brain and liver of urine-exposed mice, but this increase is smaller than that observed in apparatus-exposed mice.

DISCUSSION

The usual way to measure the incorporation of a radioactive precursor into a molecule is to determine the specific activity (dpm/moles) of both precursor and product. This corrects for variability due to injections, precursor uptake, and recovery of product. Using standard methods, we have not been able to assay free fucose or glycoproteins quantitatively in a single mouse brain. In addition such methods do not lend themselves well to behavioral studies where many animals must be examined. Thus we have used a simplified method to obtain estimates of relative radioactivity (RR).

Control experiments (Damstra-Entingh *et al.*, manuscript in preparation) have confirmed reports [16,20] that for at least 1 hr after a subcutaneous injection of ^{3}H-fucose, over 90% of the dried acid-soluble radioactivity (dpm D-dpm W), and 90% of the acid-insoluble radioactivity (dpm W) remains in fucose. In addition, 90% of the acid-insoluble radioactivity (dpm W) was solubilized by pronase and recovered as glycopeptides. Thus, dpm D and dpm W measure the radioactivity in total and bound fucose respectively. DPM T indicates the radioactivity in tritiated water + dpm D. As shown in Table 2, dpm D/dpm T, the proportion of dpm not in tritiated water is about 70% regardless of the behavioral treatments of the mice. Therefore, since D/T is constant, no change would be seen in the present data if T were used instead of D in the calculations. Since most laboratories thoroughly dry samples to remove tritiated water, dpm D was used here as an estimate of total radioactivity in free and bound fucose, and dpm D-dpm W as the pool correction factor.

Thus RR is a reasonable measure of the incorporation of fucose into glycoproteins. But this measure can be affected by changes in the rate of delivery and uptake of precursor, by pool changes and compartmentalization phenomena, and by the rate of catabolism of glycoproteins. Further research is necessary to clarify the mechanisms involved here. Conceivably, an increase in the specific activity of the precursor pool could occur early in the pulse such that the end point measurement would not provide an adequate correction. Increased dpm in free fucose were observed in stimulated mice (Table 1), but the increases of dpm in bound fucose in glycoproteins were even greater.

When intra-cranial injections of ^{3}H-fucose were given, there were no significant differences between quiet mice and apparatus-exposed mice in the RR of brains or livers (unpublished observations). This indicates that the observed incorporation changes are dependent upon a relatively undisturbed brain state, or that the differences seen after subcutaneous injections are due to changes in precursor uptake in the blood or brain.

The changes observed in RR of brain and liver appear to be related to certain types of sensory input, rather than to instrumental learning. The exact nature of this sensory input is unclear. One explanation of the data is that mild stress, such as exposure to the apparatus elevates the RR, whereas increasing amounts of stress (10-40 sec of footshock) reduce the ^{3}H-fucose incorporation (see [15]). However, a number of distinct phenomena may be responsible for the increased and decreased incorporation levels. Olfactory cues may be partially responsible for the increased RR in the apparatus-exposed mice.

Bogoch [2] has observed a marked increase of ^{14}C-glucose incorporation into the brain mucoids of pigeons during the first 10–20 min of operant conditioning. However, Holian *et al.* [8] found no significant differences in the amount of ^{14}C-glucosamine incorporated into the glycoproteins of control rats and rats trained on a one trial passive avoidance task. They injected ^{14}C-glucosamine 15 min prior to the training experience, and sacrificed the rats 1 hr or 24 hr later. We have found (unpublished data) that mice injected with ^{3}H-fucose just prior to 15 min of jump-

TABLE 3

THE EFFECTS OF VARIOUS BEHAVIORAL TREATMENTS ON THE INCORPORATION OF ^{3}H–FUCOSE INTO BRAIN AND LIVER

Treatment	N	dpm D	dpm W	RR	% Change in RR Relative to Quiet
			BRAIN		
Quiet	6	4,665 ± 1,000	202 ± 42	0.046 ± 0.005	–
Apparatus Exposed	6	5,355 ± 1,475	360 ± 119	0.072 ± 0.005	+56.0‡
Urine Exposed	6	5,001 ± 832	277 ± 59	0.059 ± 0.009	+28.0*
Handled	6	4,746 ± 816	237 ± 59	0.053 ± 0.006	+16.0
			LIVER		
Quiet	6	41,446 ± 7,635	2,006 ± 384	0.051 ± 0.005	–
Apparatus Exposed	6	51,930 ± 19,190	3,671 ± 1,065	0.079 ± 0.011	+54.0‡
Urine Exposed	6	45,409 ± 18,588	2,739 ± 2,058	0.061 ± 0.024	+20.0
Handled	6	48,492 ± 18,724	2,475 ± 1,106	0.054 ± 0.004	+ 6.0

Results are the means ± standard deviations. DPM are for 0.1 ml aliquots of 4 ml total homogenate.

Mice were injected and treated as described in the Methods. Radioactivity was determined in various homogenate fractions as described in the Methods.

$$RR = \frac{\text{dpm W}}{\text{dpm D} - \text{dpm W}}$$

*$p<0.05$, two-tailed

†$p<0.01$, two-tailed

‡$p<0.001$, two-tailed

up active avoidance training, and sacrificed 165 min later showed no differences from the RR of brains of quiet mice. Further time course studies are in progress, but it appears that the maximal effects occur when the animals are killed very soon after the completion of stimulation.

The decreased incorporation following moderate amounts of footshock is unique in our laboratory to fucose. It does not occur with the incorporation of uridine into RNA [21], phosphate into nuclear proteins [12], or amino acids into proteins [18]. As a matter of fact, Rees [18] has shown a small increased incorporation of ^{3}H-lysine into brain and liver proteins of mice and rats subjected to 20 sec of footshock. Brogan (unpublished data) found no change in the incorporation of ^{3}H-lysine into brains of mice given footshock during the isotope pulse. Irwin [9], however, found a decrease in the specific radioactivity of non-glycolipid brain carbohydrate 24 hrs after an intra-cranial injection of C^{14}-glucosamine as a result of behavioral stimulation. Thus different biochemical changes following environmental stimulation are dissimilar with respect to timing parameters and the specific experiential input.

It is conceivable that with the present methods the data are due to changes in blood glycoproteins, cerebral blood flow, or precursor uptake phenomena instead of changes in brain glycoproteins. Further experiments that examine the underlying physiological and biochemical mechanisms responsible for these changes are in progress, but particular features of these changes suggest that certain aspects of the biochemical mechanisms involved may be difficult to elucidate. (1) Since the concentration of free fucose in the brain is small, accurate measurements of the specific radioactivity of fucose in the brain are difficult. (2) The failure to detect statistically significant metabolic changes after intracranial injections of ^{3}H-fucose makes the use of a peripheral route of injection necessary. Peripheral injections, during the brief incorporation periods used here, deliver only limited amounts of radioactive fucose to the brain, making extensive subcellular fractionation studies difficult. Peripheral injections also render the injected ^{3}H-fucose sensitive to possible changes in blood circulation and to altered metabolism by peripheral organs, thus giving rise to a complex kinetic situation, and to the possibility that the primary

origin of the observed effects is in a tissue other than brain. (3) Small amounts of blood are trapped in the brain tissue at the time of sacrifice. The relative contributions to the measured radioactivity in unbound fucose from the blood and the brain may be difficult to distinguish. Nevertheless a more detailed characterization of these phenomena should give valuable insight into the nature of the biochemical responses of the organism to environmental stimuli.

REFERENCES

1. Barondes, S. H. Brain glycomacromolecules and interneuronal recognition. In: *The Neurosciences, Second Study Program,* edited by F. O. Schmitt. New York: Rockefeller University Press, 1970, pp. 747–760.
2. Bogoch, S. *The Biochemistry of Memory with an Inquiry into the Function of Brain Mucoids.* New York: Oxford University Press, 1968.
3. Bondareff, W. and J. Sjostrand. Cytochemistry of synaptosomes. *Expl. Neurol.* **24:** 450–458, 1969.
4. Brunngraber, E. G. Glycoproteins in the nervous system. In: *Handbook of Neurochemistry,* Vol. 1, edited by A. Lajtha. New York: Plenum Press, 1969, 223–244.
5. Coffey, J. W., O. N. Miller and Z. O. Sellinger. The metabolism of L-fucose in the rat. *J. biol. Chem.* **239:** 4011–4017, 1964.
6. Dische, Z. The informational potentials of conjugated proteins. In: *Protides of the Biological Fluids,* Vol. 13, edited by H. Peeters. Amsterdam: Elsevier Publishing Co., 1966, pp. 1–20.
7. Ginsburg, V. and E. F. Neufeld. Complex heterosaccharides of animals. *Ann. Rev. Biochem.* **38:** 371–388, 1969.
8. Holian, O., E. G. Brunngraber and A. Routtenberg. Glycoproteins and memory consolidation. *Life Sci.* **10:** 1029–1034, 1971.
9. Irwin, L. N. and F. E. Samson. Content and turnover of gangliosides in rat brain following behavioural stimulation. *J. Neurochem.* **18:** 203–211, 1971.
10. Kaufman, R. L. and V. Ginsburg. The metabolism of L-fucose by hela cells. *Expl. Cell Res.* **50:** 127–132, 1968.
11. Louisiot, P. Biosynthesis of brain glycoproteins. *Adv. Expl. Med. Biol.* **25:** 73–99, 1972.
12. Machlus, B. Phosphorylation of nuclear proteins during behavior in rats. Ph.D. Thesis, University of North Carolina, Chapel Hill, 1971.
13. Mans, R. J. and G. D. Novelli. Measurement of the Incorporation of Radioactive Amino Acids into Protein by a Filter-Paper Disc Method. *Archs. Biochem. Biophys.* **94:** 48–53, 1961.
14. McKean, D. B. and J. Pearl. Avoidance box for mice. *Physiol. Behav.* **3:** 795–796, 1968.
15. Pevzner, L. Z. Nucleic acids in the neuron-neuroglia unit in various functional states of the nervous system. In: *Macromolecules and the Function of the Neuron,* edited by Z. Lodin and S. P. R. Rose, John Wiley and Sons, 1968, pp. 353–362.
16. Quarles, R. H. and R. O. Brady. Synthesis of Glycoproteins and Gangliosides in Developing Rat Brain. *J. Neurochem.* **18:** 1809–1820, 1971.
17. Rambourg, A. and C. P. Leblond. Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. cell biol.* **32:** 27–53, 1967.
18. Rees, H. Brain protein metabolic correlates of sensory stimulation and behavior in mice. Ph.D. Thesis, University of North Carolina, Chapel Hill, 1972.
19. Revel, J. P. and S. Ito. The surface components of cells. In: *The Specificity of Cell Surfaces,* edited by B. D. Davis and L. Warren: Prentice-Hall, 1967, pp. 211–234.
20. Zatz, M. and S. H. Barondes. Fucose Incorporation into Glycoproteins of Mouse Brain. *J. Neurochem.* **17:** 157–163, 1970.
21. Zemp, J. W., J. E. Wilson, K. Schlesinger, W. O. Boggan and E. Glassman. Brain funtion and macromolecules. I. Incorporation of uridine into RNA of mouse brain during short-term training experience. *Proc. natn. Acad. Sci.* **55:** 1423–1431, 1966.