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Separate Biochemical Actions of Inhibitors of Short- and Long-Term Memory[1]

M. E. GIBBS

Department of Psychology, La Trobe University, Bundoora, Victoria, 3083 Australia

P. L. JEFFREY AND L. AUSTIN

Department of Biochemistry, Monash University, Clayton, Victoria, 3168 Australia

AND

R. F. MARK

Department of Physiology, Monash University, Clayton, Victoria, 3168 Australia

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GIBBS, M. E., P. L. JEFFREY, L. AUSTIN AND R. F. MARK. *Separate biochemical actions of inhibitors of short- and long-term memory.* PHARMAC. BIOCHEM. BEHAV. 1(6) 693–701, 1973.–*In vitro* effects of the antibiotics – cycloheximide, puromycin and chloramphenicol, and the sodium pump blockers – ouabain, lithium and copper, on protein systhesis in the crude mitochondrial, postmitochondrial and synaptosomal fractions of chicken forebrain were studied. In the synaptosomal fraction there was a correlation between the inhibitions produced by the sodium pump blockers, of Na^+/K^+ ATPase activity, ^{14}C-leucine uptake and the incorporation of ^{14}C-leucine into protein; whereas the antibiotics only inhibited leucine incorporation into protein. The effects of these drugs on the *in vivo* incorporation of ^{14}C-leucine into protein in various fractions of chicken forebrain were also studied. The results suggest that there is a biochemical link involving a sodium pump and the transport of amino acids, between nerve impulse activity and neuronal protein synthesis. This link may also interconvert short-term and long-term storage of memory.

Sodium pump inhibition   Protein synthesis inhibition   Amino acid transport inhibition   Ouabain   Lithium   Cycloheximide   Short-term memory   Long-term memory

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OUABAIN and other inhibitors of the active transport of sodium and potassium ions across cell membranes selectively interfere with an early phase of memory storage that is not susceptible to agents that inhibit brain protein synthesis [36,37]. This was first reported for a one trial passive avoidance learning task in day old chickens and has now been confirmed in an appetitive task and in operant learning for heat reinforcement in slightly older birds. ([31], Rogers, L. J., R. Ottinger and R. F. Mark, in preparation).

Besides blocking the behavioural expression of memory as it exists for a short period after training, ouabain may also interfere with the subsequent transformation of short-term memory into the long-term memory that is dependent upon the synthesis of new proteins [25]. In avoidance learning ouabain prevents the formation of long-term memory provided it is injected into the brain before or up to 20 sec after learning (Gibbs, M. E. in preparation). Protein synthesis inhibitors which prevent the formation of long-term memory are effective even when injected up to 10 min after learning [12,25].

This paper describes the biochemical effects of various substances on enzymes responsible for Na transport and on the incorporation of amino acid into protein in chicken brain. These substances include those that have previously been shown to inhibit the two successive stages of memory storage. The relationship between inhibition of ion transport and protein synthesis has been studied on a synaptosomal fraction isolated from the forebrain. Findings on this preparation have been confirmed in experiments on the effects of the same drugs on the incorporation of labelled amino acid into protein in the intact brain. A preliminary account of this work has appeared [35].

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METHOD

In Vitro Method

Preparation of homogenized forebrain tissue. Day-old white leghorn-black australorp cockerels were decapitated, the forebrain removed, weighed and placed in ice cold homogenization medium. The tissue was homogenized in a ten fold dilution with 10 up and down strokes at 500 rpm in a glass teflon homogenizer (clearance 0.15 mm).

Subcellular fractionation in 0.32 M sucrose containing 10 mM TRIS, pH 7.2 was carried out to isolate nuclear, crude mitochondrial, postmitochondrial, microsomal and soluble fractions as described by Austin and Morgan [3]. The crude mitochondrial fraction was further separated into myelin, synaptosomal and mitochondrial fractions using the method of Kurokawa, Sakamoto and Kato [16].

Measurement of leucine incorporation. Preliminary experiments were carried out on the postmitochondrial and the crude mitochondrial fractions. The isolation was carried out in medium M of Campbell, Mahler, Moore and Tewari [8] containing 250 mM sucrose, 20 mM TRIS pH 7.6; 10 mM magnesium acetate; 40 mM NaCl; 100 mM KCl and 5 mM 2-mercapto-ethanol, until the postmitochondrial fraction was obtained. The crude mitochondrial fraction was then suspended in an ionic medium -- medium TMN of Morgan and Austin [26] containing 125 mM NaCl; 25 mM KCl; 15 mM $MgCl_2$ and 10 mM TRIS pH 7.4.

The postmitochondrial fraction was incubated with 0.1 mM GTP and 5 mM ATP in medium M in a final volume of 1.0 ml at 37°C for 30 min. The reaction was commenced by the addition of 0.5 μCi of L-(U-^{14}C) leucine (Amersham 310 mCi/mM) after 10 min preincubation. The crude mitochondrial and synaptosomal fractions were incubated in 1 ml of medium TMN in the same manner as the postmitochondrial fractions. Reactions were terminated by the addition of an equal volume of cold 10% w/v TCA.

The above fractions were preincubated for 10 min with the following inhibitors: chloramphenicol (0.3 mM); cycloheximide (0.1 mM); ouabain (1.0 mM); puromycin (0.05 mM); lithium chloride (1.5 mM) or cupric chloride (0.05 mM). These concentrations were chosen on the basis of studies by Morgan and Austin [26]. The brackets refers to the final concentration of the inhibitor in the incubation mixture. These incubations were then treated as above.

Counting. The samples were prepared for counting and counted as described by Austin and Morgan [3]. The efficiency of aqueous ^{14}C counting was 70%.

Protein estimation. Samples were taken for protein estimation by the method of Lowry *et al.* [21].

Estimation of free amino acid uptake. Using ^{14}C-leucine, the free amino acid uptake was determined in parallel with protein synthesis studies as described by Morgan and Austin [27]. All incubations were for 30 min.

Estimation of enzymes. All samples for enzyme assay were subjected to freeze-thaw cycling before assay except the Mg^{++} and Na/K ATPase assay which was carried out on freshly prepared samples. Acetylcholinesterase activity (30 mM acetyl-β-methyl choline as substrate) was determined by the method of Aldridge and Johnson [1]. Cytochrome c reductase was determined by the method of Smith [33], Lactic dehydrogenase was determined by the method of Neilands [28]. Monoamine oxidase was determined by the method of McCaman *et al.* [22] using H^3-tyramine as substrate. The Mg^{++} and Na/K stimulated ATPase was determined as previously described [26].

In Vivo Method

Incorporation of ^{14}C-leucine into normal forebrain tissue. Day old chickens were injected intracranially as previously described [25], with 1.0 μCi of ^{14}C-leucine in 0.05 ml of saline, 5 min before decapitation. The forebrains were removed and homogenized in 20 ml of 0.32 M sucrose containing 5 mM TRIS and 10 mM leucine (pH 7.0). The homogenate was spun at 800 g for 10 min in an International PR-2 refrigerated centrifuge. The pellet was suspended in 10 ml of sucrose-TRIS medium and rehomogenized. The combined supernatants obtained from the centrifugations at 800 g for 10 min were fractionated to obtain the various subcellular fractions [3]. The level of leucine incorporation and protein estimations were carried out as described for the *in vitro* studies.

Inhibition of ^{14}C-leucine incorporation. The inhibitors cycloheximide, ouabain, lithium and chloramphenicol, were injected into each side of the forebrain in 0.05 ml of 0.9% w/v NaCl (0.025 ml × 2) in the concentrations used in the behavioural experiments, 55, 25 and 5 min before the injection of ^{14}C-leucine. One chicken was used for each time interval. A 5 min pulse of ^{14}C-leucine was used, the chickens were then decapitated and treated as described as above.

Inhibition of Na/K ATPase activity. Chickens were injected with 0.365 μgm of ouabain as for the behavioural experiments, killed 5, 30 or 60 min later. The forebrains were removed within 30 sec of this time and placed in 2 ml of 0.32M sucrose, and then homogenized. After 2 min preincubation, the ATPase activity of the total homogenate was measured in the manner described in the *in vitro* methods.

RESULTS

In Vitro Determinations

Biochemical characteristics of subcellular fractions. Evaluation of the effectiveness of the procedure described for the fractionation of the forebrain homogenate into various subcellular fractions was made by assaying for six marker enzymes. The results are shown in Table 1.

Lactic dehydrogenase (LDH) was used as a marker for soluble material. There was a large amount of activity associated with the crude mitochondrial fraction, which would be in accord with the presence of synaptosomes. LDH was concentrated in the synaptosomal fraction in terms of percentage distribution and there was some activity in the soluble fraction obtained from the crude mitochondrial fraction.

Monoamine oxidase (MAO), an outer mitochondrial membrane marker which indicates the intactness of the mitochondria, and cytochrome c reductase an inner mitochondrial membrane marker, were used as the markers for mitochondria. Both these activities were found to be concentrated in the crude mitochondrial fraction, but when this fraction was further purified on a discontinuous Ficoll gradient [16], they were concentrated in the purified mitochondrial fraction.

Acetylcholinesterase (AChE) is generally taken as a marker for membrane fragments, whether derived from the endoplasmic reticulum or the plasma membrane. AChE was found to be distributed in the crude mitochondrial, microsomal and soluble fractions. In the crude mitochondrial subfractions, AChE was found on percentage activity to be

TABLE 1

BIOCHEMICAL CHARACTERIZATION OF CRUDE MITOCHONDRIAL SUBFRACTIONS

Fraction	Protein	LDH	MAO	Cytochrome C Reductase	AChE	Na^{+}/K^{+}-ATPase Activity	Mg^{++}-ATPase Activity
Soluble	10.4	12.3 (1.19)	1.3 (0.13)	3.8 (0.36)	16.6 (1.59)	2.9 (0.28)	16.6 (1.60)
Myelin	5.52	6.6 (1.19)	1.1 (0.20)	2.0 (0.36)	5.7 (1.04)	37.0 (6.70)	4.8 (0.87)
Synaptosomal	58.44	58.1 (0.99)	38.0 (0.65)	29.0 (0.50)	43.2 (0.74)	82.2 (1.41)	52.8 (0.90)
Mitochondrial	28.90	20.9 (0.72)	26.8 (0.93)	32.2 (1.11)	7.8 (0.27)	0	44.3 (1.53)
% Recovery	103.26	97.9	67.2	67.0	73.3	122.1	118.5

Biochemical characterization of subfractions of the crude mitochondrial pellet expressed as percentages of the crude mitochondrial pellet. In this set of data the crude mitochondrial fraction comprised 30.8% of the total homogenate protein. Percentage distribution of activity and relative specific activity (in brackets).

LDH – lactic dehydrogenase, MAO – monoamine oxidase, AChE – acetylcholinesterase

mainly in the synaptosomal fraction.

Na/K ATPase activity, a plasma membrane marker, was predominantly in the crude mitochondrial fraction. The myelin fraction from the crude mitochondrial fraction had a very high RSA but on percentage activity this enzyme was nearly all in the synaptosomal fraction.

Incorporation of ^{14}C-leucine into fractions of chicken forebrain.

Postmitochondrial fraction. The microsomal and soluble fractions comprising the postmitochondrial fraction were capable of incorporating 4.16 μμmoles leucine/mg protein into hot acid insoluble protein over a 30 minute period. For protein synthesis, an energy source was necessary as shown by a dependence on ATP. There was 97.9% suppression of protein synthesis when ATP was omitted from the incubation medium. When GTP was omitted there was only 19.4% suppression, whereas when both ATP and GTP were left out there was a suppression of 98.6%.

Crude mitochondrial fraction. When the crude mitochondrial fraction was incubated under these conditions in Medium TMN there was 1.70 μμ moles of leucine incorporated/mg protein/hr.

Synaptosomal fraction. When the synaptosomes were incubated in Medium TMN there was 1.72 μμ moles of leucine incorporated/mg synaptosomal protein/hr. When labelled leucine is added, in view of the content of amino acids within the synaptosomes, there will be a consequent dilution of the labelled amino acid. The low labelling of the synaptosomal fraction observed could have represented a higher rate of protein synthesis [23] as the transfer of amino acids across the synaptosomal membrane may be the rate limiting step. The highest rate of synaptosomal protein synthesis has been observed using Medium TMN which is similar in ionic composition to the extracellular fluid.

Inhibition of ^{14}C-leucine incorporation into fractions of chicken forebrain. The concentrations of the inhibitors used in these *in vitro* biochemical studies were higher than those used in the behavioural experiments to account for dilution of the fractions. In Table 2 a comparison is made between the concentrations used *in vitro* and the concentration that might occur *in vivo*. This concentration is calculated on the basis of the chicken forebrain occupying a volume of 1 ml, which is also the volume used for the incubations. The inhibitions of incorporation are set out in Table 3.

Postmitochondrial fraction. Ouabain has no inhibiting effect on the incorporation of ^{14}C-leucine into the microsomal and soluble proteins. Both copper and lithium ions inhibited incorporation.

CXM inhibited leucine incorporation by 80.7% in the postmitochondrial fraction. Puromycin also inhibited to a large extent in the postmitochondrial fraction. Chloramphenicol (CAP), on the other hand, inhibited incorporation in this fraction by only 8.7%. This antibiotic specifically inhibits mitochondrial protein synthesis in eukaryotic cells [9].

Crude mitochondrial fraction. In the crude mitochondrial fraction ouabain inhibits leucine incorporation. The other inhibitors also reduced the incorporation of ^{14}C-leucine into protein, copper and lithium ions inhibited by 30 to 40%. CXM produced a 30.9% inhibition compared to 80.7.% in the postmitochondrial fraction. Puromycin had a less inhibiting effect in this fraction. CAP on the other hand, produced 47.3% inhibition in accord with the presence of mitochondria.

Synaptosomal fraction. Ouabain, copper and lithium

TABLE 2

APPROXIMATE CONCENTRATIONS OF INHIBITORS IN *IN VITRO, IN VIVO* BIOCHEMICAL AND BEHAVIOURAL EXPERIMENTS

Drugs	Behavioural Experiments Concentration	Biochemical Experiments Concentration	
		In Vivo	*In Vitro*
Ouabain	0.075 mM 0.548 μg	0.075 mM	1 mM
Copper	0.1 mM 0.33 μg		0.05 mM
Lithium	5.8 mM 245.0 μg	5.8 mM	1.5 mM
Cycloheximide	0.13 mM 37.5 μg	0.13 mM	0.1 mM
Puromycin	0.007 mM 18.0 μg		0.05 mM
Chloramphenicol	0.15 mM 48.5 μg	0.15 mM	0.3 mM

The concentrations for behavioural and *in vivo* biochemical experiments were calculated on the assumption that the substances were distributed in a volume of 1 ml, which is the volume of the chicken forebrain. The figures therefore serve for comparison of approximate doses used in behavioural and biochemical *in vivo* experiments and do not accurately reflect concentrations at the sites of action. The amount of the drug (in μg) injected into the forebrain in a volume of 0.05 ml is also shown. The *in vitro* concentrations were based on results of Morgan and Austin [26].

inhibited protein synthesis more in this fraction than in the crude mitochondrial fraction. In this fraction there was approximately the same degree of inhibition of protein synthesis with the sodium pump inhibitor ouabain, as with the ribosomal type inhibitor CXM and CAP. Puromycin and copper showed a more marked effect.

Amino acid uptake. Free leucine uptake into synaptosomes was investigated as an approximate measure of amino acid transport across the synaptosomal membrane (Table 4). The sodium pump inhibitors ouabain, copper and lithium, reduced the free leucine uptake into synaptosomes by 30 to 40% whereas the antibiotics CSM, CAP and puromycin, showed no significant reduction.

Na/K ATPase activity and inhibition The effect of the sodium pump inhibitors and the antibiotics on ATPase activity was examined [11] (Table 5). Ouabain, copper and lithium all inhibited the Na/K ATPase activity, whereas the protein synthesis inhibitors did not.

The results of the effects of free leucine uptake and from the inhibition of the ATPase activity are consistent with the hypothesis that sodium pump inhibitors block leucine incorporation by preventing the uptake of leucine. The Na/K ATPase is known to be concentrated in the synaptosomal membrane [14].

In Vivo Determinations

Pulse labelling. The injection of the labelled leucine directly into the brain during a 5 min pulse has the advantage of minimizing the effects of utilization that immediately follows intravenous or oral administration. For the purposes of these experiments, single pulse labelling of short duration was required in order to compare with time intervals similar to those used in the behavioural experiments. The pulse labelling time was kept constant at 5 min and comparisons of inhibitions made with the normal controls.

The addition of 10 mM cold leucine to the homogenizing medium meant that any further synthesis that might have occurred during the fractionation would result in very little further incorporation of the label.

The percentage of labelled leucine incorporated into the brain fractions was 5.3% during the 5 min pulse. The major part of the incorporated label was found in the soluble fraction (Table 6). The nuclear and mictosomal fractions contained less of the incorporated label but more than the synaptosomal and mitochondrial fractions which contained about the same amount. Incorporation into myelin fragments was very low.

TABLE 3

INHIBITION OF ^{14}C-LEUCINE INCORPORATION INTO FRACTIONS OF CHICKEN FOREBRAIN

Inhibitors	Postmitochondria	Crude Mitochondria	Synaptosomes
Ouabain (1.0 mM)	1.9 ± 15.2	22.2 ± 10.8	35.9 ± 10.6
Copper (0.05 mM)	48.4 ± 9.6	38.1 ± 3.4	86.0 ± 8.6
Lithium (1.5 mM)	67.5 ± 3.5	34.5 ± 14.0	61.4 ± 9.7
Cycloheximide (0.1 mM)	80.7 ± 4.2	30.9 ± 7.2	39.4 ± 13.6
Puromycin (0.05 mM)	96.5 ± 0.9	51.6 ± 14.1	70.8 ± 8.4
Chloramphenicol (0.3 mM)	8.7 ± 12.6	47.3 ± 17.7	33.3 ± 11.3

Inhibition of ^{14}C-leucine incorporation into fractions of chicken forebrain. The % inhibitions ± S.D. were obtained from normalized cpm/mg protein based on control values for each of 6 determinations for crude and postmitochondrial fractions and 19 determinations for the synaptosomal fractions.

Inhibition of ^{14}C-leucine incorporation into fractions of chicken forebrain by drugs administered in vivo. Cycloheximide significantly inhibited the incorporation of label into all fractions (Table 7). Maximum inhibition appeared to occur at 30 min after the injection of the antibiotic. The inhibition was severe in all fractions. The mitochondrial fraction was the least inhibited 10 min after injection of the antibiotic, but 30 min after the activity had fallen to 2.6% of the control, close to the activity observed in the microsomes. Most mitochondrial proteins are synthesized on the ribosomes and transferred to the mitochondria, which could be the reason for the mitochondria incorporating so little label at 30 min [20]. Geller *et al.* [12], have reported that there was 94% inhibition of cerebral protein synthesis 30 min after the injection of CXM but by 3 hr there was only 54% inhibition, so presumably the inhibitory action of the antibiotic is starting to decrease 60 min after the injection.

Ouabain inhibited the incorporation of the label into all fractions (Table 7). The inhibition of the mitochondrial incorporation was less than for the other fractions. The microsomal and soluble fractions, which were not inhibited *in vitro* (Table 3) were down to less than 9% of control incorporation.

The inhibition shown by lithium was not as great as that shown by ouabain (Table 7). Because lithium possibly does not attach to the carrier molecule in its inhibition of the sodium pump, it can diffuse out of the cell and hence the concentration is not maintained for as long as the inhibition obtained with ouabain.

Mitochondrial incorporation was inhibited *in vivo* by CAP (Table 7) more so than would have been expected on the basis of the *in vitro* studies (Table 3). The nuclear and microsomal fractions were about 50% of the control values for about 30 min but the incorporation into the soluble fraction was inhibited by 74 to 86%. This indicated that something else was being inhibited besides the mitochondrial protein synthesis. This could be explained on the basis of the findings that CAP at concentrations of 1.4–1.6 mM inhibits respiration and protein synthesis in mouse ascites cells by 50% [13], and the oxidation of NADH by beef heart mitochondria by 50% [11]. Thus it could be affecting protein synthetic mechanisms through a decrease in the levels of ATP.

ATPase activity inhibition in vivo. The Na/K ATPase activity was investigated only in the total homogenate following the injection of ouabain *in vivo* because the time required for further fractionation would allow the ouabain to diffuse away and be less effective. Even so, 5 min after the injection of ouabain, the activity was down to 48.7% of the activity of the control (Table 8). This increased by 10% as the time between injection of ouabain and measurement of activity was extended to 60 min. Thus the inhibition *in vivo* could have been greater than that actually measured. The dose which gave 51.3% inhibition of ATPase activity had a final concentration which was less than that which inhibited the protein synthesis *in vivo*.

DISCUSSION

The aim of these experiments was to observe the effects in chicken brain of inhibitors of protein synthesis and of

TABLE 4

SYNAPTOSOMAL LEUCINE UPTAKE

Inhibitor	cpm/mg Protein		% Inhibition
Control	7742	± 1452	
Ouabain (1.0 mM)	4303	± 1980	44.4
Copper (0.05 mM)	5177	± 2448	33.1
Lithium (1.5 mM)	4465	± 895	42.3
Cycloheximide (0.1 mM)	8664.	± 2553	−11.9
Puromycin (0.05 mM)	9222	± 1517	−28.4
Chloramphenicol (0.3 mM)	8592	± 1574	10.9

^{14}C-leucine uptake into the synaptosomes and the effect of various inhibitors. The synaptosomal fraction was incubated for 30 min with ^{14}C-leucine in conjunction with studies on the incorporation of ^{14}C-leucine into synaptosomal protein (Table 3). Means and standard deviations of 8 determinations.

Na/K ATPase that were used in previous behavioural experiments on inhibition of memory.

The actions on protein synthesis were estimated first on crude mitochondrial and postmitochondrial fractions prepared from the forebrain. Only cell nuclei had been removed from the homogenate and the fractionation procedure separated soluble and particulate fractions from membrane bound fragments. The incorporation of ^{14}C-leucine into protein in the postmitochondrial (soluble and particulate) fraction was reduced by antibiotics, by copper and lithium but not by ouabain even in a concentration greater than would be expected in the brain in the behavioural experiments.

A comparison between the effects of the drugs was made, using a synaptosomal fraction, on the incorporation of ^{14}C-leucine into protein and on the activity of the Na/K ATPase. In this system ouabain, copper and lithium all inhibited the Na/K ATPase but in contrast to the effects on the postmitochondrial preparation, also greatly decreased the rate of incorporation of leucine into protein. The uptake of ^{14}C-leucine was also measured and found to be inhibited along with the Na/K ATPase and the reduction in incorporation into protein was in proportion to the decreased uptake. The antibiotics inhibited leucine incorporation without inhibiting Na/K ATPase or amino acid transport as measured by leucine uptake.

Comparing these results with the effects of the same agents on memory, ouabain and other drugs which blocked the early stages of memory formation all inhibited Na/K ATPase but antibiotics which only blocked long-term memory formation did not. Antibiotics which inhibited long-term memory did inhibit the incorporation of ^{14}C-leucine into protein in synaptosomal preparations and so did ouabain, lithium and copper. Since ouabain in particular was without effect on protein synthesis in the post-mitochondrial fraction, the inhibition of protein synthesis in synaptosomal fractions was presumably via reduction in the membrane transport.

Other studies of the capacity of the synaptosomal fraction to synthesize protein have also shown up the importance of membrane transport. Synaptosomal protein synthesis is affected by many ionic environments. Na and K appear to affect activity and the ratio of these ions is of particular importance [2,27]. The Na and K concentrations necessary to achieve maximal protein synthesis, oxygen uptake and potassium accumulation are also those that give maximal Na/K ATPase activity. Furthermore, varying the concentrations of Na or K yielded parallel alterations in the Na/K ATPase activity, protein synthesis and potassium transport [2,10]. The maximum rate of protein synthesis occurs in a medium that most resembles extracellular fluid, suggesting that the ionic effects on protein synthesis are through a transport mechanism linked to the sodium pump. Coupled transport system for amino acids and sodium may not be directly linked to energy-yielding metabolic processes (for review see: Schultz and Curran [32]) but the sodium pump, in maintaining an electrochemical gradient across the cell membrane, may be the source of metabolic energy for the active transport of amino acids. Whatever the processes involved in the transport of amino acids across cell membranes, it appears to be involved with the Na/K ATPase activity and is inhibited by drugs which inhibit sodium transport. Inhibition of protein synthesis in the heart by ouabain has also been reported [17] and in this tissue also it appears to be due to a reduction in membrane transport of amino acids [18].

In a preparation of highly purified synaptic membranes Ramirez, Levitan and Mushynski [29] have shown that leucine incorporation into protein is sensitive to CAP and removal of Na, K ions or both, but not to CXM or ouabain. It appears that there is a CAP sensitive protein synthesizing system located in the membranes of synaptosomes as well as in the mitochondria. However, they found whole synaptosomes to be insensitive to removal of Na and K ions, CXM and ouabain which may reflect the long and sometimes unphysiological preparation [19], but on the other hand the concentration of ouabain was ten times less than the concentration used in the present experiments.

Recent work [34] has shown sensitivity of Na/K ATPase, Na and K content in guinea pig cortex to *in vivo* ouabain treatment (0.1 mg). In the intact brain where membrane transport through both glial cells and neurones might be thought to be of great importance for protein synthesis, ouabain was found in the present experiments to inhibit the incorporation of ^{14}C-leucine into protein.

Relationship of these Results to a Working Hypothesis of Biochemical Mechanisms of Memory

Recently active neurones accumulate Na ions, the active extrusion of which may well alter electrophysiological properties, perhaps by hyperpolarization of the membrane which may render it less excitable [30]. The process may also be associated in time with the post-activity increase in

TABLE 5

INHIBITION OF Mg^{++} ATPASE AND Na^{+}/K^{+} ATPASE ACTIVITIES IN THE SYNAPTOSOMAL FRACTION FROM CHICKEN FOREBRAIN

Inhibitors	Mg^{++} ATPase Activity		Na^{+}/K^{+} ATPase Activity	
	μ Moles Pi/mg/hr	% Inhibition	μ Moles Pi/mg/hr	% Inhibition
Control	2.33 ± 0.39		2.21 ± 0.12	
Ouabain (1.0 mM)	2.24 ± 0.10	3.9	0.57 ± 0.07	74.2
Copper (0.05 mM)	1.23 ± 0.20	47.2	0.22 ± 0.11	90.1
Lithium (1.5 mM)	2.20 ± 0.45	5.6	0.93 ± 0.19	57.9
Cycloheximide (0.1 mM)	2.19 ± 0.59	6.0	2.35 ± 0.92	-6.33
Puromycin (0.05 mM)	2.24 ± 0.26	3.9	2.04 ± 0.18	7.7
Chloramphenicol (0.3 mM)	2.06 ± 0.27	11.6	1.90 ± 0.36	14.0

Inhibition of Mg^{++} ATPase and Na^{+}/K^{+} ATPase activity in the synaptosomal fraction from chicken forebrain. Activities in each fraction presented as μ moles of inorganic phosphate released/mg protein/hour. Medium – TRIS (30 mM) pH7.1; ATP (5 mM); $MgCl_2$ (7.5 mM) for Mg ATPase; plus NaCl (120 mM) and KCl (20 mM) for total activity. Na^{+}/K^{+} ATPase activity being the difference between total and Mg^{++} ATPase activities. Means and standard deviations from 4 determinations.

TABLE 6

IN VIVO INCORPORATION OF ^{14}C-LEUCINE INTO CHICK FOREBRAIN FRACTIONS

Fraction	^{14}C-Leucine Incorporation counts/min/fraction	
Nuclear	19,029	±3293
Microsomal	17,934	±6502
Soluble	33,400	±5259
Myelin	1,841	±1130
Synaptosomal	5,058	±1315
Mitochondria	5,337	±1079

^{14}C-leucine incorporation into fractions of chick forebrain following injection of 1.0 μCi leucine 0.05 ml as a pulse label 5 min before sacrifice. Means (and standard deviations) of 7 experiments.

efficacy of synaptic transmission through the parallel accumulation of calcium ions [15]. Whatever the mechanism of excitability or synaptic changes which may underlie short-term memory, the extent of impulse activity of neurones will determine the electrophysiological consequences including the activation of Na extrustion over a period of minutes, extending perhaps for half an hour [7]. These are the effects that are blocked by ouabain and such changes could contribute largely to short-term memory storage that is not blocked by protein synthesis inhibitors.

Na pump activity and leucine uptake are both blocked by ouabain and lithium and the incorporation of leucine into protein suffers correspondingly. If the converse holds and amino acid transport is facilitated by Na pump activity then the accumulation of Na could control the avilability of amino acids for protein synthesis.

Neuronal protein synthesis may be modified by the type or amount of amino acid transport into those recently active neurones that are in the process of extruding accumulated sodium ions. It has been shown that the highest specific radioactivity following the incorporation of labelled amino acids into brain appears in membrane fractions [4, 5, 6, 26]. Perhaps some proteins whose synthesis may be modulated by the availability of intracellular amino acids through a mechanism linked to the sodium pump are

TABLE 7

INHIBITION OF ^{14}C-LEUCINE INCORPORATION *IN VIVO* INTO CHICKEN FOREBRAIN PROTEIN

Fractions	% Inhibition of Control											
	Cycloheximide			Ouabain			Lithium			Chloramphenicol		
	10 min	30 min	60 min	10 min	30 min	60 min	10 min	30 min	60 min	10 min	30 min	60 min
Nuclear	90.1	94.0	87.5	84.2	82.3	87.3	72.1	36.2	57.3	49.6	51.9	56.4
Microsomal	96.2	97.6	94.7	95.9	91.1	94.5	85.7	68.0	82.8	49.9	55.2	79.9
Soluble	99.1	99.5	98.4	96.8	94.2	96.3	87.3	77.4	87.1	85.7	77.7	74.3
Myelin	95.6	97.7	92.2	91.4	85.6	91.6	68.1	36.5	66.2	7.9	−35.1	30.0
Synaptosomal	95.5	97.5	95.3	96.2	91.1	94.4	81.5	63.7	84.7	52.3	55.4	73.3
Mitochondria	87.7	97.4	90.1	86.1	68.8	79.7	75.3	68.3	77.2	58.2	57.4	65.1

Inhibition of ^{14}C-leucine incorporation into fractions of chicken forbrain by cycloheximide – 0.13 mM; ouabain – 0.75 μM; lithium 5.8 mM; and chloramphenicol – 0.15 mM at 10, 30 and 60 min after the injection of the inhibitor. 1.0 μCi leucine/0.05 ml injected as a pulse label 5 min before sacrifice.

TABLE 8

INHIBITION OF Na^+/K^+ ATPASE ACTIVITY *IN VIVO* BY OUABAIN

Time After Injection	μ Moles/ml Extract/hr	% of Control
0	19.7 ± 1.1	
5 Min	9.6 ± 1.9	48.7%
30 Min	11.3 ± 2.8	57.4%
60 Min	11.5 ± 0.2	58.4%

Na^+/K^+ ATPase activity and % of control activity after administration of 0.365 μg of ouabain *in vivo*. Means and standard deviations of 4 determinations. The activity was measured in the total homogenate.

components of membranes that may play some role in the long term regulation of synaptic connectivity [24]. This would provide a link between the ionic mechanisms of initial memory and the structural change of permanent memory.

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