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Biochemical Studies of Synapses *in Vitro*. II. Potassium Transport*

Antonio V. Escueta† and Stanley H. Appel‡

ABSTRACT: Previous studies of rat brain synaptosomes *in vitro* demonstrated modulation of protein synthesis by ionic constituents and endogenous energy availability, and suggested that isolated nerve endings may support active ion transport. Utilizing a rapid filtration technique to measure intrasynaptosomal potassium accumulation, the various parameters influencing uptake of cold potassium and ^{42}K were studied at 23°. Immediately after isolation, synaptosomes contained an average of 0.092 μmole of potassium/mg of protein. At 23° with 100 mM sodium and 10 mM potassium in the external medium, the synaptosomal potassium increased to approximately 0.14–0.25 μmole/mg of protein after 8 min. The optimal potassium accumulation occurred with 50 mM sodium present in the medium. With 50 mM sodium, potassium content rose to 0.20–0.30 μmole/mg of protein after 8 min. At this optimal level of sodium (50 mM), potassium concentrations greater than 10 mM produced minimal increase in potassium accumulation. Neither adenosine triphosphate, adenosine diphosphate, glucose, increased oxygen concentration, nor the substrates α-ketoglutarate, succinate, fumarate, and glutamate influenced potassium accumulation. Ouabain was an effective inhibitor only of the sodium-dependent potassium accumulation. In sodium-free medium ouabain had essentially no effect on potassium ion accumulation. In the presence of 50 mM sodium and ouabain (10^{-4} M), the total potassium content of the synaptosomes declined by 54%.

The extent of inhibition by ouabain was equivalent to the extent of activation by sodium. Both 2,4-dinitrophenol and potassium cyanide inhibited potassium accumulation. Total potassium and ^{42}K uptake by synaptosome decreased as a function of time after isolation with the most marked reduction in the ouabain-inhibitable component. Total synaptosomal volume was measured with the use of *N*-methyl-[^{14}C]antipyrene, and the potassium content per milligram of protein was expressed in synaptosomal potassium concentrations. With 10 mM potassium and 50 mM sodium, there was an increase of intrasynaptosomal potassium concentration from approximately 71 mM following isolation to 103 mM, representing a significant accumulation of potassium against a concentration gradient. The present studies demonstrate that isolated rat brain synaptosomes support active potassium transport.

The key to the organization and function of the nervous system is given by the pattern of synaptic connections between cells and the efficiency of such synapses in interneuronal communication. In chemical synapses ionic fluxes mediate the release of neurotransmitter from the presynaptic terminal and determine the consequence of the neurotransmitter–receptor interaction in the postsynaptic terminal. Thus, in both presynaptic and postsynaptic terminals a detailed understanding of the factors underlying the movement of ions is essential for an understanding of synaptic function.

The usefulness of synaptosomes isolated from rat brain cortex as a model for studying the effects of energy availability and ions upon synaptic metabolism has recently been demonstrated (Appel and Autilio, 1969). Such synaptosomes were demonstrated to synthesize proteins *in vitro*. The protein synthesis was enhanced fourfold by 100 mM sodium and 10 mM potassium and was inhibited by ouabain as well as dinitrophenol, oligomycin, and potassium cyanide. It was not enhanced by an exogenous supply of energy or substrate. In essence,

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† Fellow in Neurobiology, Division of Neurology, Duke University Medical Center, Durham, N. C. 27706.

‡ Research career development awardee of the U. S. Public Health Service. Inquiries regarding this article should be made to this author: Division of Neurology, Duke University Medical Center.

the findings demonstrated a coupling of membrane events and macromolecular synthesis and suggested the possibility that isolated nerve endings may support active ion transport *in vitro*.

The present paper demonstrates that synaptosomes isolated from rat brain cortex may accumulate potassium against a concentration gradient. Such potassium accumulation occurs at a rapid rate, is dependent upon the presence of sodium, and is inhibited by ouabain as well as by substances interfering with mitochondrial function.

Material and Methods

Ficoll was obtained from Pharmacia, ouabain from Sigma Biochemicals, potassium cyanide from J. T. Baker Chemical Co., cesium chloride from Fisher Scientific Co., and ^{42}K (specific activity 160–170 mCi/g of potassium) from Cambridge Nuclear Co. *N*-Methyl-[^{14}C]antipyrene (4.2 mCi/mmole) and [^{3}H]methoxy-inulin (350.9 mCi/g) were obtained from New England Nuclear Corp.

Preparation of Synaptosomes. Synaptosomes were prepared from 18-day-old rat brain cortices according to techniques previously established in our laboratory (Autilio *et al.*, 1968). The method consisted primarily of mild homogenization with a Teflon glass homogenizer, removal of the crude nuclear fraction by centrifugation at 1000*g*, and isolation of a crude mitochondrial fraction by centrifugation at 14,500*g* for 20 min. The resuspended crude mitochondrial fraction was then layered on discontinuous Ficoll gradients. Following centrifugation in a SW-25.2 Spinco rotor at 25,000*g* for 45 min, the synaptosome fractions were isolated and washed twice in 0.32 M sucrose to remove any Ficoll present. The synaptosome fractions were kept in 0.32 M sucrose and used as soon as possible. The maximum number of hours elapsed between the time of isolation and the actual experimentation was 2 hr, unless otherwise specified.

Synaptosomes were identified as enriched populations of nerve endings by electron microscopy and enzyme specific activity. The average yield of synaptosomal protein was approximately 2–3 mg/g wet weight of cerebral cortex.

Incubations and Assessments of Potassium Accumulation. Incubations were performed at 23° in 2 ml containing 0.033 M Tris-Cl buffer (pH 7.5), 0.1 M sucrose, 2.8–3.2 mg of synaptosomal protein, and varying concentrations of sodium and potassium. This incubation medium was optimal for synaptosomal protein synthesis (Autilio *et al.*, 1968) and was, therefore, employed for the potassium transport studies. Tubes were agitated mildly at the start of the experiment and were not disturbed subsequently, except for sampling. When sodium chloride was omitted from the medium, choline chloride in equimolar concentration was added.

Samples (0.2 ml) were removed from the reaction mixture at various times, pipetted into 5.0 ml of ice-cold 0.25 M sucrose, rapidly mixed by inversion, and immediately poured on a moist Millipore filter mounted on a suction flask. The filters were 25 mm in diameter and 0.45 μ pore size. They were mounted on a fritted disk with a 25-ml capacity funnel attached above. The mixture of sample (0.2 ml) plus sucrose (5.0 ml) was suctioned through the filter until a minimal layer of fluid remained above the filter (approximately 0.2 ml). An additional 25 ml of ice-cold 0.25 M sucrose was added, and the suction was continued until the filter was dry. The time required for the filtration operation depended upon the synaptosomal concentration. The higher the concentration the longer the time required. In these experiments approximately 45 sec elapsed on the average between the original pipetting and complete washing of the synaptosomes on the filters. Filters were removed from their mounts and were placed on polypropylene conical test tubes. The cations were extracted by addition of 2 ml of 1% HNO_3 containing 4 mM cesium chloride to the tubes. The potassium content of the 2 ml of extract was measured approximately 16–24 hr later in a Perkin-Elmer atomic absorption spectrophotometer Model 303[1] with an Osram potassium lamp. A standard curve was established by addition of known concentrations of potassium in 1% HNO_3 containing 4 mM cesium chloride. Cesium chloride was added to correct the interference with potassium emission by sodium during atomic absorption spectrophotometry.

Proteins were determined by the method of Lowry *et al.* (1951) with crystalline bovine plasma albumin as standard. The potassium content was expressed in micromoles of potassium per milligram of protein of synaptosomes.

Measurement of ^{42}K Content in Synaptosomes. In experiments with ^{42}K procedures similar to those described above were employed. Incubations were performed at 23° in 2 ml containing 0.033 M Tris-Cl buffer (pH 7.5), 0.1 M sucrose, 2.8–3.2 mg of synaptosomal protein, 10 mM potassium, tracer amounts of ^{42}K (0.5 μCi), and varying concentrations of sodium. In most experiments ^{42}K was added at the start of incubation. In several experiments the ^{42}K was added after the synapses had been preincubated for 10 min at 23°. At appropriate times 0.2-ml samples were removed from the reaction mixture, pipetted into 5.0 ml of ice-cold sucrose, and suctioned through a 0.45-μ Millipore filter as described above. The filters were washed with an additional 25 ml of 0.25 M sucrose and suctioned until dry. The filters were completely dried under an infrared lamp, placed directly into vials with a liquid scintillation mixture, and counted in a Packard Tri-Carb scintillation spectrometer. From another aliquot of the total reaction mix or filtrate the potassium content was assessed directly in the Perkin-Elmer atomic absorption spectrophotometer. The volume of synaptosomes was so small compared with the incubation medium that during the experiment there was no significant change in potassium in the medium, and the potassium concentration and specific activity were identical in the reaction mix and filtrate. The ^{42}K radioactivity could thus be directly converted into total potassium by determining the content of potassium and the radioactivity in the total reaction mix or filtrate. The radioactivity of the

[1] We thank Dr. D. Tosteson for the use of this machine.

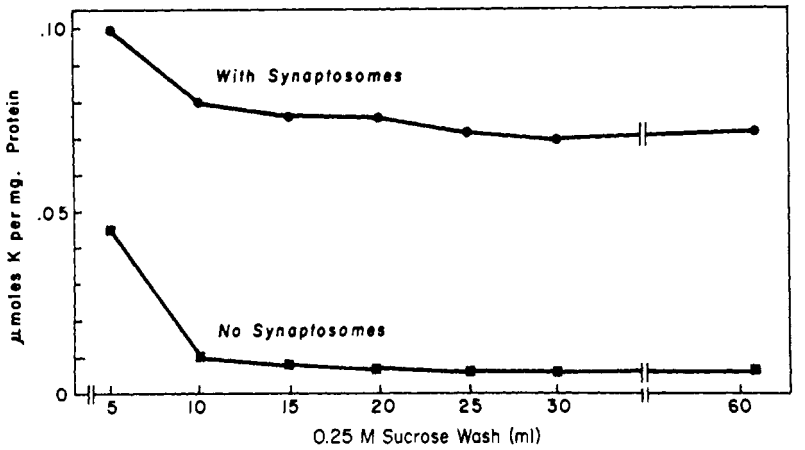

FIGURE 1: Potassium recovered with 0.25 M sucrose wash in the presence and absence of synaptosomes. Medium consisting of 10 mM potassium, 0.033 M Tris-Cl (pH 7.5), and 0.1 M sucrose was incubated at 23° for 8 min with and without 2.8 mg of synaptosomal protein. Aliquots (0.2 ml) were removed, diluted to 5 ml with 0.25 M sucrose, and washed with increasing volumes of sucrose. The filters were processed for potassium content as described in Methods.

individual time points could then be expressed directly as amount of potassium by μmoles of potassium/mg of protein = (^{42}K cpm/0.2 ml)(0.2 ml/mg of synaptosomal protein)/(^{42}K cpm/2 ml)(2 ml/total potassium), where total potassium = amount of potassium in the whole reaction mixture assessed through the Perkin-Elmer, 2 ml = the total volume of the reaction mixture, and 0.2 ml = the volume of the reaction mixture filtered and washed. Since 0.2 ml in the numerator and 2 ml in the denominator cancel each other, a shorter expression of the equation is μmoles of potassium/mg of protein = (^{42}K cpm/mg of synaptosomal protein)/(^{42}K cpm/total potassium).

Measurement of N-Methyl-[^{14}C]antipyrene Space. The total synaptosomal volume was estimated with the use of *N*-methyl-[^{14}C]antipyrene. The 2-ml incubation mixtures consisted of 0.033 M Tris-Cl buffer (pH 7.5), 0.1 M sucrose, 2.8–3.2 ml of synaptosomal protein, and 1 μCi of *N*-methyl-[^{14}C]antipyrene. Following incubation at 23°, 0.2-ml samples were withdrawn at 3, 5, 8, and 12 min. The 0.2-ml aliquots were diluted in 5 ml of ice-cold 0.25 M sucrose, filtered on Millipore filters, and washed with 25 ml of sucrose. The filters were suctioned, completely dried under an infrared lamp, and then counted in a Triton–toluene–1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene–2,5-diphenyloxazole scintillation mixture in a Packard liquid scintillation spectrometer. Aliquots of the reaction mix and filtrate were pipetted onto Millipore filters, dried without suctioning under an infrared lamp, and counted in the scintillation mix in the Tri-Carb spectrometer. By determining the radioactivity of a given volume of the reaction mix, the equivalent volume of the radioactive antipyrene caught in the Millipore filter could be calculated.

In each experiment the potassium content was expressed in micromoles of potassium accumulated per milligram of synaptosomal protein. By application of the [^{14}C]antipyrene data, the synaptosomal protein could be expressed in terms of total volume. The potassium could then be expressed as a concentration rather than potassium content per milligram of synaptosomal protein: potassium concentration in mmoles/l. = (potassium content in micromoles/mg of protein)/(total volume of synaptosomes/mg of protein), where total volume of synaptosomes/mg of protein = [cpm of [^{14}C]antipyrene/(0.2 ml/mg of protein)][(0.2 ml/mg of protein)/(cpm of [^{14}C]antipyrene/ml of reaction mixture)].

Results

Initial experiments determined the amount of sucrose necessary to wash contaminating potassium from cellulose nitrate Millipore filters following incubation with 10 mM potassium in the presence and absence of synaptosomes. As demonstrated in Figure 1, in the absence of synaptosomes, small increments of sucrose employed in the wash rapidly decreased the potassium accumulated on the cellulose nitrate filter. With 20–30 ml of sucrose wash a plateau was reached at less than 15% of the potassium content observed with minimal washing. In the presence of synaptosomes and 10 mM potassium a plateau was reached at 0.076 μmole of potassium accumulated. Washing with an additional 30 ml had no further effect upon the potassium accumulated. Therefore, in all subsequent experiments 0.2 ml of incubation medium was diluted to 5 ml, and then the filters were washed with an additional 25 ml for a total of 30 ml of wash. These washes effectively minimized the extrasynaptosomal potassium including that contaminating the cellulose nitrate filter.

At 23° in 100 mM sodium and 10 mM potassium the amount of potassium ions in synaptosomes reached a level of approximately 0.24 μmole/mg of synaptosomal protein after about 6 min (Figure 2). The rate of potassium accumulation in synaptosomes appeared to be reasonably linear over the first several minutes. The 30-sec time point served as the initial point for determining the extent of time-dependent enhancement of potassium accumulation. After approximately 9 min the potassium content of synaptosomes began to fall slowly. At 37° the accumulation of potassium ions reached its peak in 3 min. The initial accumulation was much more rapid

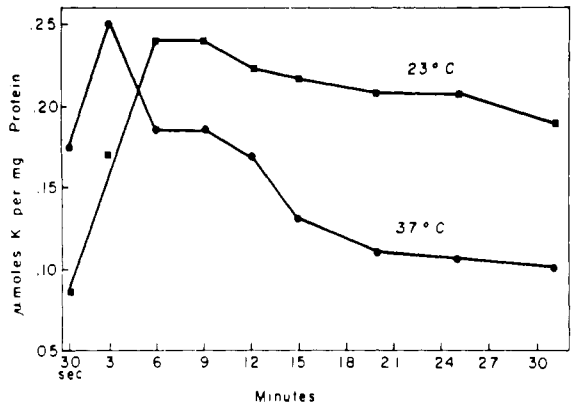

FIGURE 2: Potassium accumulation in synaptosomes at 23 and 37°. Reaction mixtures as indicated in Methods were incubated at 23 and 37°. Sodium (100 mM) and potassium (10 mM) were present in the external medium.

than at 23°, and there was a more rapid decline in potassium content over the next 20 min. The peak level, therefore, appeared to represent an unstable equilibrium. At 23° the potassium accumulation was sufficiently reproducible to permit study of the various parameters influencing uptake. However, from synaptosomal preparation to preparation there was considerable variation in the initial potassium content and the extent of potassium uptake.

ATP, ADP, glucose, and increased oxygen content of the medium had essentially no effect on either the level of accumulation achieved or the rate of decrease of potassium content in synaptosomes incubated at 23° (Table I). Furthermore, α-ketoglutarate, succinate, fumarate, and glutamate produced no significant enhancement of potassium accumulation.

The accumulation of potassium within the synaptosomes was found to be dependent upon the sodium concentration of the medium (Figure 3). The optimal potassium accumulation occurred at 50 mM sodium. With this concentration of sodium in the external medium, an average increase of 52% in potassium accumulation was observed compared to potassium accumulation in the absence of sodium (Table II). At 100 mM sodium the extent of potassium accumulation was found to decrease compared with 50 mM and resembled more closely that achieved with 10 or 25 mM sodium (Figure 3).

Ouabain, which inhibits the synaptosome sodium–potassium-activated ATPase (Abdel-Latif *et al.*, 1967; Festoff and Appel, 1968), inhibited potassium accumulation (Table III). In sodium-free medium, ouabain had very little effect on potassium ion accumulation (Figure 4). With 100 mM sodium and 10 mM potassium, an average 34% decline of total potassium content was produced by ouabain (Table III). In the experiment represented in Figure 4, ouabain produced a 40% inhibition in potassium accumulation with 50 mM sodium and 10 mM potassium in the incubation medium. In four other experiments with 50 mM sodium and 10 mM potassium, ouabain produced an average 54% inhibition of potassium accumulation (Table III). The 0.5-min points were comparable in all control experiments. They represented a significant increase in potassium content when compared with the amount of potassium present in synaptosomes assessed immediately upon isolation (0.09 μmole of potassium/mg of protein). The 0.5-min points were not comparable for control and ouabain experiments. With one exception (expt 7, Table III), the lower potassium content with ouabain suggested that its inhibitory effects were already apparent by 0.5 min. The per cent inhibition was calculated by comparing the 8-min results in control and ouabain experiments. The potassium accumulation insensitive to ouabain appeared essentially unchanged over the range of sodium employed. Ouabain appeared to inhibit to the extent that sodium activated.

TABLE I: Relation of Potassium Accumulation to Substances That Enhance Mitochondrial Function, Glucose, and High-Energy Compounds.[a]

	Potassium Accumulation (μmole/mg of synaptosomal protein)		
	0.5 min	5 min	8 min
Control	0.12	0.16	0.16
+ATP (10^{-4} M)	0.11	0.16	0.14
+ADP (10^{-4} M)	0.12	0.16	0.16
+Glucose (10^{-3} M)	0.12	0.16	0.15
+α-Ketoglutarate (5×10^{-3} M)	0.11	0.16	0.16
+Succinate (5×10^{-3} M)	0.12	0.15	0.15
+Fumarate (10^{-3} M)	0.11	0.16	0.15
+Glutamate (10^{-3} M)	0.12	0.16	0.15

[a] Synaptosomes were incubated as described in Methods and the filtration washing technique was utilized in assessing potassium intrasynaptosomal accumulation. Sodium (50 mM) and potassium (10 mM) were present in the external medium with the above additions, with the figures in parentheses representing the final concentrations. The potassium content as expressed above represent 30-sec, 5- and 8-min incubation points in experiments performed with the same synaptosomal preparation.

At the optimal sodium concentration, 50 mM, increments in the external potassium concentration resulted in increased potassium content of synaptosomes (Figure 5). However, at external potassium concentrations greater than 10 mM, minimal increase in potassium accumulation within synaptosomes was noted. The data yielded essentially the same results when the potassium accumulations at 3-, 6-, or 10-min points were selected to represent initial rates.

2,4-Dinitrophenol (10^{-4} M) and potassium cyanide (10^{-4} M) inhibited the accumulation of potassium 58 and 68%, respectively.

^{42}K Experiments. The effects noted with cold potassium accumulation were confirmed employing ^{42}K as a radioactive tracer. The accumulation of ^{42}K within the synaptosomes appeared to be maximal in the presence of 50 mM sodium (Figure 6). At 23° the entrance of ^{42}K

into synaptosomes was extremely rapid. At 0.5 min there already appeared to be a definite difference in ^{42}K accumulation depending upon the sodium concentration of the media. Since the potassium content prior to incubation is constant for a given synaptosomal preparation, the differences noted at 0.5 min with different sodium concentration may possibly reflect differences in initial rates of uptake. The potassium accumulation appeared to be maximal in the presence of 50 mM sodium (Figure 6). The ^{42}K accumulation was inhibited 41% by ouabain (10^{-4} M), 47% by dinitrophenol (10^{-4} M), and 48% by potassium cyanide (10^{-4} M) (Figure 7). The inhibitory effects of these compounds were also apparent by 0.5 min. In the absence of sodium, ouabain had es-

TABLE II: Effects of Sodium on Potassium Accumulation.[a]

	8-min Points		
Expt	No Sodium (μmole of potassium/mg of protein)	50 mM Sodium (μmoles of potassium/mg of protein)	% Stimulation
1	0.13	0.23	77
2	0.20	0.27	30
3	0.21	0.28	30
4	0.23	0.29	26
5*	0.12	0.20	66
6*	0.12	0.22	83
		Mean ± s.e.	52 ± 11%

[a] Synaptosomes were incubated and processed as described in Methods. Incubation medium consisted of 10 mM potassium and with 0 mM sodium or 50 mM sodium. In the experiments designated with *, tracer ^{42}K was added. Equimolar choline chloride was present when sodium was omitted. Six experiments with six different synaptosomal preparations are represented.

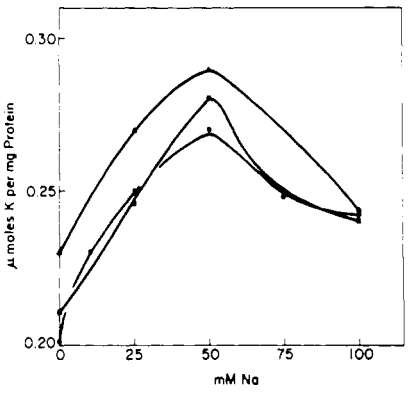

FIGURE 3: Relation of potassium accumulation in synaptosomes to external sodium concentration. Reaction mixtures were incubated as described in Methods. Potassium (10 mM) and varying concentrations of sodium were present in the external medium. The connected points represent the peak accumulation points (8 min) of three separate experiments plotted individually.

TABLE III: The Effect of Ouabain upon Potassium Accumulation.[a]

	Control (μmole of potassium/mg of protein)		Ouabain (μmole of potassium/mg of protein)		
Expt	0.5 min	8 min	0.5 min	8 min	% Inhib
		I. 50 mM Sodium and 10 mM Potassium			
1	0.12	0.22	0.08	0.07	68
2	0.15	0.25	0.10	0.09	64
3*	0.12	0.30	0.12	0.15	50
4*	0.15	0.30	0.10	0.20	33
					Mean + std dev 54 ± 6
		II. 100 mM Sodium and 10 mM Potassium			
1	0.10	0.15	0.105	0.08	46
2	0.11	0.20	0.08	0.14	30
3*	0.10	0.16	0.08	0.10	37
4*	0.08	0.15	0.08	0.12	20
5*	0.09	0.25	0.08	0.19	24
6*	0.11	0.20	0.08	0.10	50
7	0.12	0.24	0.18	0.16	33
					Mean ± std dev 34 ± 4

[a] Synaptosomes were incubated and processed as described in Methods. In both cold potassium and ^{42}K experiments (*) 10 mM potassium was present in the external medium. Ouabain was present at 10^{-4} M, when added. Eleven experiments with eleven different synaptosomal preparations are represented.

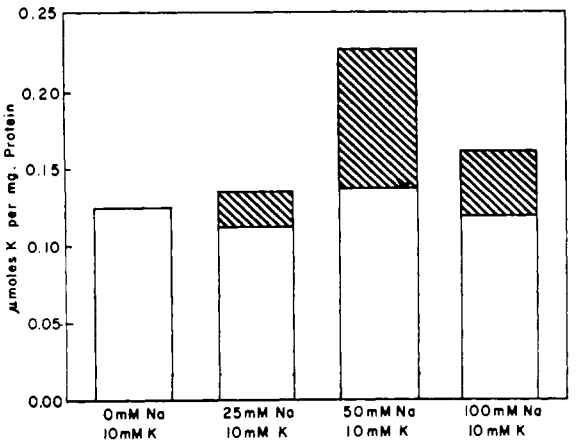

FIGURE 4: The relation of ouabain inhibition to sodium concentration. The shaded portion of the blocks represents the ouabain-inhibitable fraction of potassium accumulation. The values represent the potassium accumulated at 8-min incubation. The reaction mixture is that described in Methods with varying concentrations of sodium, 10 mM potassium, and ouabain (10^{-4} M). The total osmolarity (sum of sodium chloride and choline chloride) was held constant.

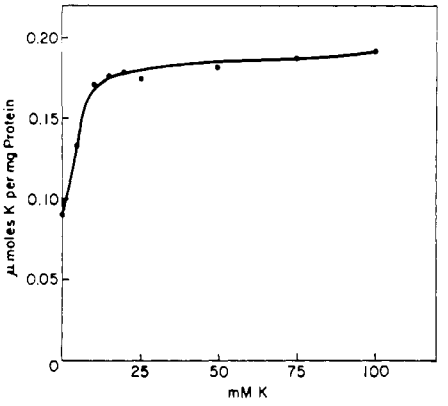

FIGURE 5: The effect of potassium concentration in the external medium on potassium accumulation in synaptosomes. Sodium (50 mM) and varying potassium concentrations are present in the reaction mixtures. Values obtained represent 8-min incubation points. The total osmolarity was held constant by addition of varying concentrations of choline chloride.

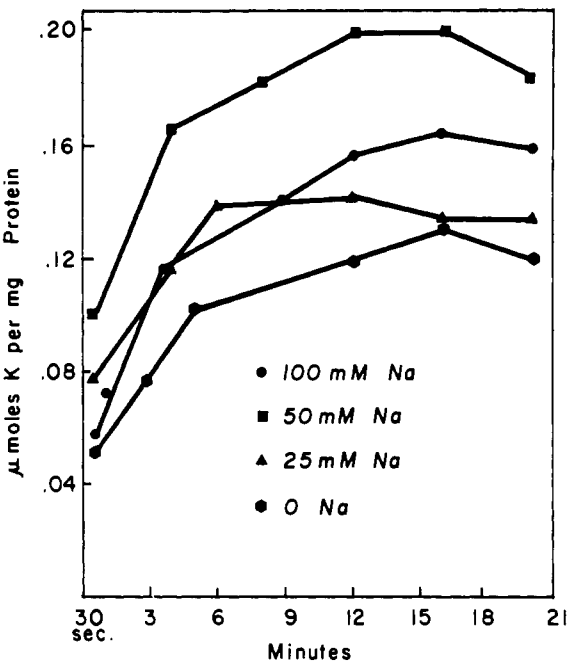

FIGURE 6: Relation of ^{42}K accumulation in synaptosomes to external sodium concentration. Incubation as described in Methods; 10 mM potassium was present in the medium, while sodium concentration varied at 0, 25, 50, and 100 mM. ^{42}K accumulation expressed in micromoles of potassium per milligram of protein is plotted as a function of time.

TABLE IV: Relation between ^{42}K Accumulation and the Time Elapsed after Isolation of Synaptosome Fraction from Ficoll Gradients and Sucrose Washing.[a]

Time Elapsed (hr)	% of Act. of ^{42}K in 7 Expt
<1.5	100
1.5–3	81 ± 4
3	41 ± 7

[a] Incubation with similar reaction medium plus tracer amounts of ^{42}K were as described in Methods. Sodium (50 mM) and potassium (10 mM) were present in the external medium. At the times listed above incorporation studies were performed with the same synaptosomes and the results expressed as mean per cent activity plus and minus standard error compared with the experiments performed at earlier times. Seven experiments with four different synaptosomal preparations are represented.

sentially no effect on ^{42}K influx, whereas at 50 mM sodium it demonstrated the greatest inhibitory effect.

Total potassium ion movements and ^{42}K uptake were maximal immediately after the isolation of the synaptosomal fractions. At 1.5–3 hr after isolation an average of 85% of the activity was demonstrated (Table IV). It was also of interest that the potassium accumulation inhibitable by ouabain decreased as a function of time after isolation (Figure 8). After more than 3 hr the per cent inhibition by ouabain was only 21–24% (Figure 8), whereas at 1–1.5 hr it averaged 54% (Table III). The potassium accumulation insensitive to ouabain decreased 30%, while the potassium accumulation inhibitable by ouabain declined 70% when the data from 1 hr and that from more than 3 hr were compared. The major effect, therefore, of any delay in the use of the synaptosomes was a decline in the fraction of potassium uptake which was inhibited by ouabain. The fraction of potassium accumulation inhibitable by ouabain was also correlated closely with the fraction of potassium enhanced by sodium.

The question must be raised as to the extent of extracellular potassium obscuring the determination of intra-

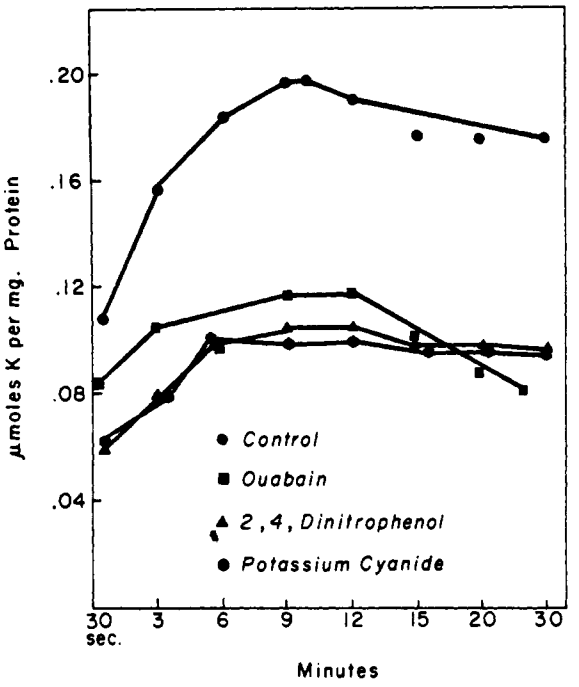

FIGURE 7: The inhibition of ^{42}K accumulation in synaptosomes by ouabain, 2,4-dinitrophenol, and potassium cyanide. Reaction mixture is as described in Methods with 100 mM sodium and 10 mM potassium, and ouabain (10^{-4} M), or 2,4-dinitrophenol (10^{-4} M), or KCN (10^{-4} M). The accumulation of ^{42}K expressed in micromoles of potassium per milligram of protein is plotted as a function of time.

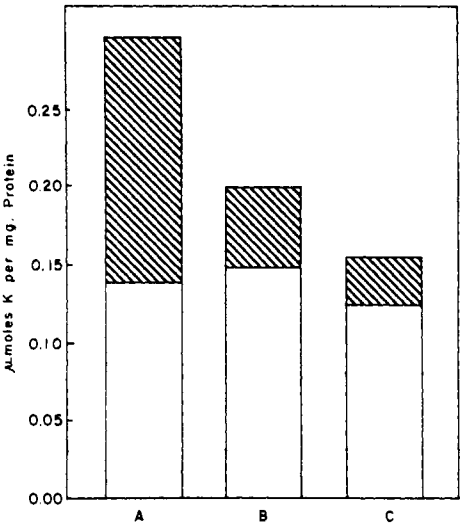

FIGURE 8: The relation of ouabain-inhibitable potassium accumulation to the time elapsed after isolation of the synaptosome fractions from Ficoll gradients. The shaded portions of the figures represent the ouabain-inhibitable fraction of potassium accumulation. (A) 1–1.5 hr after isolation. (B) 2–3 hr after isolation. (C) More than 3 hr after isolation. Values represent 8-min incubation points. The reaction mixture as described in Methods contained 50 mM sodium, 10 mM potassium, and 10^{-4} M ouabain.

synaptosomal accumulation of potassium. It appeared unlikely that extrasynaptosomal potassium contributed significantly to potassium accumulation for the following reasons. (1) The ability to accumulate potassium was almost completely saturated at levels of 50 mM potassium or greater. If extrasynaptosomal potassium made a significant contribution to the total potassium accumulated, we should expect to see a significant increase when the incubation medium was increased from 50 to 100 mM potassium. (2) The time-dependent increase in potassium accumulation at 6 min was compared to 30 sec and demonstrated a definite increase despite being pipetted from the same tubes with presumably the same extracellular contamination.

Intrasynaptosomal Volume. Volume measurements of the different compartments of the synaptosomes were initially performed with the use of *N*-methyl-[^{14}C]antipyrene and methoxy-[^{3}H]inulin, corresponding, respectively, to the total space and the extrasynaptosomal space. The difference between the total space and the extrasynaptosomal space was intended to give the volume of the intrasynaptosomal compartment. However, the results of inulin space measurements were variable with different preparations and yielded estimates of the extrasynaptosomal space from 30 to 100% of the total antipyrene space. These data presumably reflected the ability of the inulin to penetrate the synaptosomes, and are in accord with the recent observations of Cohen *et al.* (1968) that inulin distributes itself in brain slices in a complex pattern. We, therefore, relied upon [^{14}C]antipyrene as a measure of the total synaptosome volume on the Millipore filter. As in the potassium experiments, the filters were washed with 25 ml of sucrose. With no ionic additions to the medium, the [^{14}C]antipyrene volume equalled 1.16 μl/mg of synaptosomal protein. With 50 mM sodium and 10 mM potassium, the volume was 1.7 μl/mg of synaptosomal protein. The potassium content per mg of synaptosomal protein immediately after isolation (0.09 μmole/mg of synaptosomal protein) could then be converted into a synaptosomal potassium concentration of approximately 71 mM. In the same preparation the potassium accumulation at 50 mM sodium and 10 mM potassium (0.175 μmole/mg of synaptosomal protein) represented a potassium concentration of 103 mM. Since the external potassium concentration was 10 mM, the increase from 71 to 103 mM represented a significant accumulation of potassium against a concentration gradient.

The amount of extracellular potassium contamination was felt to be minimal because of the extensive washing of the particles on the filter. The optimal accumulation of potassium (0.28 μmole/mg of synaptosomal protein or 50 μmoles/0.2 ml of the incubation mixture) occurred in the presence of 50 mM sodium and 10 mM potassium (Figure 3). The volume measurement with [^{14}C]antipyrene under the same conditions was 0.8 μl/0.2 ml of the incubation mixture. As a result of the initial dilution of 0.2 ml of the incubation mixture in 5 ml, the extracellular potassium concentration had decreased from 10 to 0.4 mM. Assuming that as high as 50% of the antipyrene space is extrasynaptosomal, then the actual extracellular contamination would be 0.4 mμmole/μl × 0.8 μl × 50% = 0.16 mμmole, which is neg-

ligible compared to the 50 mμmoles of potassium accumulated in the same volume.

Our estimates of potassium content per milligram of protein of the freshly isolated synaptosomes were variable from preparation to preparation. Similarly, the volume measurements were variable and depended upon the synaptosome preparation and the time after isolation that the experiments were performed. The concentration indicated may, therefore, not accurately reflect the true values present.[2] However, in every experiment it was clear that potassium was accumulated against a significant concentration gradient.

Discussion

The success of the present experiments relates primarily to the filtration technique which allowed the assessment of potassium with short periods of incubation and with processing time of less than 1 min. Both the length of the incubation and the time elapsed from the isolation of the synaptosome fraction to the time of actual experimentation were critical. The peak accumulation of potassium in freshly isolated synaptosomes occurred at 3 min following incubation at 37° and at 8 min with incubation at 23°. In both instances the synaptosomal potassium fell gradually over the ensuing 30 min. Three hours after isolation the synaptosomes accumulated approximately 50% of the potassium accumulated in freshly isolated synaptosomes, and the major loss was in the sodium-enhanced, ouabain-sensitive potassium accumulation.

After incubation *in vitro* with 10 mM potassium and 50 mM sodium there was an increase of intrasynaptosomal potassium concentration from approximately 71 mM following isolation to 103 mM. Of interest was the fact that the enhancement of potassium accumulation mediated by various sodium concentrations was approximately equivalent to the ouabain inhibition at each of these sodium concentrations. At zero sodium, ouabain was ineffective. Conversely, sodium did not enhance the ouabain-insensitive accumulation of potassium. These results suggest the possible participation of a sodium–potassium-activated ATPase. Such an enzyme system has been demonstrated in synaptosomal membranes by Albers *et al.* (1965), Hosie (1965), Kurokawa *et al.* (1965), and Abdel-Latif *et al.* (1968), and is present in high concentration in our own preparations (Festoff and Appel, 1968). Furthermore, 50 mM sodium and 10 mM potassium represent the optimal conditions for sodium-activated ATPase as well as for potassium accumulation within synaptosomes (Festoff and Appel, 1968). However, at these ionic concentrations ouabain (10^{-4} M) produces 98% inhibition of enzyme activity, whereas it produces only an average 54% inhibition of potassium accumulation. If the sodium–potassium–ATPase is, in fact, involved in the potassium transport, the figure of 54% may reflect an upper limit to its participitation. In the experiments with cold potassium accumulation, the remaining 46% was unaffected by sodium concentration.

In the presence of 2,4-dinitrophenol or potassium cyanide, structural characteristics of the membrane as well as specific ion transport mechanisms may be affected, and it may be impossible to dissociate the cell processes responsible for the transport function of the cell membrane from those which maintain its structural integrity (Brinley, 1967). Metabolic inhibition might, therefore, lead to a generalized deterioration of the cell membranes and morphological disruption of the synaptosome with fewer available particles for active as well as passive potassium accumulation.

The experiments demonstrating potassium transport in synaptosomes were similar to those demonstrating amino acid incorporation into protein (Appel and Autilio, 1969; Autilio *et al.*, 1968). Exogenous substances such as glucose, ATP, ADP, AMP, as well as α-ketoglutarate, succinate, fumarate, and glutamate, did not markedly affect either potassium accumulation or protein synthesis. Dinitrophenol and potassium cyanide inhibited protein synthesis to the same extent that they inhibited potassium accumulation, both of which may reflect their dependence upon endogenous energy presumably derived from intrasynaptosomal mitochondria and substrates. In this context, the experiments of Abood *et al.* (1968) suggest that within synaptosomes isolated in Ficoll gradients there are substantial levels of ATP derived from the enclosed mitochondria.

The present experiments provide further evidence that the synaptosomal particles are functionally as well as morphologically intact. Our previous studies with synaptosomes *in vitro* demonstrated the effect of ionic constituents on the rate of synthesis of proteins incorporated into membrane fractions. The present studies suggest that measurements of ionic fluxes may be useful in assessing the state of synaptosome membrane constituents and their function in intercellular communication.

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[2] It is of interest that using morphologic criteria (Clementi *et al.*, 1966) for synaptosomal volume, Marchbanks (1967) arrived at a similar value (88 mM) for the potassium concentration in isolated synaptosomes.

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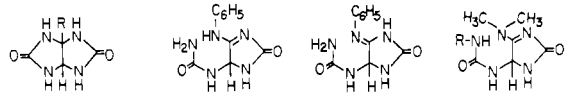
The Product of the Air Oxidation of Uric Acid. An Intermediate Formed in the Presence of Dimethylamine*

P. Heinrich Stahl

ABSTRACT: Uric and 1-methyluric acids were oxidized by air in 20% aqueous dimethylamine. The reaction is catalyzed by cupric ions or by manganese dioxide. Through nuclear magnetic resonance spectra the oxidation products have now been established as 2,5-dihydro-4-dimethylamino-2-oxo-5-ureido-1*H*-imidazole (IV) and 2,5-dihydro-4-dimethylamino-2-oxo-5-(3-methylureido)-1*H*-imidazole (V). The "isoallantoin-anilide" of Frèrejacque and Fosse (Frèrejacque, M., and Fosse, R. (1931), *Compt. Rend. 193*, 860), prepared by oxidation of uric acid in aqueous potassium hydroxide in the presence of aniline, has proved to have a similar structure, rather than the symmetrical imidazolo[4,5-*d*]imidazole structure I previously proposed.

It is apparent that the imidazole portions of uric acid and of 1-methyluric acid, respectively, remain unaffected during the formation of IV and V, in contrast to the oxidations to allantoin, *in vivo* or *in vitro*, which involve a symmetrical intermediate.

The oxidation of uric acid in aqueous alkali metal hydroxides leads to different reaction products depending upon the oxidizing agent (Schuler and Reindel, 1933). Oxidation by molecular oxygen is catalyzed by metal ions (Schuler and Reindel, 1933; Griffiths, 1952), or with charcoal present (Frèrejacque and Delepine, 1930), leading to compounds which display only end absorption or short-wavelength absorption maxima (*e.g.*, allantoin, λ_{max} 225 mμ at pH 10) in the ultraviolet region. However, in the presence of primary and secondary amines, solutions of uric acid exposed to air show an increased absorbance at about 250 mμ, and a decrease at 293 mμ at about the same rate. This oxidation, involving subsequent reaction with the amine, is catalyzed by cupric ions (Griffiths, 1952) or manganese dioxide. The oxidation product of uric acid in the presence of dimethylamine can be obtained by passing a stream of air or oxygen through a solution of uric acid in 20% aqueous dimethylamine containing suspended manganese dioxide, or with very low concentrations of cupric sulfate. The conditions are similar to those used by Frèrejacque and Fosse (1931) who oxidized uric acid in aqueous potassium hydroxide in the presence of aniline. They obtained a product to which they assigned structure I and which they called "isoallantoinanilide."



I, R = -NHC$_6$H$_5$
II, R = -N(CH$_3$)$_2$
III a
III b
IV, R=H
V, R=CH$_3$

By analogy, the oxidation product of uric acid in the presence of dimethylamine would have the structure II.

The nuclear magnetic resonance spectra of the present oxidation product of uric acid and that of "isoallantoinanilide" are compared in Table I. The results are not consistent with the symmetrical structures I and II, but favor imidazolone structures III and IV, respectively. Instead of showing two singlets, each integrating for two NH protons as to be expected for II, the spectrum of the uric acid oxidation product exhibits two singlets and a doublet integrating for a total of four exchangeable protons. One singlet integrates for two protons. Comparison with the nuclear magnetic resonance spectra of hydantoin and of the 1- and 3-methylallantoins permits assignment of all signals, so that the structure of the oxidation product of uric acid in dimethylamine solution was established as 2,5-dihydro-4-dimethylamino-2-oxo-5-ureido-1*H*-imidazole (IV, R=

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