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NUCLEOTIDE CONTENT, CALCIUM ACCUMULATION, AND PHOSPHATE METABOLISM IN SUBCELLULAR FRACTIONS OF RAT BRAIN

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SUMMARY

The distribution of nucleotides was studied in the various subcellular fractions of rat brain separated by Ficoll density gradient centrifugation. Nucleotides were found in all subcellular fractions, attached primarily to membranous components, with the highest concentration in the nerve ending fraction. By examining the *in vivo* incorporation of $^{32}P_i$ into the nucleotides of the subcellular fractions, it could be established that the mitochondrion was the primary site of ATP synthesis and that the pattern of nucleotide distribution was not an artifact of the isolation procedure. Ca^{2+} appeared to be associated with the lipid component of the fractions, and its accumulation within the fractions and isolated frog spinal ganglia did not appear to be energy-dependent.

INTRODUCTION

Although it is generally recognized that ATP must play an important role in neuronal function, its exact nature in the many aspects of neuronal activity is unknown. It is involved in the synthesis, storage, and release of chemical transmitters and in the removal of intracellular Na^+ following depolarization either in the capacity of an energy source or as a substrate for enzyme carrier such as ATPase.

For a number of years in this laboratory the argument has been advanced that the role of ATP in bioelectric phenomena was not directly related to its participation in phosphorylative and other metabolic systems but to the interactions which it can undergo with Ca^{2+}, phospholipids, and macromolecular constituents of the excitatory membrane (see ref. 1 for review). It has been proposed that ATP, by virtue of its chelating and sequestering action, may be regulating the degree of interaction of Ca^{2+} with the excitatory membrane and, thereby, controlling ionic permeability. As part of a general program to elucidate the role of ATP in bioelectric phenomena, it was essential to determine its intracellular distribution and some of the factors regulating its association with various subcellular components. NYMAN AND WHITTAKER[2] have already reported on the distribution of ATP in the various subcellular fractions of

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guinea-pig brain, but their studies concerned only this nucleotide which is largely hydrolyzed during the isolation procedure.

The objectives of the present study were (1) to examine in detail the distribution of ATP and related substances in the various subcellular fractions, (2) to examine the rate of ^{32}P incorporation *in vivo* into the ATP of the various subcellular fractions, and (3) to study the accumulation *in vitro* of ^{45}Ca and [^{14}C]ATP in the various subcellular components. One conclusion was that the distribution of the adenine nucleotides was quite ubiquitous, although the isolated nerve endings tended to have somewhat higher concentrations while the main site of ATP synthesis was the mitochondria. A further observation was made that Ca^{2+}, which is evidently bound to lipids, is also ubiquitous in its subcellular distribution and, except in the case of mitochondria, associates with the various subcellular components in the absence of energy requirements.

METHODS AND MATERIAL

Subcellular fractionation

The technique for the subcellular fractionation of whole rat brain has been described in detail elsewhere[3,4]. Up to the point where water shock was performed on the B fractions (see Fig. 1), the fractionation was similar to that described elsewhere except that a discontinuous rather than a continuous Ficoll gradient was employed. Usually 2 whole brains were used for each run. Water shock was performed by homogenizing the combined Fractions B_1 and B_2 in 20 ml of ice-cold water employing a tight-fitting (clearance about 0.1 mm) glass homogenizer. After standing at 5° for 30 min, the suspension was centrifuged at 20000 rev./min for 15 min. The residue was then homogenized in 2 ml of 0.4 M sucrose and layered on a sucrose gradient consisting of 0.6, 0.8, 1.0, and 1.2 M sucrose. From 4–5 fractions were generally obtained after centrifugation of the latter at 100000 × *g* for 30 min. After the tube was sliced with a Spinco tube slicer, the separate fractions were homogenized in 8 ml of 0.25 M sucrose and centrifuged at 50000 × *g* for 15 min. The final B subfractions were designated B_1 and B_5. All density gradient centrifugation was performed with the Spinco Model L centrifuge employing the SW 39 rotor. The Supernatant S_1 was centrifuged in the Spinco 40 rotor first at 25000 × *g* for 30 min to yield E, then at 100000 × *g* for 60 min to yield S and finally at 100000 × *g* for 120 min for Fraction R.

Radiotracer studies

In order to study $^{32}P_i$ incorporation into the various subcellular fractions, 300 μC of carrier-free $^{32}P_i$ were injected intracranially 1 h before sacrifice. To study the exchange of ^{45}Ca (carrier-free) or [^{14}C]ATP (50 mC/mmole) in the various subcellular fractions, 2 μC of the isotope were added to the P_2 residue prior to homogenation. After brief homogenation, the fractionation was continued without further delay. Special precautions were taken to perform all procedures at 0° to minimize metabolic activity within the fractions. The procedure for subsequent fractionation was identical to that described above except that the final fraction was resuspended an additional time in 8 ml sucrose and centrifuged at 100000 × *g* to help remove excess ^{45}Ca or ^{14}C-labeled nucleotides. [^{14}C]ATP was randomly labeled (98 % pure) and obtained from Nuclear-Chicago.

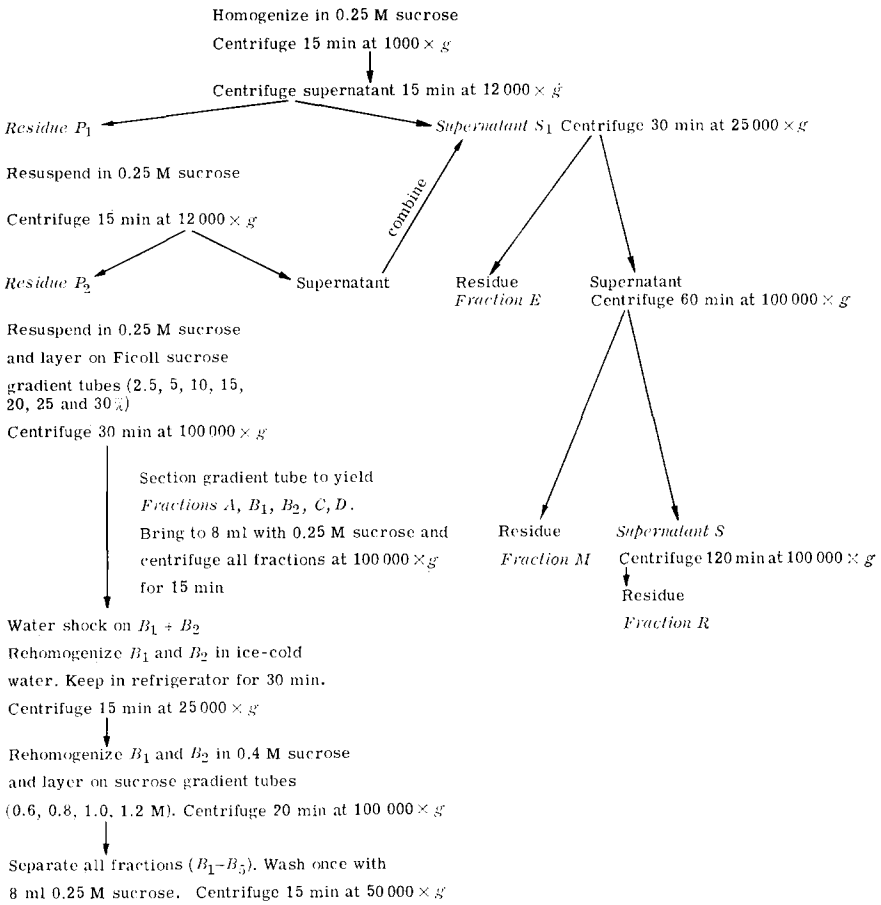

Fig. 1. Flow diagram for subcellular fractionation of whole rat brain by density gradient centrifugation. Designations of fractions same as those used in text.

Extraction of residues with chloroform–methanol

In an effort to determine the extent to which ^{45}Ca and nucleotide derivatives were adsorbed to lipids, the residues of the various subcellular fractions were extracted at 27° with chloroform–methanol (2:1, v/v), a procedure extracting all the lipids and some proteins. After evaporation of the solvent *in vacuo*, the residue was extracted with 0.5 M perchloric acid, the extract neutralized with KOH, and the supernatant obtained after centrifugation analyzed for nucleotide content. Samples of the dried chloroform–methanol residue were taken up in a small volume of chloroform–methanol and subjected to silica gel G thin-layer chromatography employing the following solvent systems: (1) chloroform–methanol–water (73:28:5), (2) chloroform–methanol–ammonium hydroxide–water (73:28:4:2), (3) chloroform–methanol–acetic acid (73:28:4) and (4) butanol–pyridine–water (45:5:20). Radioautographs were made of the plates on no-screen X-ray film. The nucleotides and their derivatives were detected by an ultraviolet lamp and the lipids by iodine vapor. ^{45}Ca or [^{14}C]ATP (0.1 μC) was also applied to a silica gel G plate in combination with a lipid

sample obtained from whole rat brain. The radiotracer was applied directly to the plate after the application of the lipid, or it was suspended in the lipid solution prior to application. Chromatography was then carried out followed by radioautography and lipid detection to determine whether either isotope migrated with specific lipids.

Nucleotide analysis

The residues were extracted with 5 ml of ice-cold 0.5 M perchloric acid to remove the acid-soluble components including ^{45}Ca. After neutralization with KOH to remove the perchlorate, the extract was freeze-dried. For thin-layer chromatography the dried residue was dissolved in 1 ml of water, and after centrifugation (to remove perchlorate), the extract was once again freeze-dried in a 2-ml centrifuge tube. After redissolving in 0.1 ml of water, the material was ready for thin-layer chromatography using polyethyleneimine, cellulose and LiCl as the solvent system[5].

Column chromatography with Dowex 1-X8 formate (100–200 mesh) was performed after the method of SCHMITZ, HURLBERT AND POTTER[6]. The samples were pooled and freeze-dried over KOH pellets to remove the formic acid and ammonium formate used for elution. The residue was redissolved in water and analyzed spectrophotometrically in a DB recording UV spectrophotometer and for radioactivity.

Nucleosides, purines, and pyrimidines were separated by Dowex 50 (100–200 mesh) chromatography by the method of COHN[7]. The eluent obtained after passing the sample through a Dowex 1 column was employed for this purpose.

Radioactive measurements were made with a liquid scintillation spectrometer (Nuclear-Chicago). The vials were prepared by adding to a 0.2-ml sample 2 ml methanol and 8 ml of solution containing 0.4 % 2,5-diphenyloxazole *plus* 0.01 % 1,4-bis-(5-phenyloxazolyl-2) benzene in toluene. Appropriate samples of ^{45}Ca and [^{14}C]ATP of known specific radioactivity were used as standards.

Calcium exchange in spinal ganglia

Spinal ganglia with the nerve roots attached were carefully dissected from frogs (*Rana pipiens*) employing a dissecting microscope. Details of the technique and methods employed to determine electrical excitability and biochemical viability in such preparations are described elsewhere[8]. From 3–4 individual ganglia (total wet weight about 1.0 mg) were then incubated at 27° for 30 min in 1 ml of the test solution containing 1 μC of ^{45}Ca. Each set of spinal ganglia was then washed by being immersed for 1 min in 3 successive beakers containing 5 ml of frog Ringer's solution (1.2 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 2 mM $NaHCO_3$ to final pH of 7.4). The ganglia were then transferred to vials and radioactivity measured as described above.

The technique for preparing specimens for electron microscopy are described in detail elsewhere[3,4].

RESULTS

Electron micrographs of the various subcellular fractions of brain prepared in Ficoll media are presented in detail in other publications[3,4]. The electron micrographs to be described were obtained after the nerve ending fractions (B_1 and B_2) were subjected to water shock and subfractionated on a discontinuous sucrose gradient

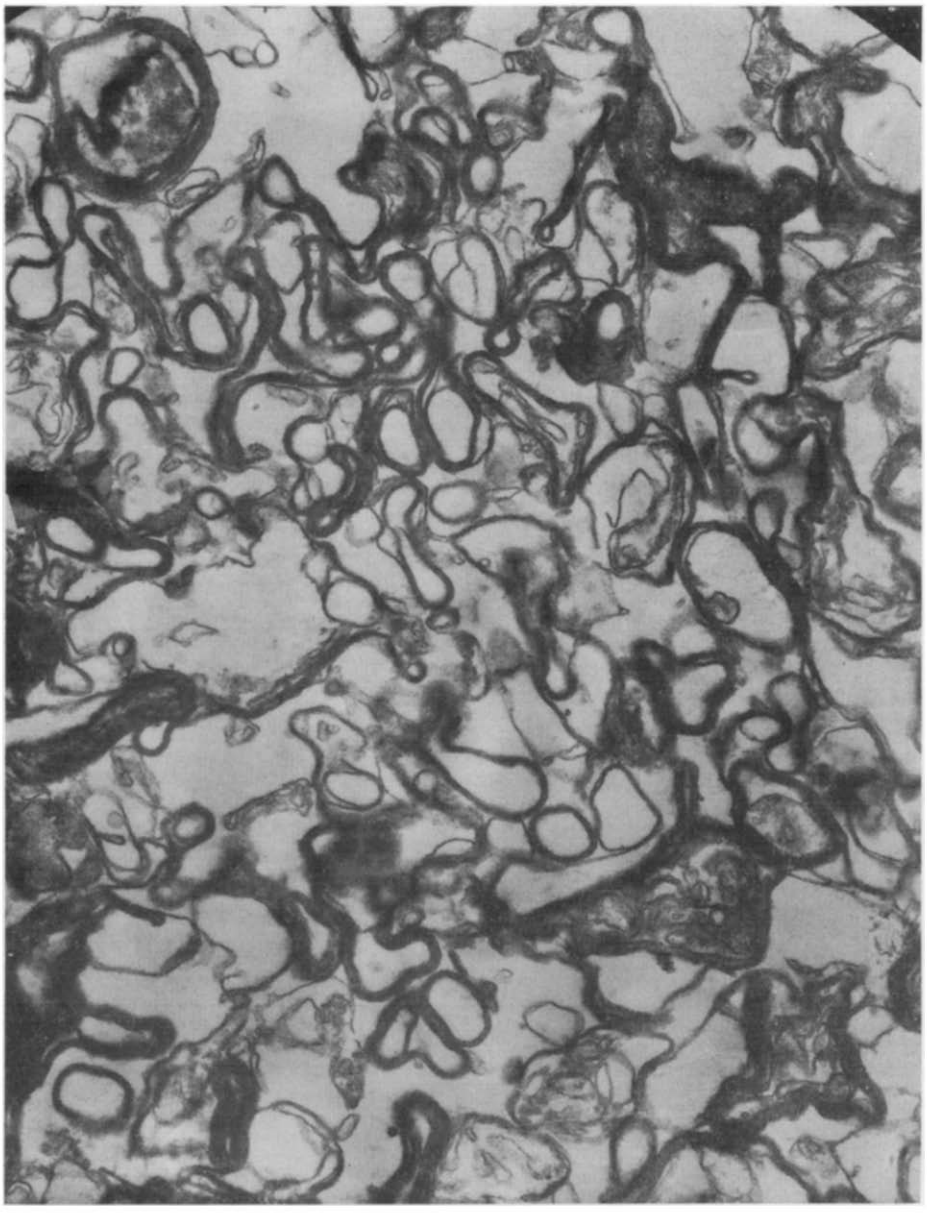

Fig. 2. Electron micrograph of subcellular Fraction A. Largely myelin fragments are present with a few fragments of nerve endings. Magnification 27 500 ×.

(Fig. 2). In Fig. 2 can be seen relatively pure myelin fragments comprising Fraction A. Fig. 3 shows the appearance of the B fractions after water shock and prior to subfractionation in a sucrose gradient. In addition to partially disrupted nerve endings, there are components derived from nerve endings such as the surrounding membranes, synaptic vesicles, and intact and partially disrupted mitochondria. Figs. 4–6

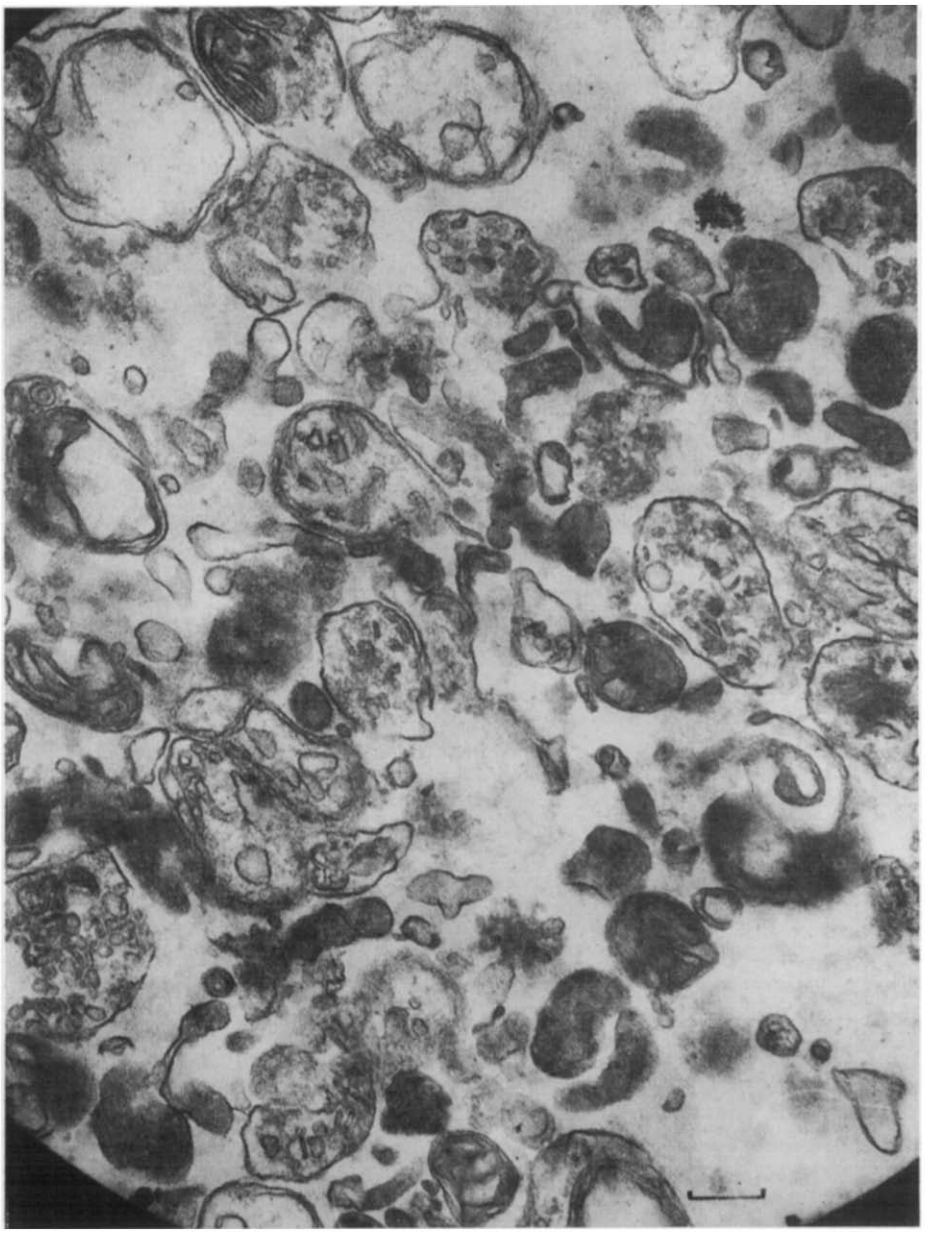

Fig. 3. Electron micrograph of Fraction B after water shock. Present are fragments of nerve endings after water shock and prior to sucrose density gradient centrifugation. These include partially disrupted endings, the surrounding membranes, mitochondria, synaptic vesicles, and a few myelin fragments. Magnification 27 500 ×.

were made from Fractions B_1, B_2 and B_4 *plus* B_5, respectively, obtained after centrifugation in a sucrose gradient. In Fig. 3 the major components are nerve ending membranes of varying size. Fraction B_3 (Fig. 5) consists largely of membranes derived

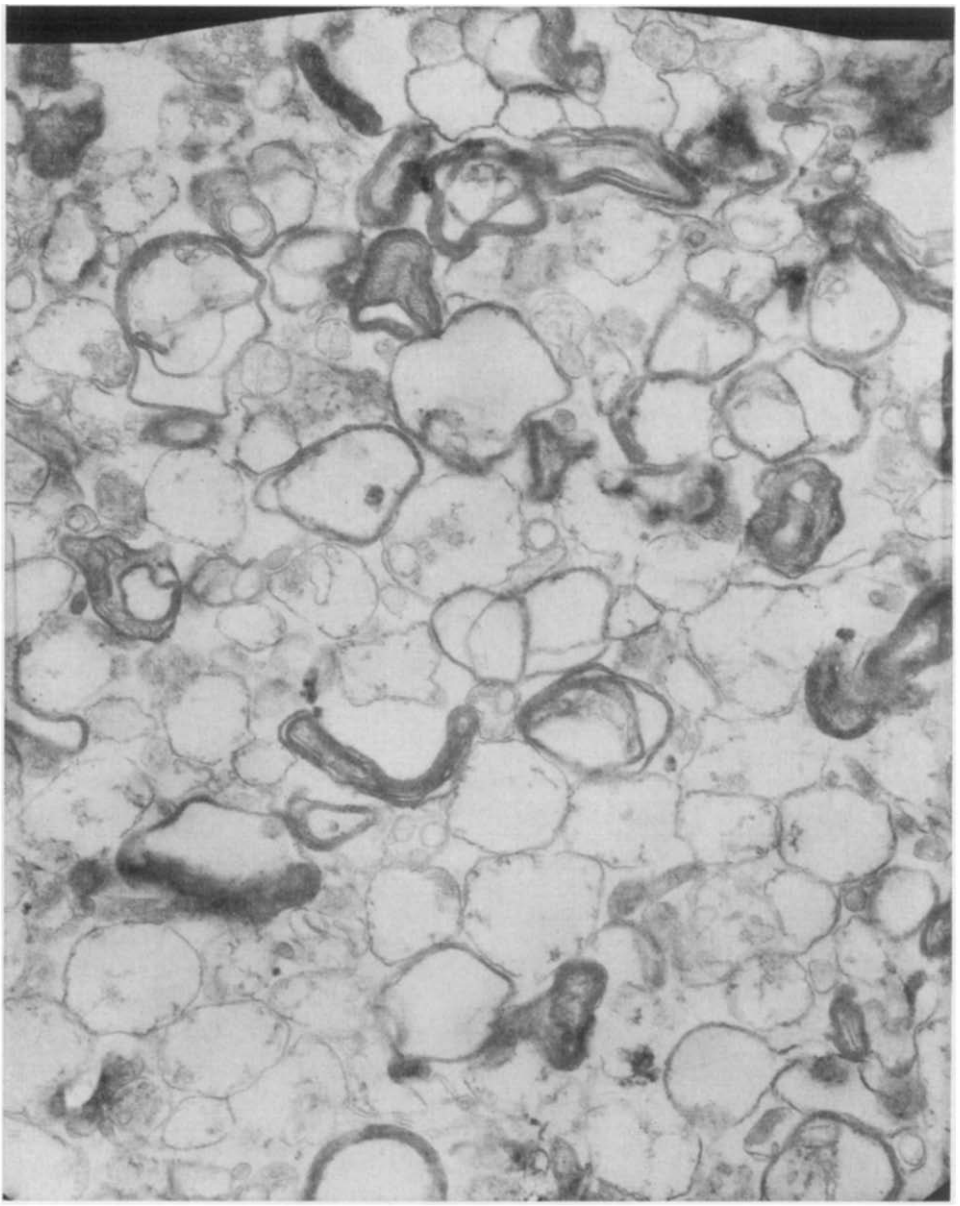

Fig. 4. Electron micrograph of Fraction B_1. Present are largely membranes of nerve endings with some myelin fragments and a few vesicles. Magnification 27 500 ×.

from synaptic vesicles along with fragments of nerve ending membranes. Fraction B_4 (Fig. 6) contains varying degrees of disrupted nerve endings, intact and swollen mitochondria, and a few synaptic vesicles.

The greatest concentration of ATP and other nucleotides was in those fractions containing largely nerve endings (Table I). A similar concentration of total nucleotides

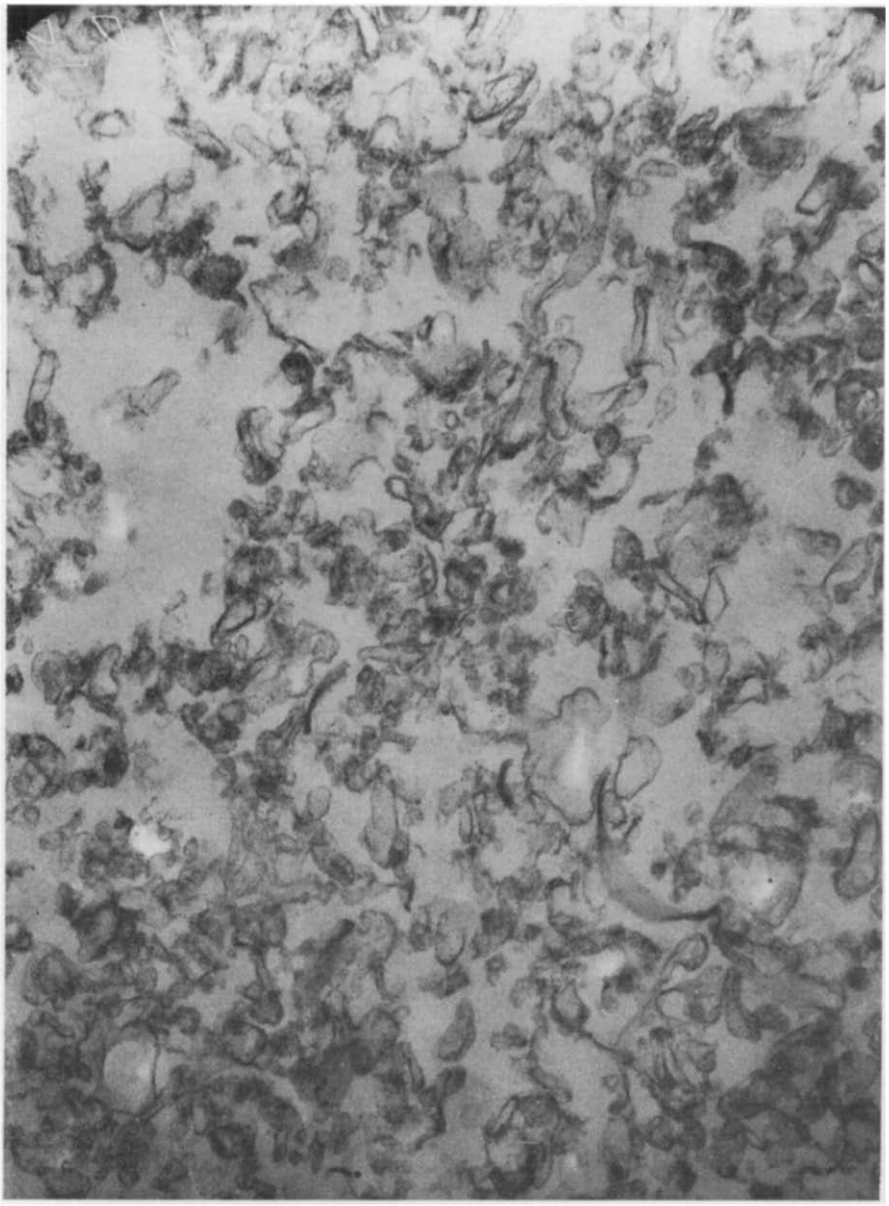

Fig. 5. Electron micrograph of Fraction B_2. Present are synaptic vesicles of varying size, a few partially disrupted small nerve endings, and membranes and small mitochondria derived from nerve endings. Magnification 55000 ×.

was observed in the microsomal fraction (E) and the relatively pure myelin (A) and mitochondrial (D) fractions; whereas, the somewhat higher concentrations of nucleotides in the A–B and C fractions may be due to the presence of nerve ending particles. Most of the nucleotide present in each fraction was in the form of either AMP or ADP. The addition of EDTA to the suspending media used to fractionate the subcellular

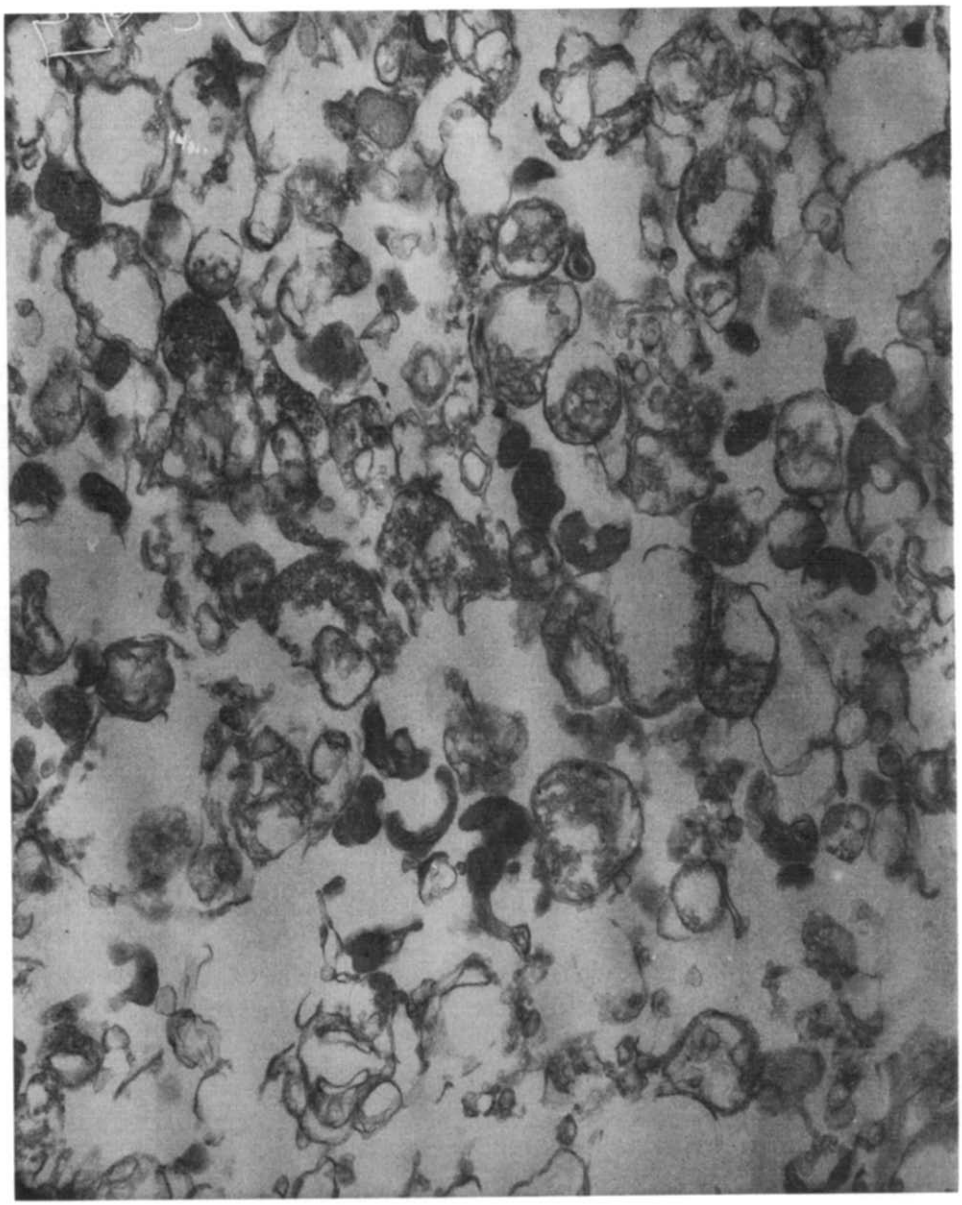

Fig. 6. Electron micrograph of Fraction B_4. Present are intact and disrupted mitochondria and some disrupted nerve endings. Magnification 27 500 ×.

components did not appear to significantly alter the total nucleotide content of the fractions.

After water shock and subfractionation of the fragmented nerve endings, the nucleotide concentration of the various nerve ending components ranged from 0.73 to 1.10 μmoles/μmole protein N (Table II). These values are about 1/2 those for

TABLE I

DISTRIBUTION OF ADENINE NUCLEOTIDES IN SUBCELLULAR FRACTIONS OF WHOLE RAT BRAIN

Values are given as mean ± S.D., based on five separate experiments.

Fraction	*ATP*	*ADP*	*AMP*	*Total*	*Total (EDTA)*
	(μmoles/μmole N × 10³)				
A	0.22 ± 0.05	0.54 ± 0.10	0.53 ± 0.08	1.29	1.50
A–B	0.17 ± 0.04	0.54 ± 0.12	1.08 ± 0.3	1.79	1.64
B_1	0.44 ± 0.07	0.88 ± 0.15	0.90 ± 0.20	2.22	2.40
B_2	0.52 ± 0.08	0.94 ± 0.20	1.18 ± 0.30	2.64	1.72
C	0.34 ± 0.10	0.59 ± 0.12	0.76 ± 0.20	1.69	1.30
D	0.18 ± 0.05	0.26 ± 0.05	0.44 ± 0.06	0.88	0.55
E	0.18 ± 0.05	0.46 ± 0.10	0.32 ± 0.10	0.96	

TABLE II

INCORPORATION OF $^{32}P_i$ *in vivo* INTO VARIOUS SUBCELLULAR FRACTIONS OF RAT BRAIN

The first column presents data obtained prior to water shock, and the next two columns after water shock in Fraction B. All data are an average of 3 experiments and the individual values generally agreed within 10%. Figures in parentheses refer to figures for subcellular fractions not given water shock.

Fraction	*Counts/min per μmole N × 10⁻⁴*	*Water shock (counts/min per μmole N × 10⁻⁴)*	*Nucleotides (μmoles)*	*% total radioactivity*		
				P_i	*AMP*	*ADP + ATP*
A	127	—	(1.50)	(55)	(20)	(15)
B	400			(70)	(15)	(8)
B_1	—	63	0.73	72	18	8
B_2	—	21	0.88	65	15	12
B_3	—	22	1.10	75	10	6
B_4	—	123	1.0	40	25	15
B_5		150	0.86	65	12	13
C–D	575	—	(1.05)	(30)	(35)	(30)
E	255	—	(1.60)	(65)	(10)	(12)
M	90	—	(1.50)	(65)	(15)	(10)
R	35	—	(1.40)	(70)	(10)	(10)

the intact nerve endings. Additional washing of the fragments in 0.25 M sucrose resulted in only a 10–20% decrement in the total nucleotide content.

The incorporation of $^{32}P_i$ *in vivo* was highest in the mitochondrial fraction and next in the nerve endings (Table II). An appreciable amount of radioactivity was also present in the small membranous fraction E and in the A fraction. After water shock, the major portion of the radioactivity was found to be present in the mitochondria (B_5) and membranous fraction (B_4) of the nerve endings. In the mitochondrial fractions, including B_5, the total radioactivity was almost equally distributed between P_i, AMP, and ADP *plus* ATP; whereas, in most of the other fractions the major portion was in the form of P_i.

The uptake of [^{14}C]ATP and ^{45}Ca by the various subcellular fractions was examined after the (separate) addition of the radiotracer to the crude particulate

Fraction R (Table III). The level of total radioactivity derived from [^{14}C]ATP was not significantly different between the various fractions with the possible exception of the Fraction A, which was about 50 % that of the mitochondria or nerve ending fraction. Most of the ^{14}C label was in the form of AMP and ADP, a condition probably due to the presence of ATPase activity associated with the various fractions[9]. ^{45}Ca uptake appeared to be greatest in the large nerve ending and the large mitochondrial fractions. Considerably less uptake occurred in the other fractions, particularly the small membranous fraction E.

In an effort to determine what nucleotides and their derivatives were associated with lipids, an analysis was made of the chloroform–methanol extract of whole rat brain (Table IV). The major constituents were adenine, adenosine, and orthophosphate. With the exception of AMP, nucleotides were present in only trace amounts. Neither ^{45}Ca nor [^{14}C]ATP migrated with any lipids during silica gel thin-layer chromatography, employing any of the solvent systems.

TABLE III

UPTAKE OF [^{14}C]ATP AND ^{45}Ca *in vitro* BY ISOLATED SUBCELLULAR FRACTIONS OF RAT BRAIN

Units for nucleotides, counts/min per μmole N and for calcium, counts/min per μmole N $\times$ 10^{-2}. The results represent an average of 3 separate experiments and the agreement was within 12 % of the average.

Fraction	*Total*	*% total radioactivity*			
		AMP	*ADP*	*ATP*	*Calcium*
A	130	40	40	20	100
A-B	195	60	30	10	72
B_1	250	45	35	20	66
B_2	240	55	30	10	470
C	245	40	35	25	162
D	180	55	30	15	410
E	—	35	45	20	42

TABLE IV

NUCLEOTIDE DERIVATIVES PRESENT IN CHLOROFORM–METHANOL EXTRACT OF WHOLE RAT BRAIN

The results are an average of 3 separate experiments

Substance	*μmole/g*
Adenine	0.055
Adenosine	0.150
AMP	0.01
ADP	0.005
ATP	0.000
DPN	0.002
P_i	0.050

The uptake of ^{45}Ca by spinal nerve roots and ganglia was examined in various media (Table V). No difference was observed between normal Ringer's and K^+-Ringer's solution. In a Ca^{2+}-free medium $5 \cdot 10^{-4}$ M ATP was without effect, while

TABLE V

Ca^{2+} ACCUMULATION IN ISOLATED FROG SPINAL GANGLIA AND NERVE ROOTS

"K^+-Ringer's": 112 mM KCl substituted for NaCl; "Ca^{2+}-free": Ringer's without Ca^{2+}; [ATP] = $5 \cdot 10^{-4}$ M; [EDTA] = $5 \cdot 10^{-4}$ M; [2,4-dinitrophenol] = 10^{-4} M; [iodoacetic acid] = 10^{-3} M. The number of experiments in each group was 5.

	Ringer's	*K^+-Ringer's*	*Ca^{2+}-free*	*Ca^{2+}-free + ATP*	*Ca^{2+}-free + EDTA*	*Ca^{2+}-free + 2,4-dinitrophenol*	*Ca^{2+}-free + iodoacetic acid*
Ventral	1050 ± 450	950 ± 320	2200 ± 520	2450 ± 450	120 ± 15	875 ± 350	980 ± 400
Dorsal	1200 ± 600	1350 ± 550	2700 ± 480	2650 ± 350	80 ± 10	990 ± 400	1000 ± 350
Ganglion	2050 ± 450	1800 ± 520	4500 ± 510	4700 ± 580	140 ± 15	1850 ± 350	1850 ± 375

a similar concentration of EDTA almost completely prevented Ca^{2+} uptake. No effect was observed in the presence of metabolic inhibitors such as 2,4-dinitrophenol and iodoacetic acid.

DISCUSSION

The present findings appear to confirm those of NYMAN AND WHITTAKER[2] that a larger concentration of nucleotides are present in the nerve endings than other subcellular fractions of rat brain. An important question is whether this difference is representative of the situation *in vivo* or is due to an artifact of the preparative procedure. Since the mitochondria are the main sites of ATP synthesis, they would be expected to contain the largest concentration of nucleotides. The lower concentration of nucleotides in isolated mitochondria would be explainable if during fractionation mitochondria are less able to retain their nucleotides than the other subcellular fragments. It should be mentioned that the mitochondria prepared in a Ficoll gradient are not only morphologically well-preserved but exhibit no diminution in their ability to carry on oxidative phosphorylation[3,9]. The mitochondrial fractions (C and D) were over 95 % pure mitochondria. The higher concentration of nucleotides in the large nerve ending fraction may then be due to the presence of mitochondria within the nerve endings which are partly shielded from disruptive and diffusional forces. If such an explanation were valid, the concentration of intramitochondrial ATP would be extremely high since the total mitochondria represented in the nerve ending fraction is a few per cent of that present in the mitochondrial fraction (C *plus* D). Furthermore, the studies with ^{32}P indicate that the transfer of mitochondrial nucleotides, either *in vitro* or during the fractionation procedure, was not sufficient to account for the intracellular distribution observed. In any case the nerve endings appear capable of accumulating and retaining relatively large concentrations of adenine nucleotides.

Since after hypotonic shock over half of the total nucleotides was retained by the membranes of the nerve endings, it is reasonable to assume that the major portion was associated with the membranous components. The nucleotides are also sufficiently well adsorbed to the membranes so that disruptive and washing procedures alone are unable to remove them. The studies with [^{14}C]ATP, however, do not suggest that any particular subcellular fraction is capable of significantly greater binding or exchange of ATP. Such studies are difficult to interpret, since the ATP undergoes con-

siderable hydrolysis even at low temperatures because of the presence of ATPase activity in the fractions[3,9]. Nevertheless, the labeled AMP and ADP, as well as ATP, remain attached to the particular fraction.

It had been previously suggested that the binding of ATP and other nucleotides to membranes may require Ca^{2+}, Mg^{2+}, or other multivalent metals[1,10]. This hypothesis was supported by results from studies of nucleotide adsorption to surface monolayers of various lipids, including brain lipids[10]. The addition of EDTA to the media during subcellular fractionation did not significantly reduce the nucleotide content of the myelin or small nerve ending fractions but did in the mitochondrial and large nerve ending fractions. One explanation for these seemingly equivocal results may be that the EDTA is unable to chelate the multivalent metals in fractions where the lipid content is high, such as in the A and B_1. Studies with surface films have shown that the presence of EDTA in the subsolution will not prevent ATP adsorption to various lipid monolayers in the presence of Ca^{2+} (ref. 10). Mitochondria, on the other hand, are freely permeable to EDTA; however, it is not certain that the diminution in nucleotide content is due to the removal of bivalent cations by EDTA or the accompanying alterations in mitochondrial structure.

The greatest accumulation of ^{45}Ca was found in the mitochondria and the large nerve ending fraction. Although mitochondria are present in the nerve endings, their number alone is insufficient to account for the ^{45}Ca accumulation in this fraction. ^{45}Ca uptake by mitochondria is known to require ATP or an energy source[11,12], but it is not known to what extent the Ca^{2+} accumulation in the present studies may be energy-dependent. From the studies with frog ganglia and nerve fibers, it does not appear as if energetic or other metabolic factors are directly involved in Ca^{2+} accumulation or exchange. Energetic factors, however, are evidently involved in the accumulation of Ca^{2+} to the various subcellular components, those fractions richest in mitochondria showing the greatest accumulation. One possible explanation for this difference between intact tissue and subcellular components is that the measurement in the intact tissues was largely due to Ca^{2+} accumulation in extracellular compartments and non-mitochondrial components for which energy is evidently not required. The availability of anionic binding sites, such as the phosphate group of phospholipids[13,14], may be the determining factor in this latter situation.

The question arises as to the significance of the nucleotide association with various membranous components of neural tissue. One possibility is that ATP and its derivatives may be involved in the structural organization of the membrane[1,10]. It has been demonstrated that the formation of membranes from a mixture of beef brain lipids in a Ca^{2+} solution is facilitated by ATP[10]. Presumably, substances that can combine with Ca^{2+} could regulate the association of Ca^{2+} with phospholipids and other components interacting with Ca^{2+} (refs. 10, 15). By studying such interfacial properties as surface potential, pressure, and adsorption of a variety of phospholipids, it has been established that complexes of phospholipids–ATP–Ca^{2+} can readily form[10,15]. At present studies are in progress to compare lipids derived from various brain subcellular fractions for their ability to form interfacial complexes with Ca^{2+} and ATP. Preliminary results reveal the least adsorption to myelin lipids and the maximal to lipids derived from the synaptic membranes. Work is in progress to determine the specific lipids responsible for the ATP adsorption.

Since virtually all of the ^{45}Ca associated with the various subcellular fractions

could be extracted by chloroform–methanol along with the lipids, it is reasonable to assume that Ca^{2+} is mainly bound to lipids. It was not possible, however, to demonstrate an association of Ca^{2+} or ATP with any particular lipid employing silica gel thin-layer chromatography in a variety of solvent systems. Apparently the affinity of the silica gel for Ca^{2+} and other highly polar materials prevents any association with lipids. Although adenine and adenosine were extracted with the lipids from the subcellular components, only trace amounts of nucleotide were removable. This finding is in agreement with the observation that the adsorption of ^{14}C-labeled adenine and adenosine to lipid monolayers occurred much more readily than with any of the adenine nucleotides[10].

It is obvious from the present studies that the distribution of ATP and its derivatives within the nervous system is ubiquitous. Even though the main production is in the mitochondria, the membranous components, presumably because of their high content of phospholipids and Ca^{2+}, appear to retain appreciably greater amounts of nucleotides. Although it has been clearly established that complexes between Ca^{2+}, ATP and brain lipids can occur, the nature and locus of the association of ATP with the membranous components has yet to be determined.

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