Studies on valinomycin inhibition of synaptosome-fraction protein synthesis

M. Anthony VERITY, M. K. CHEUNG and W. Jann BROWN

Department of Pathology (Neuropathology), Brain Research Institute and Mental Retardation Center,
UCLA Center for the Health Sciences, Los Angeles, CA 90024, U.S.A.

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The ionophore valinomycin inhibited adult and neonatal synaptosome-fraction protein synthesis with half-maximal inhibition at ~ 10 nM. Valinomycin had no effect on [3 H]leucine uptake into synaptosomes at high or low external $[K^+]$. Synaptosome-fraction protein synthesis was dependent on $[K^+]_e$ reaching a maximum at 25 mM- K^+ . Valinomycin inhibition of protein synthesis was not reversed at high $[K^+]_e$. Valinomycin failed to influence the intrasynaptosomal $[K^+]$ even at zero $[K^+]_e$. A significant increase in State-4 respiration of synaptosomal fractions was found at 5 nM-valinomycin with a decrease in the respiratory control index. At these concentrations of valinomycin there was no inhibition of the ADP-stimulated (State 3) respiration rate. Valinomycin had no effect on cerebral microsomal protein synthesis *in vitro*, which was inhibited by puromycin (100 μ g/ml) or the absence of ATP. Valinomycin, 2,4-dinitrophenol and KCN inhibition of protein synthesis was not reversed by added ATP, suggesting impermeability of the membrane to ATP. Valinomycin induced a rapid fall in synaptosome ATP content not observed with atractylate or ouabain. Valinomycin inhibition of protein synthesis under these conditions is secondary to uncoupling of mitochondrial oxidative phosphorylation with a subsequent decrease in intraterminal ATP necessary for translation.

Since the demonstration of Austin & Morgan (1967) that synaptosome-enriched fractions from rat brain synthesized protein, investigators have concluded that at least two synthesizing systems operate in these preparations: (a) the prokaryotic chloramphenicol-sensitive system in mitochondria (Gordon & Deanin, 1968; Hernandez, 1974) and (b) a eukaryotic cycloheximide-sensitive ribosomal 80S system protected by a membrane (Gambetti *et al.*, 1972; Deanin & Gordon, 1973). Although the synaptic membrane preparations of Gilbert (1972) and Ramirez *et al.* (1972) may contribute to the synthetic activity, recent studies favour a more significant contribution from glial cytoplasmic particles (Henn *et al.*, 1976; Sieghart *et al.*, 1978; Sieghart & Singer, 1979) or closed-off dendritic structures (Hefti & Lichtensteiger, 1978). Although ribosomes are generally thought to be absent from the typical presynaptic 'pinched-off nerve endings' (Palay & Palade, 1955), ribosomes have been identified in membrane-bound structures consistent with post-synaptic endings isolated from immature cortex (Jones & Revell, 1970; Verity *et al.*, 1980). The bulk of evidence therefore suggests that protein synthesis in synaptosome fractions, whether isolated from sucrose or Ficoll/sucrose gradients, resides in

membrane-bound ribosome- and mitochondria-containing particles of glial or dendritic origin. Such a heterogeneous nature for the synaptosome fraction precludes an exact identification of the sole or major site of protein synthesis. However, such synthesis has been shown to be modulated by the (Na^+ + K^+)-dependent ATPase residing in the limiting membrane, presumably of the gliosome or dendrosome (Autilio *et al.*, 1968; Appel *et al.*, 1969; Verity *et al.*, 1979). Wedege *et al.* (1977) demonstrated a stimulation of amino-acid incorporation into synaptosomal-fraction protein by increasing the external $[K^+]$ and we have recently demonstrated the role of K^+ and transmembrane ion gradients in modulating protein synthesis in this system (Verity *et al.*, 1979). Further studies by Li & White (1977) have elucidated the effects of depolarizing agents on synaptosomal Na^+ and K^+ and revealed that veratridine or valinomycin rapidly reversed the contents of these ions in the isolated fractions with a residual decline in intraterminal $[K^+]$. Valinomycin is an ionophore transporting K^+ but not Na^+ across lipid membranes (Mueller & Rudin, 1967; see also Pressman, 1976, for review), and has also been shown to uncouple mitochondrial oxidative phosphorylation (McMurray & Begg, 1959). Inter-

ference with ATP production by intrasynaptosomal mitochondria was found to inhibit protein synthesis by Morgan & Austin (1969). These findings may provide an explanation for the valinomycin-induced inhibition of synaptosomal protein synthesis (Verity *et al.*, 1976), namely a stimulation of K^+ efflux, lowering of K^+ content within the synaptosome and/or a loss of ATP needed for protein synthesis.

In the present paper we wish to examine the mechanism of valinomycin inhibition of protein synthesis and evaluate the respective contribution of ionophore-induced changes in synaptosome Na^+ and K^+ content, with that of valinomycin inhibition of intraterminal oxidative phosphorylation.

Materials and methods

Adult Sprague-Dawley rats (100–180 g) and neonatal rats (10–15 days) of either sex were used. Ultrapure sucrose was obtained from Schwarz/Mann (Orangeberg, NJ, U.S.A.). Ficoll, atractyloside, cycloheximide, valinomycin, ADP and ATP were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). L-[U- ^{14}C]Leucine (sp. radioactivity 240 Ci/mmol); [4,5- 3H]leucine (sp. radioactivity 50 Ci/mmol) and 3H_2O (sp. radioactivity 100 mCi/ml) were obtained from ICN (Irvine, CA, U.S.A.) or Amersham Radiochemical Center (Arlington Heights, IL, U.S.A.). Carboxy[^{14}C]dextran (sp. radioactivity 1–5 mCi/g) was purchased from NEN, Boston, MA, U.S.A. Soluene-100 was obtained from Packard Instruments Company.

Preparation of synaptosome-containing fractions and amino-acid incorporation into protein

Homogenization of cerebral cortex, preparation of synaptosomes and incorporation of radiolabelled leucine into protein were performed as described previously (Verity *et al.*, 1977).

Amino-acid uptake into synaptosome fraction

Approx. 0.3–0.5 mg of synaptosome protein was incubated in 1 ml of basal uptake medium of composition 10 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂ and 0.2 M-sucrose containing 10 μ M-[^{14}C]leucine. When K^+ was added, equimolar choline chloride was added to a control preparation. Samples (0.25 ml) were removed at intervals and placed on prewashed Millipore AA filter discs (diameter, 25 mm; pore size, 0.8 μ m) under negative pressure. The filters were washed rapidly three times at room temperature with 5 ml portions of basal medium minus leucine. Discs were placed in 10 ml of Beckman Filter-SolvTM and shaken for 30 min before measurement of radioactivity in a Beckman LS-250 scintillation counter (Verity *et al.*, 1979).

Respiratory activity of brain mitochondria and synaptosomal particles

Preparation of mitochondria and measurement of respiratory parameters was determined as described previously (Verity *et al.*, 1975). The respiration medium (2 ml) contained 100 mM-KCl, 5 mM-KH₂PO₄, 20 mM-Tris/HCl, pH 7.4, 1 mM-EGTA, 0.1 ml of 5% bovine serum albumin, 5 mM-glutamate and 200 μ l of mitochondria suspension. Respiration rates were obtained on a Y.S.I. oxygen electrode assembly and expressed as μ g-atoms of O₂ utilized/min per 100 mg of protein. The respiratory control index was defined as the ratio of the respiration rate in the presence of ADP to that after utilization (Chance & Williams, 1955). Appropriate additions of ethanol were used in control experiments.

Microsomal protein synthesis

Microsomal fraction ('microsomes') was prepared by homogenizing whole rat brains in 5 ml of ice-cold medium (medium M') containing 0.35 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂·6H₂O, 40 mM-NaCl and 100 mM-KCl with an Elvehjem-Potter glass homogenizer. The homogenate was centrifuged at 2064 g (r_{max}) for 1 min and the postnuclear supernatant was removed and saved. The pellet (P₁) was resuspended in 4 ml of fresh homogenization medium and re-centrifuged at 2064 g (r_{max}). The S₁ supernatants were combined and a postmitochondrial supernatant (S₂) was obtained by centrifuging at 23 000 g (r_{max}) for 4 min. The S₂ supernatant was then centrifuged in a 50 angle rotor at 127 000 g (r_{max}) for 120 min ($\omega^2 t = 12419 \text{ rad}^2/\text{s} \times 10^7$). The resulting pellet (P₃) was resuspended in 1 ml of medium M' by using a 2 ml glass homogenizer fitted with a Teflon pestle. For protein-synthesis studies, 0.2–1.0 mg of the P₃ suspension was added to 1 ml of mercaptoethanol-free M' medium supplemented with 3 mM-ATP and 0.1 mM-GTP. The reaction was started by adding 10 μ Ci of [3H]leucine. Incubations were carried out at 37°C and the reaction was stopped at appropriate time intervals by the addition of equal volumes of ice-cold 10% trichloroacetic acid. Radiolabelled leucine incorporation into trichloroacetic acid-precipitable protein was determined as described previously (Verity *et al.*, 1979).

Intrasynaptosomal K⁺ and Na⁺ concentration

Measurements of intrasynaptosomal K^+ and Na^+ concentration were performed as described previously (Verity *et al.*, 1979). Duplicate 0.5 ml portions of medium containing synaptosomes were placed in 1 ml plastic conical centrifuge tubes, one of which held 10 μ l of a mixture containing [^{14}C]dextran and 3H_2O . Both tubes were centrifuged at

30000 g for 3 min at room temperature. K^+ content was obtained on a Beckman 'KLiNa' flame photometer with an internal lithium standard and the $[K^+]$ of the intrasynaptosomal space was calculated from the dextran and water spaces respectively.

Estimation of synaptosomal-fraction ATP content

Intrasynaptosomal ATP was determined on 0.1 ml samples removed from the incubation medium added to equivolume 0.6M-HClO₄. After precipitation and neutralization with K₂CO₃, the supernatant was used for ATP determination using the firefly bioluminescence method (Lundin & Thore, 1975). The contents of a vial containing a crude luciferin-luciferase preparation (Sigma, FLE 50) were reconstituted with 5 ml of water to give a final solution of 10 mg of extract/ml in 20 mM-MgSO₄ and 50 mM-potassium arsenate buffer. Of this extract 0.1 ml was added to 1 ml of 5 mM-Tris/HCl, pH 7.4, supplemented with 5 μ g of luciferin placed in a plastic mini-scintillation vial. Of the neutralized ATP sample 5–50 μ l was added to the firefly system, shaken and the radioactivity was immediately measured in a Beckman LS-250 scintillation counter using an open window. The maximum peak chemiluminescence occurring within 15 s at 25°C was recorded. A sigmoidal standard curve of chemiluminescence versus [ATP] in a standard solution was determined daily for each vial of firefly extract. ATP was standardized spectrophotometrically at 259 nm assuming an extinction coefficient of 15.4×10^6 cm²/mol.

Protein determinations

These were made by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Effect of valinomycin on synaptosome-fraction protein synthesis

The rate of [³H]leucine incorporation into trichloroacetic acid-precipitable synaptosome-fraction protein increased with increasing external leucine concentration reaching a plateau at 4 μ M-leucine (Verity *et al.*, 1979). Valinomycin inhibition of protein synthesis was immediate, demonstrated no latent period and gave 50% inhibition at approx. 10 nM (Fig. 1). Slight stimulation of incorporation was found at [valinomycin] ~ 1 nM. Valinomycin inhibition was independent of synaptosome concentration within the range 0.2–0.6 mg of protein/ml.

Effect of valinomycin on amino-acid uptake

Synaptosomes possess carrier-mediated mechanisms for amino-acid uptake (Peterson & Raghupathy, 1972) many of which are electrogenic and Na⁺-dependent. Leucine uptake is not Na⁺-depend-

Fig. 1. Profile of valinomycin inhibition of synaptosome-fraction protein synthesis

Adult rat synaptosomes were incubated in ionic media containing various concentrations of valinomycin. [³H]Leucine incorporation was determined at 20 min and 37°C in three to four experiments. The inset shows valinomycin (50 nM) inhibition (----) as a function of time of [³H]leucine incorporation. —, control.

ent and is significantly inhibited by Na⁺ or K⁺ in the medium (Verity *et al.*, 1976). Addition of equimolar choline chloride had no effect on uptake. Valinomycin inhibition of uptake via an electrogenic mechanism may contribute to the decreased synthesis. The effect of valinomycin on leucine uptake into synaptosomes incubated in K⁺-free (choline-supplemented) or 80 mM-KCl media was examined. The initial peak uptake of leucine at 2–3 min was greater in K⁺-free medium (Fig. 2). Valinomycin (5 μ M) did not inhibit the initial maximum uptake or equilibrium values in the presence or absence of 80 mM-KCl. Pre-equilibration of synaptosomes at 37°C before addition of [¹⁴C]leucine inhibited peak uptake at 2–3 min but this was uninfluenced by valinomycin. Such experiments dissociate the valinomycin-induced inhibition of protein synthesis from a possible effect on amino-acid uptake.

Effect of external potassium ($[K^+]_e$) on synaptosome protein synthesis and the inhibitory effect of valinomycin

Appel *et al.* (1969) and Autilio *et al.* (1968) demonstrated synaptosome protein synthesis dependence on the operation of the (Na⁺ + K⁺)-dependent ATPase located in the limiting membrane and we showed previously an inhibition of synthesis by ouabain coupled to a decrease in internal $[K^+]$ (Verity *et al.*, 1979). As shown in Fig. 3, a stimulation of leucine incorporation occurred

Fig. 2. Effect of medium $[K^+]$ on valinomycin inhibition of $[^{14}C]$ leucine uptake into synaptosomes

Synaptosomes were suspended in 0.2 M-sucrose/10 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂, containing 80 mM- K^+ (■ and □) or 80 mM-choline chloride (● and ○). Valinomycin was present at 5 μM (● and ■). Uptake was determined by using the rapid Millipore filtration technique after incubation at 37°C. Values represent means for two experiments.

Fig. 3. Effect of external $[K^+]$ on synaptosome-fraction protein synthesis

$[^3H]$ Leucine incorporation (0.05 μM) was determined after 40 min in the absence (●) and presence (○) of valinomycin (50 nM).

with $[K^+]_e$ up to 25 mM, after which a decline in synthesis rate was seen. Valinomycin (50 nM) induced considerable inhibition in both the K^+ -free and the K^+ -stimulated components and there was no evidence of a K^+ reversal of valinomycin inhibition of protein synthesis.

Fig. 4. Correlation of intrasynaptosomal $[K^+]$ as a function of medium K^+ concentration

Neonatal synaptosomes were incubated in variable external $[K^+]$ for 20 min at 37°C. The intrasynaptosomal $[K^+]$ was determined from $[^{14}C]$ -dextran and 3H_2O measurements (see the Materials and methods section). ○, Control; ●, 0.5 μM-valinomycin. Data represent means for three experiments.

Valinomycin translocates K^+ across biomembranes and an outward movement of K^+ leading to decreased intraparticle $[K^+]$ could account for the decrease in protein synthesis. Our failure to demonstrate a reversal of valinomycin inhibition by increasing $[K^+]_e$ would apparently negate this hypothesis so we examined further the effect of valinomycin on intrasynaptosomal $[K^+]$ as a function of $[K^+]_e$. This study (Fig. 4) failed to reveal a significant efflux of intrasynaptosomal $[K^+]$ in the presence of valinomycin, even when the particles were suspended in K^+ -deficient media.

Action of valinomycin on respiration and oxidative phosphorylation

Valinomycin has been shown to uncouple mitochondrial oxidative phosphorylation (McMurray & Begg, 1959). In the present study we confirmed that $[valinomycin] > 1$ nM significantly stimulated glutamate-supported State-4 respiration with an accompanying decrease in respiratory control (Fig. 5). At these concentrations there was no effect on the rate of ADP or 2,4-dinitrophenol-stimulated electron transport. This uncoupling action of valinomycin on synaptosome mitochondria occurred at valinomycin concentrations found to produce inhibition of protein synthesis. If valinomycin inhibited protein synthesis via an uncoupling action on oxidative phosphorylation, then similar inhibition of synthesis should be found with other inhibitors of oxidative phosphorylation (Table 1). KCN and

Table 1. *Effect of mitochondrial inhibitors supplemented with ATP on synaptosome protein synthesis*

Synaptosomes were isolated from neonatal rats (12–14 days old). Protein synthesis was determined in media containing 0.1 M-sucrose, 50 mM-NaCl, 25 mM-KCl, 5 mM-MgCl₂, 5 mM-Na₂HPO₄, 33 mM-Tris/HCl, pH 7.4, and 4 μ M-[³H]leucine. Incorporation in control animals was 4.2 ± 0.7 ($n = 6$) pmol/mg of protein per 20 min. Results are means $\pm$ S.E.M. for the numbers of observations shown in parentheses.

Inhibitor	Concentration (μ M)	Inhibition (%)	
		no ATP	+ 1 mM ATP
2,4-Dinitrophenol	250	83 ± 3 (4)	88
KCN	500	89 ± 2 (4)	92
Attractylate	100	2 ± 3 (5)	—
Valinomycin	4.5×10^{-2}	86 ± 1 (3)	80

Fig. 5. *Effect of valinomycin on adult synaptosome fraction respiration in vitro*

Respiration medium (2.0 ml) contained 100 mM-KCl, 5 mM-KH₂PO₄, 20 mM-Tris/HCl, pH 7.4, 1 mM-potassium EGTA, 3 mg of bovine serum albumin, 4 mM-glutamate and 200 μ l of synaptosome suspension. Δ , State 4; $\circ$, State 3 measured in the presence of 0.5 μ M-ADP. The respiratory control index ($\bullet$) was determined polarographically at 30°C. Data are means $\pm$ S.E.M. ($n = 4-6$).

2,4-dinitrophenol produced comparable inhibition, which was not reversed on addition of 1 mM-ATP. In contrast, no effect on protein synthesis was observed in the presence of 100 μ M-attractylate or equimolar carboxyatractylate shown to inhibit synaptosomal respiration and oxidative phosphorylation (Verity, 1972) by inhibition of adenine nucleotide translocation across the mitochondrial inner membrane (Klingenberg, 1970).

Effect of valinomycin on ribosomal protein synthesis in vitro

The observed inhibition of the predominantly non-mitochondrial cycloheximide-sensitive synaptosomal-fraction protein synthesis by valinomycin may be related to a direct interaction of the ionophore with the protein-synthesis machinery producing a suppression of polypeptide synthesis. We found no inhibition of cerebral microsomal protein synthesis at 0.5 μ M-valinomycin (Table 2). In

Table 2. *Effect of valinomycin on cerebral microsomal protein synthesis*

Microsomes were prepared from neonatal whole brain (12–18 day-old rats) as described in the Materials and methods section. Cell-free microsomal protein synthesis was measured in medium M' (0.35 M-sucrose, 10 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 40 mM-NaCl and 100 mM-KCl) supplemented with 2.5 mM-disodium ATP and 0.1 mM-GTP. Enzyme (pH 5) was not added. The reaction was started on addition of 10 μ Ci of [³H]leucine. Results are means $\pm$ S.E.M. for the numbers of observations in parentheses.

	Protein synthesis (pmol/mg of protein per 20 min)
Control	4.65 ± 0.5 (3)
Valinomycin (0.5 μ M)	4.2 ± 0.3 (2)
Puromycin (100 μ g/ml)	0.8 ± 0.2 (2)
No ATP	0.24 ± 0.01 (3)

Fig. 6. *Recovery of synaptosome fraction ATP as a function of incubation at 37°C and valinomycin*

Synaptosomes were suspended in the protein-synthesis ionic medium containing none ($\circ$), 10 nM-valinomycin (Δ) or 0.1 μ M-valinomycin ($\bullet$). Values represent means $\pm$ S.E.M. ($n = 2-4$).

this cell-free system, protein synthesis was significantly inhibited by puromycin or the absence of ATP.

Effect of valinomycin on synaptosome-fraction ATP content

Synaptosomes incubated in the protein-synthesizing medium at 37°C lost 20–30% of their endogenous ATP during 10 min incubation (Fig. 6). Most of this loss occurred within the first 2 min. The initial value of 7.01 ± 1.07 nmol of ATP/mg of protein was approximately twice that found by Li & White (1977). Valinomycin (0.1 μ M) induced a marked decrease to <12% of the control value after 1 min incubation. Atractylate (50 μ M) and ouabain (200 μ M) had no significant effect on ATP content.

Discussion

The studies in the present paper do not answer the controversy of what components in the synaptosome fraction support protein synthesis. Agreement exists that the structure(s) responsible for protein synthesis contains cytoplasm, is bounded by a membrane, is dependent on ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity for optimal synthesis (Verity *et al.*, 1979), carries a membrane potential (Wedge *et al.*, 1977), in immature preparations reveals ribosome profiles within postsynaptic or dendrosome particles (Gambetti *et al.*, 1972; Hefti & Lichtensteiger, 1978; Verity *et al.*, 1980) and is mandatorily coupled to mitochondrial oxidative phosphorylation. We do not suggest that amino-acid incorporation *in vitro* occurs only in the presynaptic terminal, but refer to incorporation into the synaptosome-containing fraction, characterized by pre- and post-synaptic structures, and dendrosomes. The value of the preparation lies in its sensitivity to external modulation, evidenced in these studies with valinomycin, which demonstrated inhibition of protein synthesis unrelated to amino-acid uptake or external K^+ concentration. K^+ ions alone are known to increase synaptosomal protein synthesis (Morgan & Austin, 1969; Appel *et al.*, 1969; Verity *et al.*, 1979). Wedge *et al.* (1977) found a stimulation of synaptosome protein synthesis by K^+ and other depolarizing treatments, which in the case of K^+ ions was not blocked by tetrodotoxin. Lipton & Heimbach (1978) demonstrated a 2–3-fold stimulation of protein synthesis in hippocampal slices when external K^+ was varied from 1.3 mM to 6.4 mM, and suggested that an alteration in the K^+/Na^+ ratio affected this change in synthetic rates. We have shown that ouabain inhibition of synaptosome protein synthesis was coupled to decreased $[\text{K}^+]$ (Verity *et al.*, 1979). The present study confirmed that increasing the $[\text{K}^+]_e$ in the incubation medium stimulated protein synthesis and increased the intraterminal K^+ content. The stimulation of protein synthesis by K^+ may be coupled directly to increased intrasynaptosomal

K^+ content and may be mediated via the K^+ -dependent increase in the activity of peptidyltransferase in the 60S ribosome subunit (Miskin *et al.*, 1970).

Our failure to demonstrate a valinomycin-induced change in synaptosomal $[\text{K}^+]$ or reversal of inhibition by increasing the $[\text{K}^+]_e$ would apparently preclude a valinomycin-induced K^+ flux as the mechanism responsible for the inhibition of protein synthesis. Li & White (1977) have reported a fall in synaptosomal $[\text{K}^+]$ and slight gain in $[\text{Na}^+]$ in the presence of valinomycin. However, the valinomycin concentration was approx. 0.1 μ M, five times greater than the concentration needed to inhibit protein synthesis in our synaptosome preparations and their use of Krebs' medium (110 mM- Na^+ /3 mM- K^+) may have allowed for K^+ efflux. No direct effect of valinomycin was demonstrated on cell-free cerebral microsomal protein synthesis. It is therefore likely that the valinomycin-induced inhibition of synthesis was due to the uncoupling action of valinomycin on mitochondrial oxidative phosphorylation (McMurray & Begg, 1959). Mitochondria are impermeable to K^+ ions, a restriction essential for coupled mitochondrial function (Caswell, 1969; Ligeti & Fonyo, 1977). Mitochondrial membrane permeability to K^+ is increased by valinomycin (Moore & Pressman, 1964; Smith & Beyer, 1967) and the process is associated with rapid K^+ uptake, proton extrusion, swelling and loss of oxidative phosphorylation. Stimulation of State-4 respiration is expected and was shown in our experiments on synaptosomal respiration. Inhibition of oxidative phosphorylation will lead to decreased intraterminal concentrations of ATP, increased ADP and lowered energy charge accounting for the decline in synthesis rate (Brenner *et al.*, 1970; Trachsel *et al.*, 1977).

We found 0.45 μ M-valinomycin induced a rapid loss of synaptosome ATP during the first 60 s, leaving a residual content of ~ 1 nmol of ATP/mg of protein. Assuming a synaptosome volume of 3.3 μ l/mg of protein, this represents an internal ATP concentration of approx. 0.3 mM, insufficient to support protein synthesis.

What is the mechanism leading to such a precipitous decline in ATP content? In control preparations, ATP loss through breakdown and metabolic utilization was approx. 20% in 10 min in the absence of added oxidizable substrate (Fig. 6). Atractylate, known to block translocation of ADP and ATP across the mitochondrial membrane, did not affect this endogenous rate of ATP loss. The absence of an atractylate or carboxyatractylate effect on protein synthesis suggested that the supply of mitochondrial ATP was not limiting for protein synthesis, which seems unlikely in view of the effect of other mitochondrial inhibitors, or that atractylate does not diffuse to the intraterminal mito-

chondria. The failure of atractylate or the more lipophilic carboxyatractylate to permeate the limiting membrane would seem unusual in view of diffusion into myocardial muscle fibres (Shug *et al.*, 1977) or isolated hepatocytes (Stubbs *et al.*, 1978). If the Na⁺ pump played a significant role in the utilization of endogenous ATP under our medium conditions, then increased utilization of endogenous ATP should be seen in the presence of atractylate. This was not so. Moreover, 0.2 mM-ouabain did not 'protect' the endogenous decay rate of ATP. The rapid decline of ATP by valinomycin must have a dual explanation: (1) inhibition of ADP phosphorylation due to depolarization of the mitochondrial membrane potential by valinomycin plus K⁺, thus replacing the proton gradient leading to uncoupling, and (2) a simultaneous massive and rapid activation of mitochondrial ATPase that consumed the available endogenous ATP. Jurkowitz *et al.* (1974) established that valinomycin activated isolated heart mitochondrial ATPase in the presence of K⁺ ions. Intrasyntosomal mitochondria are 'surrounded' by ~60 mM-K⁺, even when synaptosomes are incubated in a K⁺-free medium. Li & White (1977) found that high concentrations of valinomycin provoked a loss of synaptosomal K⁺, smaller gain in Na⁺ and a decline in ATP most likely associated with a direct effect on mitochondrial coupling. If we accept that valinomycin inhibition of protein synthesis is directly and causally related to a decline in ATP content occasioned by activation of mitochondrial ATPase and block of oxidative phosphorylation, then addition of ATP should reverse the protein-synthesis inhibition. This was not found in our experiments and suggests either that the membrane-limited protein-synthesizing system is impermeable to high concentrations of external ATP, or that the added ATP is rapidly hydrolysed by ecto-ATPase. Another explanation of our data may be that cycloheximide-sensitive synaptosomal protein synthesis is mandatorily coupled to both an optimum concentration of ATP and a transmembrane K⁺ potential.

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