The Effect of Electrical Stimulation on the Incorporation of L-[U-¹⁴C]Valine into the Protein of Chopped Tissue from Guinea-Pig Cerebral Cortex

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1. Chopped tissue from guinea-pig cerebral cortex carried out an energy-dependent incorporation of [¹⁴C]valine into protein. 2. At all times studied the nuclear fraction of the homogenized tissue accounted for about 25% of the total labelled protein. 3. Electrical stimulation at first increased, but subsequently decreased, the rate of incorporation of [¹⁴C]valine into protein of the chopped tissue. 4. The initial increase in the incorporation of [¹⁴C]valine into protein occurred in the nuclear fraction. At later times electrical stimulation decreased the incorporation into all the subcellular fractions, but the relative contribution of the nuclear fraction to the total labelled protein increased. 5. These changes are discussed in relation to the changes in the rates of respiration, glycolysis, high-energy phosphate content and intracellular Na⁺ and K⁺ concentrations, which were measured under the same experimental conditions as those used to study protein synthesis.

Although considerable interest has been shown in protein synthesis by tissue preparations and by cell-free systems derived from the mammalian nervous system, very little attempt has been made to investigate the effect of physiological activity on protein synthesis in nervous tissue *in vitro*. The primary object of this and the following paper (Jones & Banks, 1970) is to examine the effect of electrical stimulation on protein synthesis of cerebral cortex.

However, since stimulation leads to profound changes in many areas of metabolism (McIlwain, 1966), which may be expected to have some influence on protein synthesis, this and a subsequent paper (Jones & Banks, 1970) contain data on energy metabolism, intracellular ion concentrations and [¹⁴C]valine fluxes obtained under conditions identical with those used to study protein synthesis. In the discussion the relationship between these data and the observed effects of electrical stimulation on cerebral protein synthesis is examined.

The measurement of respiration and lactate production provided indices of the viability of the tissue preparations and of the effectiveness of electrical stimulation. The information on respiration, lactate production, ion content and the concentration of ATP and creatine phosphate of stimulated brain slices is in broad agreement with, and extends, the observations made by McIlwain and his colleagues (McIlwain, 1966).

METHODS

Preparation of tissue. Male guinea pigs (375–425 g) were killed by cervical fracture followed by bleeding from the neck. Superficial slices of cerebral cortex were taken from the dissected brain and chopped in the cold-room (5°C) (McIlwain & Rodnight, 1962) to give small blocks (0.35 mm × 0.5 mm × 0.5 mm), which were suspended at 50 mg/ml in oxygenated Krebs phosphate saline (30–37°C) within 10 min of the animal's death. Samples (1 ml) of the tissue suspension were pipetted into Warburg flasks (containing a further 2.5 ml of Krebs phosphate saline in the main compartment and side-arm), which were then oxygenated and incubated at 37°C with shaking. After a 20 min preincubation period oxygen consumption was measured at 5 min intervals for a control period of 30 min. The composition of the Krebs phosphate saline was: Na₂HPO₄, 20 mM; NaCl, 124 mM; KCl, 6.7 mM; MgSO₄, 1.3 mM; CaCl₂, 0.75 mM; glucose, 10 mM. It was adjusted to pH 7.4 with 2 M-HCl.

Incubation. At the end of the 30 min control period, 1 μCi of L-[U-¹⁴C]valine (270 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the main compartment from the side arm. Simultaneously electrical pulses from a condenser-pulse generator (Specialised Laboratory Equipment Ltd., Croydon, Surrey, U.K.) were applied to those Warburg flasks fitted with gold-plated concentric electrodes (McIlwain & Rodnight, 1962). The condenser pulses, monitored on a Telequipment SB 51 oscilloscope, had a peak voltage of 18 V, a time-constant of 0.4 ms and a frequency of 100 pulses/s. The distance between the electrodes was approx. 1 cm, which should give a linear potential gradient of

about 12–15 V/cm (see Ayres & McIlwain, 1953). As the gold-plated electrodes slowly deteriorated with use, they were cleaned in KCN and replated after about six experiments. After being replated, the electrodes were annealed by passing stimulating pulses for about 15 min. If this was not done gold left the electrodes during periods of stimulation and was taken up by the suspended tissue, which turned pink on heating with 5% (w/v) trichloroacetic acid.

In experiments where the frequency of the applied pulses was varied, the reduced-frequency signal was generated by a Siemens-Ediswan Low Frequency Oscillator (type R666), amplified with a Williamson amplifier (100 Ω output) and was used to trigger the condenser-pulse generator. The condenser pulses monitored on a Tetronix 502A oscilloscope showed no significant distortion of the signal or irregularity of frequency.

Incorporation of L-[¹⁴C]valine into protein. At the end of the incubation period the flasks were placed on ice and 3 ml of ice-cold 10% (w/v) trichloroacetic acid containing 0.5% of casein hydrolysate was added to each flask. The acidified contents and further 3 ml samples of 5% (w/v) trichloroacetic acid, which were used to wash out the flasks, were pooled and homogenized. The homogenates were spun at 1500g for 5 min and the pellets were collected. These were washed three times in 6 ml of 5% trichloroacetic acid and during the third washing were incubated at 90°C for 30 min; at this stage the recovery of precipitable protein was $79 \pm 1.5\%$ (105), most of the loss probably being due to the tissue remaining in the Warburg flasks. The insoluble material was extracted at 0°C three times with 6 ml of chloroform-methanol (1:1, v/v) and finally dried with 6 ml of ether. During the chloroform-methanol extraction and ether drying, the loss of precipitable protein was $31 \pm 1.1\%$ (105); the specific radioactivity (d.p.m./mg of protein) of the lipid-extracted protein was $6.6 \pm 1.5\%$ (105), higher than that of the non-extracted protein. The dried pellet was suspended in water and its protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactivity was determined by placing protein samples in 10 ml of liquid scintillator [NE220+NE221 (1:1, v/v); Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.] containing 0.5 ml of Triton X-100 (Mann Research Laboratories, New York, N.Y., U.S.A.) and counting in a Nuclear-Chicago liquid-scintillation counter. Counting efficiency, as determined by the channels-ratio method, was about 75% and the results were corrected to 100% counting efficiency.

ATP and creatine phosphate determinations. At the end of the incubation the flasks were placed on ice and treated at 0°C with 0.5 ml of 2.1 M-HClO₄ and this, together with 0.5 ml of 0.3 M-HClO₄ used to wash the flasks, was homogenized. The homogenate was treated with 0.1 ml of 40 mM-EDTA (to prevent Ca²⁺ precipitating ATP) and the ice-cold homogenate was centrifuged for 3 min at 1500g. The pellet was washed twice with 5% trichloroacetic acid, and protein was determined. The supernatant was brought to about pH 7.8 with 2 M-KHCO₃ since creatine phosphate is most stable at this pH (Ord & Stocken, 1959) and the precipitate of KClO₄ formed during the subsequent 30 min at 0°C was removed by centrifuging for 3 min at 1500g. The supernatant was retained for determination of lactate, creatine phosphate and ATP.

ATP and creatine phosphate were determined by using

hexokinase, creatine phosphokinase and glucose 6-phosphate dehydrogenase by the fluorimetric method of Lowry, Passonneau, Hasselberger & Schulz (1964). The appearance of NADPH was measured on a chart recorder attached to an Aminco-Bowman spectrofluorimeter by exciting the test solution at 340 nm. NADPH gave a linear increase in fluorescence from 0 to 5 μ M at 475 nm. The concentration of NADPH measured in the assay was approx. 1–1.5 μ M.

The fluorescence produced by the presence of ATP showed a rapid rise and the reaction was almost complete within about 5 min. After that time the reaction rate was several orders of magnitude lower than the initial rate. The rate of reaction with creatine phosphate was much lower than that with ATP, and it was difficult to decide when the reaction was complete since even after 30 min the rate was still about 15% of the initial rate. In both cases the concentration of the energy-rich compound was calculated from the intersection point for the initial and final rates. This procedure was straightforward in the ATP determination but difficulty was experienced in determining the exact position of the intersection during the determination of creatine phosphate.

The quantity of tissue recovered from the flasks at the end of each incubation was estimated from the protein content by using the previously determined value of 8.28 ± 0.88 (18) mg of protein/mg wet wt. of tissue.

Lactate determination. Lactate was determined with lactate dehydrogenase as described by Hohorst (1963).

Determination of intracellular Na⁺ and K⁺. Chopped tissue was incubated as described above in Krebs phosphate saline containing 1 μ Ci of [*carboxy*-¹⁴C]inulin (7.85 mCi/mmol; The Radiochemical Centre). At the end of the incubation the flask contents were filtered under vacuum through Whatman no. 40 filter paper placed on a micro Hirsch funnel. The tissue chops were removed from the filter paper with a stainless-steel spatula and homogenized in 5 ml of glass-distilled water. Protein in the homogenate was precipitated from a 1 ml sample with 5 ml of 10% trichloroacetic acid, washed three times with 5% trichloroacetic acid and determined as described above. Radioactivity in the homogenate and filtrate were determined in a liquid-scintillation counter. The remaining 3 ml of the homogenate was made 0.1 M with respect to HNO₃, left overnight, and any precipitate that formed was spun down. The Na⁺ and K⁺ concentrations in the resulting supernatant and in the filtrate obtained after the chopped slices had been filtered were determined with a Unicam SP.90 Atomic Absorption Spectrophotometer fitted with a flame emission burner. The emission from Na and K was measured at 589 nm and 766.5 nm respectively.

The determination of protein allowed an estimate of the quantity of tissue used for each extraction. On the assumption that inulin is localized exclusively extracellularly, the determination of radioactivity allowed an estimate to be made of the distribution of inulin between the slices and the incubating medium, and thus permitted the volume of the inulin space (i.e. extracellular space and suspending medium adhering to each slice) to be calculated. Then, from knowledge of the quantity of ion in each extract and the concentration of the ion in the Krebs phosphate saline, the intracellular ion concentration could be calculated by using a formula of the following type:

$\mu\text{equiv. of Na}^+/\text{g wet wt. of tissue} =$

$$\frac{\mu\text{equiv. of Na}^+ \text{ in tissue extract} - (\mu\text{equiv. of Na}^+/\text{ml of medium} \times \text{ml of inulin space})}{\text{g wet wt. of tissue}}$$

Tissue fractionation. The preparation, incubation and electrical stimulation of the tissue were carried out as described above. At the end of the incubation with L-[^{14}C]valine the chopped tissue (from three separate incubations) was spun down at 500g for 15s, washed at 0°C with 5 ml of 0.32 M-sucrose and resedimented by spinning for 1 min at 1500g. (At this stage a fine fibrous network of tissue and capillaries was left floating on the top of the sucrose.) The sedimented pellet of tissue was resuspended in 2.5 ml of 0.32 M-sucrose (tissue/fluid ratio approx. 5%, w/v) and gently homogenized in a Potter-Elvehjem homogenizer fitted with a Teflon pestle (clearance on the radius 0.14 mm). A sample (0.5 ml) of the homogenate was taken and the remainder was centrifuged. The nuclear fraction was collected by centrifuging for 10 min at 600g and 0°C, and the resulting pellet was washed at 0°C with a further 2 ml of 0.32 M-sucrose and re-spun. The pooled supernatants were then centrifuged in a MSE Superspeed 50 centrifuge (rotor no. 2418) for 20 min at 10000g (r_{av} , 11 cm) at 0°C to provide a crude mitochondrial pellet. The supernatant was further centrifuged in a Superspeed 50 centrifuge (rotor no. 2410) for 45 min at 155000g (r_{av} , 6.4 cm) at 0°C to provide a crude microsomal fraction. The protein in each fraction was precipitated at 0°C with 5 ml of 10% trichloroacetic acid containing casein hydrolysate (0.5%) and, after standing on ice for 60 min, collected by spinning for 5 min at 1500g. The pellets were washed three times with 5% trichloroacetic acid before their protein content and radioactivity were measured. The recovery of precipitable proteins after the fractionation was $102.8 \pm 1.2\%$ (30).

Succinate dehydrogenase determination. Succinate dehydrogenase activity was determined in each fraction by the method of Arrigoni & Singer (1962), which makes use of phenazine methosulphate and 2,6-dichlorophenol-indophenol.

DNA determination. The subcellular fractions from two separate homogenates were each suspended in 1 ml of water and dialysed at 5°C overnight against several changes of water to remove sucrose. The corresponding fractions from the two homogenates were then pooled to provide one set of subcellular fractions. Then solid trichloroacetic acid was added to each fraction to give a final concentration of 10% (w/v). Each acidified fraction was then extracted at 100°C for 30 min and centrifuged. The resulting pellet was extracted at 100°C again with 1 ml of 10% trichloroacetic acid. The deoxyribose in the pooled supernatants was determined by the method of Burton (1956).

Results. These are expressed as means $\pm$ S.E.M. with the numbers of observations in parentheses.

RESULTS

Effect of electrical stimulation on respiration and lactate production. The respiratory rate of control slices suspended in normal Krebs phosphate saline remained almost constant at about 1600 μl of O_2/h per g wet wt. for 2 h after the preincubation period.

The application of electrical pulses (100/s; 18 V; time constant 0.4 ms) caused a $78.9 \pm 3.2\%$ (60) increase in the respiratory rate for the first 30 min. During the next hour the response to electrical stimulation diminished so that after a total of 90 min exposure to stimulation the rate of respiration did not differ significantly from the control. The application of pulses failed to stimulate the respiration of homogenates of chopped cerebral cortex tissue or to cause an increase in oxygen uptake in vessels containing Krebs phosphate saline alone. It would appear therefore that the increase in respiration of the tissue evoked by electrical stimulation is dependent on the structural integrity of the tissue.

Resting respiration fell by 30% when the Na^+ concentration of the medium was decreased from 164 mM to 40 mM. Electrical stimulation had no significant effect on respiration at 40 mM- Na^+ . During the first 50 min of incubation lactate production by the chopped tissue was 32 $\mu\text{mol/h}$ per g wet wt. During the next 30 min the rate fell to 10 $\mu\text{mol/h}$ per g wet wt. in the control slices, but rose to 61 $\mu\text{mol/h}$ per g wet wt. when electrical pulses (100/s) were applied.

The rates of respiration and lactate production at rest and during periods of electrical stimulation indicate that the chopped tissue was in a satisfactory condition. This view is also confirmed by the ATP and ion content of the chopped tissue (see below).

Evidence for the incorporation of L-[^{14}C]valine into protein. The radioactivity recovered in the trichloroacetic acid-precipitated lipid-extracted pellet can be equated with the incorporation of an equivalent amount of L-[^{14}C]valine into protein for the following reasons. (1) Repeated washing with 5% trichloroacetic acid containing casein hydrolysate (0.5%) before lipid extraction did not significantly alter the specific radioactivity (d.p.m./mg of protein) of the precipitated material. (2) The specific radioactivity of the trichloroacetic acid-precipitated lipid-extracted pellet did not differ significantly from that of the non-extracted protein. (3) Digestion of the purified acid-precipitable material with pronase liberated 93% of the radioactivity, indicating that most of the label was located in protein molecules. (4) Terminal incorporation of [^{14}C]valine, determined with 2,4-difluoro-1-nitrobenzene, was only $0.53 \pm 0.16\%$ (4), indicating that a true synthesis of protein was occurring rather than terminal addition of labelled valine. (5) Incorporation of [^{14}C]valine was linear over the first hour after an initial lag of 5 min (Fig. 1). (6) Zero-time

Fig. 1. (a) Incorporation of [¹⁴C]valine into the protein of chopped cerebral cortex at rest and during periods of electrical stimulation. Labelled valine was added and electrical stimulation began at zero time. (b) First 15 min of incorporation on an expanded scale. Δ, Control; ▲, electrically stimulated (18 V; 100 pulses/s; time-constant 0.4 ms). Each point represents the mean $\pm$ S.E.M. (represented by vertical bars) of four to six experiments.

incorporation measured by adding label and acid simultaneously was only 0.78 ± 0.95 (8) d.p.m./mg of protein compared with 640 ± 54 (4) d.p.m./mg of protein after 7 min. (7) Incorporation of [¹⁴C]valine was decreased by 64.3% after 90 min when 2,4-dinitrophenol (0.2 mM) and sodium fluoride (5 mM) were added together with the label, thus demonstrating that incorporation of label was energy-dependent. (8) The incorporation of label was inhibited by about 65% in the presence of puromycin (10 and 100 μ g/ml) or cycloheximide (100 μ g/ml) over a period of 90 min (Jones & Banks, 1969a). Furthermore preincubation with cycloheximide (100 μ g/ml) for 10 min followed by a 5 min incubation with [¹⁴C]valine gave a 77% inhibition of incorporation into protein. Chloramphenicol (100 μ g/ml) caused only a 7.7% inhibition over 90 min. In addition to supporting the view that true protein synthesis is being observed, these results also suggest that the incorporation of label cannot have been the consequence of bacterial contamination, since bacterial protein synthesis is much more sensitive to inhibition by chloramphenicol than by cycloheximide (Brock, 1961; Hall, Kavanagh & Asheshov, 1951).

Effect of electrical stimulation on protein synthesis. After the addition of [¹⁴C]valine to control flasks its rate of incorporation into protein rose exponentially for about 5 min to a rate that was maintained for about an hour. Thereafter the specific radioactivity of the protein did not change (Fig. 1a). Application of 100 pulses/s from the moment of adding the label increased the rate of incorporation of [¹⁴C]valine transiently (Fig. 1b); this increase was

followed by a progressive inhibition of incorporation for the remainder of the experiment. The transient increase in incorporation induced by electrical stimulation is most clearly seen when it is expressed as a percentage of the control value (Fig. 2). The reason for this is that the percentage change gives a much better indication of the statistical significance of the altered incorporation than does a comparison of the mean values of the specific radioactivities of the protein. The increased incorporation was apparent 2 min after electrical stimulation commenced [$15.9 \pm 8.3\%$ (4)] and reached $85.6 \pm 9.8\%$ (4) after 3.5 min. The incorporation of valine into protein, expressed as a percentage of the control value, then fell exponentially to a final value about 30% below that of the control after 30 min.

The incorporation of label into protein over a period of 3.5 min was measured at a variety of different stimulating frequencies. Incorporation was not increased significantly at 5 pulses/s, but rose by 24.8% at 10 pulses/s, by 50% at 20 pulses/s and by 85.6% at 100 pulses/s. The inhibition of incorporation observed after 15 min at 100 pulses/s was not seen after stimulation for 15 min at 20 pulses/s. Electrical stimulation (100 pulses/s) had no effect on the rate of incorporation of label into the protein of homogenates of superficial layers of guinea-pig cerebral cortex.

Tissue fractionation: purity of the collected fractions. The distribution of protein between the fractions showed that by far the greatest amount was recovered in the mitochondrial fraction; the percentages of the total homogenate protein

Fig. 2. Percentage change in the incorporation of [^{14}C]-valine into protein of chopped cerebral cortex produced by electrical stimulation. Labelled valine was added and electrical stimulation began at zero time. Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four to six experiments.

recovered in the various subcellular fractions were: nuclear, $8.0 \pm 0.4\%$ (30); mitochondrial, $70.4 \pm 1.4\%$ (30); microsomal, $17.4 \pm 0.7\%$ (30); supernatant, $8.8 \pm 0.4\%$ (30).

Analysis of the DNA content and succinate dehydrogenase activity of the fraction indicated that the nuclear and mitochondrial fractions were greatly enriched with nuclei and mitochondria respectively, although some cross-contamination occurred (Fig. 3). These conclusions were confirmed by electron microscopy.

Effect of electrical stimulation on the incorporation of [^{14}C]valine into proteins of various cell fractions. (a) Nuclear fraction. The incorporation of [^{14}C]valine into proteins of the nuclear fraction was much higher than in any other fraction studied (see also Austin & Morgan, 1967) and was sigmoidal in nature. A lag in the incorporation of label into protein also occurred in unfractionated tissue. Electrical stimulation at a frequency of 100 pulses/s initially increased the rate of incorporation of label into protein of the nuclear fraction. However, after 7 min there was no significant change in the specific radioactivity of the protein of the nuclear fraction from electrically stimulated slices, and this resulted

Fig. 3. Distribution of (a) DNA and (b) succinate dehydrogenase between subcellular fractions isolated from chopped cerebral cortex. The DNA values represent the means of one determination on the pooled fractions from four fractionations: □, % of total DNA; ■, μg of DNA/mg of protein. The succinate dehydrogenase values are the means $\pm$ S.E.M. (represented by vertical bars) of ten experiments: □, total activity; ■, activity/mg of protein.

in an inhibition of incorporation compared with the control (Fig. 4a). Thus 3.5 min after adding the label and applying electrical pulses the incorporation of [^{14}C]valine into protein of the nuclear fraction was 1188 ± 187 (2) d.p.m./mg of protein compared with 593 ± 52 (2) d.p.m./mg of protein in the control, whereas after 30 min it was 2322 ± 178 (2) and 5072 ± 325 (2) d.p.m./mg of protein in the stimulated and control tissue respectively.

(b) Mitochondrial fraction. The rate of increase in the specific radioactivity of proteins from the mitochondrial fraction of control tissue was about one-sixth of that observed in the nuclear fraction. As with the nuclear fraction, the incorporation of label showed sigmoidal kinetics, indicating an initially low rate of incorporation. Electrical stimulation had no effect on the rate of incorporation for the first 3.5 min. After 7 min the specific radioactivity of the mitochondrial fraction protein from stimulated tissue ceased to change, although that of the control continued to rise. Consequently, 30 min after adding the label and applying pulses the incorporation into protein of the mitochondrial fraction was 253 ± 23 (2) d.p.m./mg of protein

Fig. 4. Effect of electrical stimulation on the incorporation of [^{14}C]valine into (a) nuclear, (b) mitochondrial, (c) microsomal and (d) supernatant fractions of chopped cerebral cortex. The fractions were isolated as described in the Methods section. Electrical stimulation began from the time of adding the labelled valine. Δ , Control; $\blacktriangle$, electrically stimulated (18 V; 100 pulses/s; time-constant 0.4 ms). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of two experiments on the pooled tissue from three separate incubations.

compared with 843 ± 34 (2) d.p.m./mg of protein in the control (Fig. 4b).

(c) Microsomal fraction. Incorporation of label into proteins from control tissue was almost linear over the 30 min incubation period and about one-fifth of that found in the nuclear fraction. The incorporation showed no lag phase, and electrical stimulation of the tissue did not effect incorporation significantly until the tissue had been incubated for 30 min, when the specific radioactivity of the protein precipitated was 1280 ± 60 (2) d.p.m./mg of protein in the control compared with 1020 ± 52 (2) d.p.m./mg of protein in the electrically stimulated tissue (Fig. 4c).

(d) Supernatant fraction. The pattern of incorporation of label into the supernatant proteins from control tissue was similar to, although two- to three-fold higher than, that found in the microsomal

fraction. Electrical stimulation of the tissue progressively decreased incorporation compared with the controls (Fig. 4d), so that at 30 min the values for the control and electrically stimulated tissue were 3199 ± 290 (2) and 1553 ± 72 (2) d.p.m./mg of protein respectively.

These results indicate that electrical stimulation inhibited the rate of incorporation of [^{14}C]valine into protein in all the subcellular fractions examined, although with the nuclear fraction the inhibition was preceded by a brief period of enhanced incorporation. Since the degree of inhibition varied from fraction to fraction, it follows that electrical stimulation changed the contribution made by each fraction in the total labelled proteins of the homogenate. Thus at all time-intervals electrical stimulation increased the contribution made by the nuclear fraction to the total radioactivity of the

homogenate protein, whereas it had the opposite effect on the contribution made by the protein of the mitochondrial fraction. At all time-intervals at least 25% of the label incorporated into the protein

of the control tissue was accounted for by protein from the nuclear fraction.

Effect of electrical stimulation on the Na⁺ and K⁺ contents of the slices. After a 50min period of pre-incubation in 164mm-sodium chloride, the Na⁺ and K⁺ contents of the slices remained essentially constant for a further 30min at about 22 and 60 μ equiv./h per g wet wt. respectively. The application of pulses (100/s) throughout this 30 min period led to a rapid rise in the intracellular content of Na⁺ and a rapid fall in that of K⁺ (Figs. 5 and 6). Thus after 15min of stimulation the intracellular concentrations of Na⁺ and K⁺ were 56.5 ± 3.7 (4) and 41.3 ± 7.3 (4) μ equiv./h per g wet wt. respectively.

Lowering of the external Na⁺ concentration diminished the transmembrane concentration gradient of Na⁺ at rest and diminished the effect of electrical stimulation on the internal Na⁺ and K⁺ concentrations (Table 1).

Fig. 5. Effect of electrical stimulation on the internal Na⁺ concentration of chopped cerebral cortex. Δ , Control; $\blacktriangle$, electrically stimulated (18V; 100 pulses/s; time-constant 0.4ms). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four experiments.

Fig. 6. Action of electrical stimulation on the internal K⁺ concentration of chopped cerebral cortex. Δ , Control; $\blacktriangle$, electrically stimulated (18V; 100 pulses/s; time-constant 0.4ms). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four experiments.

Fig. 7. Concentrations of ATP in chopped cerebral cortex after preparation, preincubation and electrical stimulation. Preparation was achieved in approx. 10min after which the tissue was oxygenated in saline at 30–37°C for 5min. Then the tissue was preincubated for 20min at 37°C before the control respiratory rate was measured for 30min. Finally, 65min after death, electrical stimulation began. Δ , Control; $\blacktriangle$, electrically stimulated (18V; 100 pulses/s; time constant 0.4ms). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of three to six experiments.

Table 1. *Effect of the external Na⁺ concentration ([Na⁺]_o) on the intracellular concentrations of Na⁺ ([Na⁺]_i) and K⁺ ([K⁺]_i) at rest after 30min of electrical stimulation*

Conditions were as described in the Methods section; electrical stimulation was 18V, at 100 pulses/s and time-constant 0.4ms. Each value (μ equiv./g wet wt.) is the mean of four experiments.

[Na ⁺] _o ...	40mm		80mm		164mm	
	[Na ⁺] _i	[K ⁺] _i	[Na ⁺] _i	[K ⁺] _i	[Na ⁺] _i	[K ⁺] _i
Control	31.3 $\pm$ 3.9	40.1 $\pm$ 3.4	28.3 $\pm$ 4.2	53.6 $\pm$ 6.7	24.5 $\pm$ 4.4	64.9 $\pm$ 3.2
Electrically stimulated	40.3 $\pm$ 5.4	35.2 $\pm$ 2.1	58.6 $\pm$ 6.7	40.1 $\pm$ 3.7	69.8 $\pm$ 3.5	40.6 $\pm$ 5.0

Fig. 8. Concentration of creatine phosphate in chopped cerebral cortex after preparation, preincubation and electrical stimulation. Conditions were as described in Fig. 7. Δ , Control; $\blacktriangle$, electrically stimulated (18 V; 100 pulses/s; time-constant 0.4 ms). Each point is the mean $\pm$ s.e.m. (represented by vertical bars) of three to six experiments.

ATP and creatine phosphate contents of the tissue. The ATP content of the tissue rose from a value of 0.67 ± 0.06 (3) $\mu\text{mol/g}$ wet wt. immediately after preparation to 1.19 ± 0.12 (3) $\mu\text{mol/g}$ wet wt. after incubation for 50 min at 37°C . During the next 60 min it showed a small, but scarcely significant, fall (Fig. 7). The creatine phosphate content rose during the first 50 min of incubation from 0.67 ± 0.11 (3) to 1.41 ± 0.15 (3) $\mu\text{mol/g}$ wet wt. Thereafter it remained constant for about 30 min before falling after a further 30 min to 0.92 ± 0.11 (3) $\mu\text{mol/g}$ wet wt. (Fig. 8).

At the end of the preincubation the continuous application of electrical pulses (100/s) caused a rapid fall in the concentrations of both ATP and creatine phosphate, so that after 7 min they were 71.5 and 79.1% respectively of the control values. Half an hour after beginning electrical stimulation the ATP and creatine phosphate contents had fallen to 64 and 58.6% respectively of the control values (Figs. 7 and 8).

DISCUSSION

The rate of protein synthesis by cortical slices from guinea-pig brain has been found to exceed that of liver slices (Mase, Takahashi & Ogata, 1962), and experiments carried out *in vivo* (Gaitonde & Richter, 1956; Lajtha, 1964) have shown that brain proteins have a relatively high rate of turnover. How this large capacity for protein synthesis is related to the overall functioning of the central nervous system is not understood, although some of it is probably concerned with maintaining the secretory activity of the neurones. The extent to which the rate of protein synthesis by the brain is modified by func-

tional activity has been the subject of several investigations. Bennett, Diamond, Krech & Rosenzweig (1964) found that prolonged exposure of rats to a 'complex' environment increased the weight and protein content of the cerebral cortex, whereas other workers have shown that the rate of incorporation of labelled amino acids into brain proteins is enhanced by activities such as swimming (Krawczynski, 1961) or enforced treadmill exercise (Altman, 1963). In this context it is noteworthy that Appel, Davis & Scott (1967) have found that exposure of dark-adapted rats to light increased the brain's capacity for protein synthesis and its content of polyribosomes. The liver was not affected similarly under these conditions. However, enforced muscular activity is also reported to result in the loss of protein from the cell bodies of some neurones (Hyden, 1943; Gomarito, 1954).

In contrast with results obtained by using physiological stimuli, convulsive activity induced by electroshock or by drug treatment appears to cause a fall in cerebral protein synthesis and a disaggregation of brain polyribosomes (Sikdar & Gosh, 1964; Vesco & Guiditta, 1968; Kolousek & Babicky, 1961). Further, spreading cortical depression after the topical application of potassium chloride is characterized by a decrease in the incorporation of labelled amino acid into protein (Bennett & Edelman, 1969).

Few systems have been described in which protein synthesis by isolated neural tissue can be studied at rest and during periods of activity. Stimulation of the giant axon of squids leads to an increased incorporation into protein of amino acids introduced into the axoplasm by micro-injection (Fischer, Cellino, Gariglio & Tellez-Nagel, 1968). Before the present study the effect of electrical stimulation on protein synthesis by slices from cerebral cortex has been the subject of only one investigation (Orrego & Lipmann, 1967). In this study the application of sinusoidal electrical pulses (60 Hz) led to a marked inhibition in the incorporation of [^{14}C]valine into protein over periods of from 15 to 30 min. It is probable that the method of stimulation adopted in our experiments and in those of Orrego & Lipmann (1967) excited all the neural elements in the tissue, rather than triggering the complex patterns of electrical activity normally associated with the cerebral cortex. Thus the metabolic changes occurring under the present conditions could possibly be likened to those occurring *in vivo* during periods of convulsive activity.

Under the conditions described in this paper electrical stimulation at 100 pulses/s had a biphasic effect on the incorporation of [^{14}C]valine into protein. The enhancement of incorporation observed during the first 5 min was replaced by an inhibition at later times. The inhibition of incorporation noted

after 15 min, although somewhat smaller, is in general agreement with that observed by Orrego & Lipmann (1967).

Despite the rapid fall in the intracellular concentrations of ATP, creatine phosphate and K^+ there was a transient rise in the incorporation of labelled valine into protein on electrical stimulation. This is probably accounted for by the initial increase in the intracellular content of valine in electrically stimulated tissue compared with the controls (Jones & Banks, 1970).

The reasons for the fall in incorporation after 15 min are almost certainly many and complex. One factor could well be the fall in the concentrations of ATP and creatine, which reached their maximum extents after about 15 min. The decrease in the ATP and creatine phosphate concentrations probably reflects the increased work load placed on the sodium pump after the increased influx of Na^+ and efflux of K^+ and the consequent elevation of the internal Na^+ concentration and the diminution of the internal K^+ concentration. The fall in the ATP content and the consequent rise in the ADP content both act as positive-feedback signals to stimulate glycolysis and oxidative phosphorylation. These aspects of the changes induced by electrical stimulation are reflected in the elevated rates of lactate production and oxygen consumption.

The importance of Na^+ influx in regulating respiration is also shown by the respiratory rates of slices incubated in media having a low Na^+ concentration (Table 1). It would appear that the maintenance of the transmembrane Na^+ gradient accounts for about 30% of the total respiration during rest and about 70% during periods of electrical stimulation.

It is also possible that the K^+ loss accompanying electrical stimulation could lead to an impairment of protein synthesis, since several workers have shown that protein synthesis in other systems is dependent on the K^+ concentration in the cells or cell-free systems used (Sachs, 1957; Lubin, 1964; Conway, 1964; Coleman, 1969). Further, as the internal Na^+ concentration rises the stability and rate of production of aminoacyl-tRNA species might be lowered, as shown for *Escherichia coli* tRNA by Smith (1969).

The dependence of amino acid uptake on the transmembrane ion gradient is well characterized (Crane, 1965, 1967; Christensen, 1967; Stein, 1968), and evidence is presented in the following paper (Jones & Banks, 1970) that, whereas [^{14}C]valine uptake is at first increased by electrical stimulation, at later time-intervals there is a net loss of label from the tissue so that the concentration of labelled valine in the slices falls below that in the control. This net loss is probably related to the diminution in the transmembrane gradients of Na^+ and K^+ . Such a

fall in the intracellular [^{14}C]valine content would obviously decrease the availability of label for incorporation into protein compared with the control tissue.

In addition to these closely related factors tending to diminish protein synthesis, electrical stimulation is also reported to alter the state of aggregation of polyribosomes (Vesco & Guiditta, 1968) and to increase protease activity (Ungar, Ascheim, Psychoyos & Romano, 1957), although the mechanisms of these changes are not understood.

The nuclear fraction from stimulated slices always contained a higher percentage of the total radioactive protein in the tissue than did nuclei from control tissue. This suggests that the incorporation of label into the nuclear fraction is less sensitive to the inhibitory action of electrical stimulation than is incorporation of label into the other fractions studied. This may be a consequence of the nuclear pool of ATP being little affected by the increased activity of the sodium pump.

A further possibility is that the increase in the intracellular concentration of Na^+ may facilitate [^{14}C]valine uptake by the nuclei (Allfrey, Meudt, Hopkins & Mirsky, 1961; Navon & Lajtha, 1969). The possibility that the amino acid uptake by nuclei may be regulated by the intracellular concentration of Na^+ has been suggested in a preliminary communication (Jones & Banks, 1969b).

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