The Effect of Electrical Stimulation and Ouabain on the Uptake and Efflux of L-[U-¹⁴C]Valine in Chopped Tissue from Guinea-Pig Cerebral Cortex

By C. T. JONES AND P. BANKS

Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

(Received 30 January 1970)

1. Chopped tissue from guinea-pig cerebral cortex carried out an energy-dependent accumulation of L-[¹⁴C]valine. 2. The uptake was dependent on the extracellular concentration of Na⁺ and was markedly inhibited by ouabain (20 μ M). The extent of the inhibition of uptake by ouabain was also Na⁺-dependent. 3. The accumulation of labelled valine was not directly dependent on the ATP and creatine phosphate contents of the slices. 4. Electrical stimulation increased the rate of [¹⁴C]-valine uptake at first but ultimately led to a net loss of the label so that the amount of label present in the tissue was lower than in the controls. 5. The rate of loss of label during prolonged stimulation was dependent on the extracellular concentration of Na⁺. 6. The efflux of labelled valine from slices preloaded at 164 mM-Na⁺ was studied at 164, 80 and 40 mM-Na⁺ with and without electrical stimulation or ouabain. 7. Lowering the Na⁺ concentration or adding ouabain increased the rate of efflux. 8. Electrical stimulation had little effect on the rate of efflux at first but ultimately led to a more complete loss of label from the tissue than occurred in the control. A kinetic analysis of the efflux curves was attempted.

Electrical stimulation was found to have a biphasic effect on the incorporation of L-[¹⁴C]valine into the protein of chopped tissue from guinea-pig cerebral cortex (Jones & Banks, 1970); incorporation was first enhanced but later it was inhibited. Since both the rate of uptake and the intracellular concentration of labelled valine are likely to influence the rate of incorporation, it was decided to investigate the effect of electrical stimulation on amino acid uptake and efflux. Because incubation with ouabain, like electrical stimulation, increases the intracellular concentration of Na⁺ and decreases the intracellular concentration of K⁺, its effects on amino acid uptake and efflux were also studied.

METHODS

[¹⁴C]Valine uptake into chopped tissue. Chopped tissue from guinea-pig cerebral cortex was prepared, preincubated and incubated, and electrically stimulated as described by Jones & Banks (1970); however, at the beginning of the incubation 0.1% inulin was added to the Krebs phosphate saline. At the end of the incubation the chopped tissue was rapidly filtered under vacuum on Whatman no. 40 filter papers supported on micro Hirsch funnels. The tissue was removed from the filter paper with a stainless-steel spatula, suspended at 0°C in 3 ml of 5% (w/v) trichloroacetic acid and homogenized. The homogenate was spun at 1500 g for 5 min, the supernatant

was retained and the precipitate was washed with a further 3 ml of 5% trichloroacetic acid. Radioactivity in the pooled supernatant and in the filtered medium was determined with a Nuclear-Chicago liquid scintillation counter. Inulin in the supernatant and in the filtered medium was determined as described by Varon & McIlwain (1961). It is assumed that the inulin in the tissue was restricted to the extracellular space and therefore that the determination of inulin in the tissue gives a value for the quantity of extracellular space and adhering medium. From this, together with the quantity of label in the suspending saline and the total acid-soluble radioactivity of the tissue, the intracellular content of [¹⁴C]-valine was calculated. Determination of protein (Lowry, Rosebrough, Farr & Randall, 1951) allowed the quantity of tissue homogenized in the preparation of each extract to be calculated, since 1 g of tissue contained 82.8 $\pm$ 8.8 (18) mg of protein.

Two-dimensional chromatography on silica gel (Brenner, Niederwieser & Pataki, 1965) of the acid-soluble radioactivity recovered from tissue incubated with [¹⁴C]valine for 60 min gave a radioautograph indistinguishable from that given by standard [¹⁴C]valine, suggesting that no significant formation of acid-soluble metabolites of [¹⁴C]valine occurred during the incubation.

The uptake of [¹⁴C]valine into the tissue was expressed in terms of the concentration ratio, defined as:

$$\frac{\mu\text{Ci of intracellular L-[}^{14}\text{C]valine/g of initial tissue wet wt.}}{\mu\text{Ci of L-[}^{14}\text{C]valine/ml of saline}}$$

It should be noted that this concentration ratio is based on the intracellular [^{14}C]valine/g of tissue and not on the labelled valine/ml of intracellular water. Consequently the concentration ratio as defined above will be lower than the 'true' concentration ratio (see the Appendix) defined as:

$$\frac{\mu\text{Ci of L-}[^{14}\text{C}]\text{valine/ml of intracellular water}}{\mu\text{Ci of L-}[^{14}\text{C}]\text{valine/ml of saline}}$$

In this work no estimate of cell swelling could be made; this will not affect the interpretation of the results from the control and stimulated tissues as electrical stimulation does not lead to tissue swelling (Varon & McIlwain, 1961). However, in the case of tissue treated with ouabain the occurrence of swelling may cause the true concentration ratios to be lower than is suggested by the values quoted in this paper.

To obtain the rate constants for valine efflux by using the equation discussed in the Appendix it was necessary to know the amounts of [^{14}C]valine in the intracellular and extracellular compartments. This required a knowledge of the true concentration ratio defined above. Consequently a value for the inulin space of the chopped tissue not involving a contribution from adhering medium was required. To obtain this value, chopped tissue was preincubated for 50 min and then incubated for a further 15 min. Inulin was present from the beginning of the preincubation. The conditions of incubation were identical with those used to determine the concentration ratio after 15 min of incubation in the presence of [^{14}C]valine. At the end of the incubation the chopped tissue was spun for 15 s at 500 g and the resulting pellet was spread on dry filter paper. The tissue was immediately removed from the paper with a stainless-steel spatula, homogenized in 3 ml of 5% trichloroacetic acid and the supernatants obtained after spinning and a further washing with 3 ml of 5% trichloroacetic acid, as described above, were pooled. The inulin in the extract and saline was determined as described by Varon & McIlwain (1961) and gave an inulin space of $62.4 \pm 1.9\%$ (12) of the total wet weight of the chopped tissue.

The zero-time uptake of [^{14}C]valine by the chopped tissue determined after the simultaneous addition of acid, labelled valine and tissue to the micro Hirsch funnels, was $5.3 \times 10^4 \pm 1.9 \times 10^4$ (4) d.p.m./g wet wt. of tissue equivalent to a concentration ratio of 0.09 ± 0.03 (4).

Efflux of [^{14}C]valine from preloaded chopped tissue. After preincubation, chopped tissue was preloaded with label by incubation for 15 min at 37°C in 3.5 ml of oxygenated Krebs phosphate saline containing $1 \mu\text{Ci}$ of L- $[^{14}\text{C}]$ valine. At the end of the incubation the chopped tissue was centrifuged for 15 s at 500 g and resuspended in the centrifuge tubes, by using a vortex mixer, with 3.5 ml of saline. Immediately after mixing, a $10 \mu\text{l}$ sample of the saline was taken and its radioactivity was determined. This gave the zero-time concentration of label in the medium. After the zero-time sample had been taken the tissue suspension was immediately tipped into a 25 ml conical flask or into a Warburg flask fitted with concentric electrodes (see Jones & Banks, 1970) and incubated with oxygenation and shaking at 37°C . A $10 \mu\text{l}$ sample of the medium was taken at various times during the incubation and the radioactivity in the samples was determined.

ATP and creatine phosphate. ATP and creatine phosphate were determined as described by Jones & Banks (1970).

RESULTS

Uptake of L- $[^{14}\text{C}]$ valine by control tissue. At a Na^+ concentration of 164 mM the uptake of label by the chopped tissue rose rapidly to achieve a concentration ratio (see the Methods section for definition) of 3.66 ± 0.28 (4) at 15 min and 4.52 ± 0.32 (4) at 30 min. During the next 30 min it fell to a value of 3.55 ± 0.42 (4) (Fig. 1).

Energy-dependence of the uptake of L- $[^{14}\text{C}]$ valine. Preincubation with 2,4-dinitrophenol (0.2 mM) and sodium fluoride (5 mM) for 50 min before the addition of label greatly decreased the ability of the tissue to take up [^{14}C]valine. When dinitrophenol and sodium fluoride were added at the same time as the label uptake proceeded normally for 3.5 min. Thereafter a net loss of labelled valine occurred, so that after 15 min the concentration ratio for

Fig. 1. Effect of electrical stimulation, ouabain and 2,4-dinitrophenol plus NaF on the uptake of [^{14}C]valine by chopped tissue from cerebral cortex. The incubations were carried out, as described in the Methods section, in saline containing 164 mM- Na^+ . (○), Control; (●), electrical stimulation (18 V; 100 pulses/s; time-constant, 0.4 ms) after the addition of [^{14}C]valine; (■), ouabain (20 μM) added together with radioactive valine; (△), dinitrophenol (0.2 mM) and NaF (5 mM) added at the same time as labelled valine; (▲), dinitrophenol (0.2 mM) and NaF (5 mM) added 50 min before the addition of [^{14}C]valine. Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four experiments.

Fig. 2. Effect of electrical stimulation and ouabain on the uptake of $[^{14}\text{C}]$ valine by chopped tissue from cerebral cortex suspended in saline containing 80mm-Na^+ . (○), Control; (●), electrically stimulated (18V ; 100 pulses/s ; time-constant, 0.4ms); (△), ouabain ($20\mu\text{M}$). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four experiments.

Fig. 3. Effect of electrical stimulation and ouabain on the uptake of $[^{14}\text{C}]$ valine by chopped tissue from cerebral cortex suspended in saline containing 40mm-Na^+ . (○), Control; (●), electrically stimulated (18V ; 100 pulses/s ; time-constant, 0.4ms); (△), ouabain ($20\mu\text{M}$). Each point is the mean $\pm$ S.E.M. (represented by vertical bars) of four experiments.

$[^{14}\text{C}]$ valine did not differ significantly from that observed when the tissue was preincubated with dinitrophenol and sodium fluoride (Fig. 1).

These results demonstrate that an energy-dependent uptake of valine occurred in the preparation. Preincubation with dinitrophenol and sodium fluoride decreased the supply of energy

Fig. 4. Appearance of the Na^+ -dependence of $[^{14}\text{C}]$ valine uptake with time. A series of values for control uptakes at different times taken from Figs. 1, 2 and 3 are shown. (■), 1 min; (●), 3.5 min; (○), 7 min; (▲), 15 min; (△), 30 min.

available at the time of addition of the label; after simultaneous addition of the label and inhibitors, accumulation proceeded normally for a short period before the tissue was depleted of high-energy phosphates.

Na^+ -dependence of the uptake of $\text{L-}[^{14}\text{C}]$ valine. With Na^+ concentrations of 40 and 80mm in the incubation medium the initial rates of accumulation of label were identical although they were approx. 45% lower than at 164mm-Na^+ . After 7min no further accumulation of label occurred at 40mm-Na^+ although it continued to increase at 80 and 164mm-Na^+ ; thus 30min after adding the label the concentration ratios at 40 , 80 and 164mm-Na^+ were 1.85 ± 0.5 (4), 2.76 ± 0.09 (4) and 4.52 ± 0.32 (4) respectively (Figs. 2 and 3). That is, the Na^+ -dependence of the $[^{14}\text{C}]$ valine uptake became more evident as time progressed (Fig. 4).

It seemed possible that the failure to observe a clearcut difference between uptake at 40 and 80mm-Na^+ at short time intervals was a consequence of the Na^+ -dependent accumulations being masked by a Na^+ -independent uptake of label. Such a Na^+ -independent uptake might be related to the equilibration of label between the tissue and the incubating medium observed at 164mm-Na^+ , in tissue preincubated with dinitrophenol and sodium fluoride (Fig. 1), and which was complete after about 7min .

Effect of electrical stimulation on the uptake of $\text{L-}[^{14}\text{C}]$ valine. Electrical stimulation at a frequency of 100 pulses/s (18V ; time-constant, 0.4ms) from the time of adding the label increased the rate of uptake of $[^{14}\text{C}]$ valine by $73.1 \pm 12.3\%$ (4), in tissue incubated with 164mm-Na^+ . After 3.5min the concentration ratio in the stimulated tissue was 3.29 ± 0.46 (4) compared with 2.16 ± 0.12 (4) in the controls. This difference in uptake was maintained

for a further 3.5 min, after which the stimulated tissue began to lose some of its accumulated [^{14}C]valine, whereas the control tissue continued to take up label. Thus 30 min after adding the label the concentration ratios in the control and stimulated tissue were 4.52 ± 0.32 (4) and 1.69 ± 0.08 (4) respectively (Fig. 1).

At 80 mM- Na^+ , electrical stimulation increased the initial rate of uptake of label by $38.4 \pm 10.1\%$ (4) but had no significant effect on the initial rate of uptake at 40 mM- Na^+ . The pattern of uptake at 40 and 80 mM- Na^+ was similar to that at 164 mM- Na^+ , in that the initial uptake of [^{14}C]valine gave way to a loss of label at later times, although the magnitude of the changes diminished as the concentration of Na^+ was lowered (Figs. 2 and 3).

These results indicate that the initial rate of uptake of [^{14}C]valine is increased by electrical stimulation and that the magnitude of the increased rate of uptake is dependent on the Na^+ concentration of the incubation medium. The initial increase in uptake gives way to a net loss of label as time progresses. The first-order rate constant for the net loss of label is also dependent on the external concentration of Na^+ (Fig. 5).

Effect of ouabain on the uptake of L-[^{14}C]valine. Ouabain (20 μM) inhibited the uptake of the label at all concentrations of Na^+ tested. However at 40 mM- Na^+ the extent of the inhibition after 15 min was only $59.9 \pm 1.7\%$ (4) compared with $90.9 \pm 0.24\%$ (4) and $86.7 \pm 4.0\%$ (4) at 80 and 164 mM- Na^+ respectively. That is, valine uptake became less sensitive to inhibition by ouabain as the

external concentration of Na^+ was lowered. Such a fall in sensitivity might be expected if the uptake of valine consists of at least two components; one ouabain-sensitive, probably driven by the transmembrane concentration gradient of Na^+ , and another a passive component, which may increase its relative contribution to the total uptake as the external concentration of Na^+ falls. The possibility that the passive component might also be sensitive to ouabain cannot be ruled out since ouabain (20 μM) is able to decrease the uptake of label by tissue preincubated for 50 min with dinitrophenol and sodium fluoride by about 50% after 3.5 min.

At all three concentrations of Na^+ used, treatment with ouabain eventually led to a loss of label that had been accumulated during the first few minutes after the addition of [^{14}C]valine and the drug. Thus after 30 min the concentration ratios had fallen from near unity to 0.42 ± 0.04 (4), 0.14 ± 0.01 (4) and 0.59 ± 0.03 (4) at 164, 80 and 40 mM- Na^+ respectively. This indicates that in the presence of ouabain there is a net loss of free valine from the tissue.

Independence of ATP concentration and [^{14}C]valine uptake. At 15 min after adding labelled valine to chopped tissue incubated at 164, 80 and 40 mM- Na^+ , the concentration ratios were 3.66 ± 0.28 (4), 2.13 ± 0.03 (4) and 1.71 ± 0.08 (4) respectively, although the ATP contents of the tissues did not differ significantly (Table 1). Further, although 20 μM -ouabain caused a profound inhibition of valine uptake after 15 min in 40 and 80 mM- Na^+ , it had no significant effect on the ATP contents of the tissues incubated in those concentrations of Na^+ (Table 1). At 164 mM- Na^+ 20 μM -ouabain inhibited valine uptake and decreased the tissue content of ATP and creatine phosphate.

Efflux of [^{14}C]valine from preloaded slices. The loss of labelled valine from preloaded slices took place rapidly and appeared to follow an exponential time-course. Usually such washout curves are reduced to two exponential components on the assumption that the amount of label remaining in the tissue at time t , (Yt), is given by the expression:

$$Yt = A_0 e^{-k_1 t} + B_0 e^{-k_2 t} \quad (1)$$

(Winegrad & Shanes, 1962; Ames & Nesbitt, 1966; Treherne, 1966; Hertz, 1968). However, Huxley (1960) (see also Schoffeniels, Hagemeijer & Rorive, 1966) has pointed out that this method is mathematically unsound. Consequently an attempt was made to represent the efflux curves by an equation of the form:

$$C = C_0 + A_0(1 - e^{-k_1 t}) +$$

$$B_0[(1 - e^{-k_2 t}) - \frac{A_0/B_0}{1 - k_2/k_1} (e^{-k_2 t} - e^{-k_1 t})] \quad (2)$$

Fig. 5. Na^+ -dependence of the loss of [^{14}C]valine from chopped tissue from cerebral cortex, which became apparent approx. 7 min after the simultaneous addition of labelled valine and application of electrical pulses. The efflux of [^{14}C]valine is represented by its first-order rate constant (K_s) and was determined by the least squares method from the values for [^{14}C]valine content shown in Figs. 1, 2 and 3 from 7 min onwards.

Table 1. *Absence of a correlation between [^{14}C]valine accumulation and high-energy phosphate content of chopped tissue incubated for 15 min at different Na^+ concentrations with and without ouabain.*

The ATP and creatine phosphate concentrations are expressed as $\mu\text{mol/g}$ wet wt. and are the means $\pm$ S.E.M. of three to six experiments. Abbreviations: A_1/A_0 , concentration ratio (see the Methods section); CP, creatine phosphate.

Concn. of NaCl ...	164 mm			80 mm			40 mm		
	ATP	CP	A_1/A_0	ATP	CP	A_1/A_0	ATP	CP	A_1/A_0
Control	1.16	1.18	3.66	1.13	1.38	2.13	1.05	1.19	1.71
	± 0.08	± 0.07	± 0.28	± 0.09	± 0.1	± 0.03	± 0.08	± 0.07	± 0.08
Ouabain-treated (20 μM)	0.71	0.73	0.49	1.01	1.78	0.18	0.99	1.34	0.65
	± 0.05	± 0.16	± 0.1	± 0.04	± 0.11	± 0.01	± 0.05	± 0.1	± 0.01

Table 2. *Kinetic constants, k_1 and k_2 , for the efflux of [^{14}C]valine for chopped tissue at various Na^+ concentrations and during periods of electrical stimulation or treatment with ouabain*

Electrical stimulation was at 18 V, 100 pulses/s and time-constant 0.4 ms. The constants were determined as described in the Appendix, are expressed as min^{-1} and are the means $\pm$ S.E.M. of nine determinations.

Concn. of NaCl ...	164 mm		80 mm		40 mm	
	k_1	k_2	k_1	k_2	k_1	k_2
Control	0.146	2.26	0.281	3.36	0.292	2.94
	± 0.01	± 0.15	± 0.02	± 0.27	± 0.013	± 0.049
Ouabain-treated (20 μM)	0.526	2.41	0.295	2.43	0.34	2.06
	± 0.04	± 0.25	± 0.014	± 0.033	± 0.21	± 0.008
Electrically stimulated	0.175	2.43	0.171	3.14	0.281	1.97
	± 0.013	± 0.17	± 0.008	± 0.23	± 0.008	± 0.085

which describes the amount of C produced with time by the process: $A \xrightarrow{k_1} B \xrightarrow{k_2} C$ (see the Appendix). In the context of the present experiments A_0 represents the amount of [^{14}C]valine in the intracellular space at zero time, B_0 represents the amount of label in the extracellular space of the tissue at zero time and C represents the amount of label appearing in the bulk phase ($C_0 = C$ when $t = 0$). This model assumes that label leaving the intracellular compartment enters the extracellular compartment before entering the bulk phase. The traditional double-exponential treatment is unsatisfactory in part because it makes the opposite assumption, namely that the label enters the bulk phase directly from the intracellular compartment.

The rate constants, k_1 and k_2 , were determined during the first half of the efflux by the graphical method of Swain (1944) as described in the Appendix. The values obtained were essentially constant for any one efflux curve (Table 2) and were used in each case to calculate a theoretical washout curve. A comparison of the theoretically determined and experimentally determined curves (Fig. 6) shows, in all cases, at 164, 80 and 40 mm- Na^+ (values for efflux at 80 and 40 mm- Na^+ are not shown), that the first half of the efflux can be defined by eqn. (2).

In some cases there is also a close fit between the experimental and theoretical curves during the second half of the efflux although in other cases the agreement is poor.

In the control slices k_2 was some 15 times k_1 (Table 2) indicating, as might be expected, that the rate of efflux from the extracellular compartment was very much higher than the rate of efflux from the intracellular compartment. Since the first half of the efflux of [^{14}C]valine can be described by equation (2) it is possible, knowing k_1 and k_2 , to determine the changes taking place in the [^{14}C]valine content of the intracellular and extracellular spaces for at least 4 min after resuspension of the preloaded tissues in unlabelled saline by calculating the 'reduced concentrations' A/A_0 and B/B_0 that are kinetically defined as (see the Appendix):

$$A/A_0 = e^{-k_1 t} \quad (3)$$

and

$$B/B_0 = e^{-k_2 t} + \frac{A_0/B_0}{1 - k_2/k_1} (e^{-k_2 t} - e^{-k_1 t}) \quad (4)$$

Such an analysis shows that after resuspension at 164 mm- Na^+ (Figs. 7 and 8) the amount of labelled valine in the extracellular compartment fell by half within 40 s, whereas more than half the

labelled valine was still present in the intracellular compartment after 4 min. If material lost from compartment *A* did not pass through compartment *B*, the extracellular compartment would have been almost depleted of label after 2 min since in that case B/B_0 would be defined by $e^{-k_2 t}$, which would be 0.01 at 2 min.

After approx. 50% of the previously accumulated labelled valine had been lost from the control tissue the efflux was no longer described by eqn. (2), as the value of k_1 had diminished.

Efflux of L-[14C]valine from preloaded chopped tissue during periods of electrical stimulation or incubation with ouabain at 164 mM-Na⁺. When preloaded tissue was resuspended in saline containing ouabain (20 μ M) and 164 mM-Na⁺ the efflux of [14C]valine from the tissue was much faster than in the absence of ouabain (Fig. 6b). Table 2 shows that this increased rate of efflux was associated with an increase in the value of k_1 whereas k_2 remained unchanged. The effect of this large increase in k_1 is that the extracellular compartment is 'flooded' with [14C]valine from the intracellular compartment (Figs. 7 and 8) and for nearly 1 min after resuspension the extracellular compartment contains more [14C]valine than it did at zero time. Labelled valine then disappears very rapidly from compartment *B*.

After half of the [14C]valine has been lost from ouabain-treated tissue there is again some divergence from eqn. (2) (Fig. 6b). However, the theoretical curve fits the efflux curve much better than it does for the control tissue, and the divergence is in a region where the error in the determination of the percentage of the material remaining in the tissue is greatest.

When chopped tissue preloaded with [14C]valine was subjected to electrical stimulation (100 pulses/s; 18 V; time constant, 0.4 ms) from the moment of resuspension in unlabelled saline at 164 mM-Na⁺, the value of k_1 was increased by only a small amount and k_2 remained essentially unchanged (Table 2, Fig. 6c). Hence, during the first half of the efflux electrical stimulation had little effect on the shape of the washout curves. However, during the second

Fig. 6. Efflux of [14C]valine from preloaded cerebral-cortex tissue in the presence of ouabain or during electrical stimulation at 164 mM-Na⁺. —, Experimentally determined efflux curve; ----, theoretically determined efflux curve (see the Appendix) for $A \rightarrow B \rightarrow C$. (a) Control tissue: ----, theoretically determined efflux curve for $A \rightleftharpoons B \rightarrow C$ (see the Appendix and text); (b) electrically stimulated tissue (18 V; 100 pulses/s; time-constant, 0.4 ms); (c) ouabain (20 μ M)-treated tissue. Each point on the experimental curves is the mean from three experiments.

Fig. 7. Disappearance of [^{14}C]valine from the intracellular compartments of preloaded chopped cerebral-cortex tissue in the presence of ouabain or during electrical stimulation at 164mm- Na^+ . The loss over the first 4 min is expressed as the reduced concentration, A/A_0 , as defined by eqn. (3) and was calculated by using the values for k_1 given in Table 2. —, Control; ----, electrically stimulated; ····, ouabain-treated.

Fig. 8. Disappearance of [^{14}C]valine from the extracellular compartments of preloaded chopped cerebral-cortex tissue in the presence of ouabain or during electrical stimulation at 164mm- Na^+ . The loss of label over the first 4 min is expressed as the reduced concentration, B/B_0 , as defined by eqn. (4), and was calculated by using the values for k_1 and k_2 given in Table 2. —, Control; ----, electrically stimulated; ····, ouabain-treated.

half of the efflux there was rather better agreement between the experimental and theoretical washout curves than with the controls. This suggests that during the latter phase of the efflux electrical stimulation increases the value of k_1 compared with the control.

Effect of decreasing the external Na^+ concentration on the efflux of [^{14}C]valine. Decreasing the external Na^+ concentration to 80 and 40mM increased the rate of efflux of labelled valine compared with the control efflux at 164mM- Na^+ . This increase was associated with increases in both k_1 and k_2 (Table 2). Lowering the external Na^+ concentration also resulted in a better agreement between the control and theoretical washout curves than at 164mM- Na^+ . At the lower Na^+ concentrations ouabain had no significant effect on the value of k_1 when compared with the appropriate controls, although the value of k_2 fell slightly. It is of note that the value of k_1 in the ouabain-treated tissue at 164mM- Na^+ is much higher than that obtained for ouabain-treated tissue at either 80 or 40mM- Na^+ . At 80mM- Na^+ electrical stimulation caused a significant decrease in the value of k_1 while k_2 remained unchanged, but no change was apparent at 40mM- Na^+ . The fit between the theoretical and experi-

mental efflux curves for electrically stimulated slices or those treated with ouabain at 80 and 40mM- Na^+ was reasonably good.

DISCUSSION

The primary object of the experiments described in this paper was to discover whether the variations in the incorporation of L-[^{14}C]valine into the protein of guinea-pig chopped brain tissue observed during periods of electrical stimulation (Jones & Banks, 1970) could be related to changes in the intracellular content of labelled valine. The dependence of the rate of incorporation of labelled amino acids into the protein of whole cells on the cellular concentration of the radioactive amino acid has been demonstrated by several workers (Riggs & Walker, 1963; Appel, 1967; Kuchler, 1967). The present results show that the initial ($85.6 \pm 9.8\%$) enhanced incorporation of [^{14}C]valine into protein caused by electrical stimulation at 100 pulses/s correlates well with the initial increase ($73.1 \pm 12.3\%$) in the rate of uptake of labelled amino acid.

The inhibition of incorporation of label into protein which became evident after electrical stimulation for 15min may be caused in part by the tissue content of free [^{14}C]valine falling below that of the control. However, other factors, such

as a fall in the content of ATP and creatine phosphate (Jones & Banks, 1970) and changes in the ionic composition of the cells are likely to be of great importance in lowering the ability of the tissue to incorporate labelled valine into protein.

[^{14}C]Valine uptake. The experiments described in the present paper also provide further confirmation that amino acid uptake by chopped cerebral cortex at low extracellular concentrations of amino acid increases with rising extracellular concentrations of Na^+ (Takagaki, Hirano & Nagata, 1959; Abadom & Scholefield, 1962; Lahiri & Lajtha, 1964; Nakazawa & Quastel, 1968; Margolis & Lajtha, 1968) and that the Na^+ -dependent uptake is inhibited by ouabain (Fox, Thier, Rosenberg & Segal, 1964). Accumulation of labelled valine to give a concentration ratio greater than unity is dependent on the continued supply of energy since it is prevented by preincubation with dinitrophenol and sodium fluoride. These properties suggest that the uptake of valine is an active, energy-dependent, process. However, it is impossible to assess the contribution to uptake that may be made by exchange diffusion. Such a passive process could readily lead to a concentration ratio greater than unity if the intracellular concentration of amino acids able to exchange with valine were greater than the concentration of [^{14}C]valine in the medium (Heinz & Walsh, 1958; Schwartzman, Blair and Segal, 1967a). This passive exchange would be diminished by treatment with metabolic inhibitors such as dinitrophenol and sodium fluoride if they produced a net loss of amino acids from the cells (see Schwartzman, Blair & Segal, 1967b). This possibility cannot be ignored since after the simultaneous addition of dinitrophenol, sodium fluoride and [^{14}C]valine there is an initial uptake of label followed by a rapid loss.

The Na^+ -dependence of the valine accumulation also suggests that an active uptake process is involved. Here too caution must be exercised in interpreting the results since exposure to low extracellular concentrations of Na^+ enhances the efflux of valine and might decrease the amount of label taken up by exchange diffusion, by lowering the intracellular concentration of exchangeable amino acids. It has been claimed, however, that exchange diffusion in Ehrlich ascites cells is not affected by lowering the extracellular concentration of Na^+ or treatment with ouabain (Johnstone & Scholefield, 1965).

In short, it is very difficult, if not impossible, to decide whether uptake is active or passive merely on account of its sensitivity to metabolic inhibitors and its dependence on Na^+ , unless a net increase in the intracellular concentration of the amino acid in question can be demonstrated.

Despite all these qualifications it is quite possible

that the accumulation of [^{14}C]valine observed in the present experiments is an active process driven by the transmembrane concentration gradient of Na^+ , maintained by the operation of the sodium pump (Riggs, Walker & Christensen, 1958; Vidaver, 1964a,b; Crane, 1965, 1967; Christensen, 1967; Eddy, 1968). This view is supported by two observations, namely that lowering the external Na^+ concentration decreases the valine uptake without altering the ATP content of the tissue and that at 40 and 80 mm- Na^+ ouabain inhibits uptake without diminishing the ATP content of the slices. Further, the fall in the sensitivity of the uptake of [^{14}C]valine to inhibition by ouabain as the Na^+ concentration of the medium was lowered is in general agreement with such a hypothesis.

Electrical stimulation initially increases the rate of uptake of [^{14}C]valine and the extent of the enhancement is greater at an extracellular Na^+ concentration of 164 mm than at 40 mm. This observation may indicate that some of the increased Na^+ influx occurring during electrical stimulation, which would be greater at 164 mm- Na^+ than at 40 mm- Na^+ , is directly coupled with an increased active uptake of [^{14}C]valine (see Schafer & Jacquez, 1967). On the other hand it may merely be that electrical stimulation leads to a general increase in permeability of the membrane and so facilitates exchange diffusion. The dependence of the enhanced uptake on the Na^+ concentration would, on this basis, be a reflexion of the fact that nervous tissue becomes less excitable as the external Na^+ concentration is decreased (see Tasaki, 1962).

The initial uptake of label that occurs during periods of electrical stimulation gives way to a progressive net loss of label from the tissue, the rate of which is higher the greater the external Na^+ concentration. This loss may be a consequence of the increase in the intracellular concentration of Na^+ that occurs during electrical stimulation (Jones & Banks, 1970) and decreases the transmembrane concentration gradient for Na^+ . On the basis of Christensen's hypothesis (Riggs *et al.* 1958; Christensen, 1967) such a decrease in the transmembrane concentration of Na^+ would decrease the ability of the tissue to retain valine and may thus lead to a loss of some of the [^{14}C]valine accumulated a little earlier, when the gradient was higher.

[^{14}C]Valine efflux. The model adopted to describe the efflux of [^{14}C]valine from preloaded slices ignores the possibility that label could be taken up by the cells from the extracellular space. This omission might be thought to be responsible for the failure of the model to provide a perfect description of the experimental efflux curve. However, there is some evidence that the failure of the model does not lie in the fact that it substitutes a simple efflux

for what is really an unbalanced exchange of label between the intra- and extra-cellular compartments. In the presence of ouabain at 164mm- Na^+ the re-uptake of label is greatly inhibited and agreement between the experimental and theoretical curves is quite close. Under these conditions a maximum value for k_1 is obtained and it may be reasonable to assume that this represents the true constant for efflux, undiminished by re-uptake. Now at equilibrium the 'true concentration ratio' for distribution of [^{14}C]valine between the intra- and extra-cellular compartments is about 12. In other words the rate constant for uptake of label (k_{21}) must be 12 times that for efflux (k_{12}) from the cells. Hence if k_{12} can be obtained from the efflux curve in the presence of ouabain at 164mm- Na^+ (0.526min $^{-1}$, Table 2) then $k_{21} = 12k_{12}$. Having thus obtained the values of k_{12} and k_{21} it is possible to calculate a theoretical control washout curve (see the Appendix) at 164mm- Na^+ based on a model of the type:

where $k_{23} = k_2$ (see the Appendix). When this was done it was found that there was no significant difference between the theoretical curves obtained with the two models (Fig. 6a). It would appear therefore that the deviation between the experimental and theoretical curves does not lie in their failure to take into account re-uptake of label. The most likely reason for the discrepancy is that the constant k_{12} is not simple but contains some components of low value that define the rate of efflux of some 20–30% of the label present in the chopped tissue at zero time. That is, the reason for the divergence between the theoretical and experimental curves is that the models do not allow for part of the label tightly bound by the tissue.

As described above, the increase in k_1 observed during the efflux of [^{14}C]valine from chopped tissue at 164mm- Na^+ treated with ouabain could be accounted for by a diminution in the rate of re-uptake. Ouabain would be expected on the basis of the Christensen (1967) hypothesis to diminish valine uptake by increasing the internal Na^+ concentration.

In the presence of ouabain the internal Na^+ concentration rises very rapidly (C. T. Jones & P. Banks, unpublished work). Similarly, a fall in the extracellular concentration of Na^+ should increase the net loss of [^{14}C]valine from the tissue. As described above, lowering the external Na^+ concentration did cause an increased rate of efflux that was reflected by an increase in the value of k_1 .

The failure of electrical stimulation to increase the rate of efflux significantly at 164mm- Na^+ until

the greater part of the labelled valine had been lost from the slices may be related to the relatively slow rate at which the intracellular Na^+ concentration rises during periods of electrical stimulation (Jones & Banks, 1970).

The ability of ouabain, electrical stimulation and low external Na^+ concentrations to improve the fit between the theoretical and experimental efflux curves probably reflects their ability to diminish the barriers to diffusion of [^{14}C]valine. That is, the agents that facilitate the efflux of labelled valine from the tissue also decrease the ability of the tissue to bind [^{14}C]valine tightly.

The efflux of [^{14}C]valine from preloaded chopped tissue is clearly a complex process that cannot be adequately defined in terms of two compartments (intracellular and extracellular) and two simple rate constants. This is not really surprising since chopped brain tissue clearly contains a variety of cell types and a variety of intracellular and extracellular compartments. However, the results described above suggest that chopped tissue behaves more like the two-component system postulated in the model when they are treated with agents that facilitate the efflux of labelled valine from the intracellular compartments.

The difficulties described above suggest that satisfactory descriptions of amino acid efflux curves will only be obtained when the values of the rate constants for efflux from all the sites in which the label is sequestered are known.

The results described in this paper indicate that the accumulation of Na^+ and loss of K^+ from cells as a result of prolonged electrical activity might impair protein synthesis by decreasing the size of the intracellular amino acid pool. It is also possible that Na^+ influx induced by brief periods of electrical stimulation may enhance amino acid influx and consequently lead to an increase in the intracellular concentration of amino acid, provided that the Na^+ influx does not lead to a marked increase in the intracellular concentration of Na^+ .

We are grateful for the assistance of Professor W. Bartley and Dr C. B. Taylor in the preparation of this paper. C. T. J. acknowledges receipt of a Medical Research Council Studentship.

REFERENCES

- Abadom, P. N. & Scholefield, P. G. (1962). *Can. J. Biochem. Physiol.* **40**, 1693.
- Ames, A. III & Nesbitt, F. B. (1966). *J. Physiol., Lond.*, **184**, 215.
- Appel, S. H. (1967). *Trans. N.Y. Acad. Sci.* **29**, 63.
- Brenner, M., Niederwieser, A. & Pataki, G. (1965). In *Thin-layer Chromatography*, p. 399. Ed. by Stahl, E. Heidelberg: Springer-Verlag.
- Christensen, H. N. (1967). *Perspect. Biol. Med.* **10**, 471.
- Bioch. 1970, 118

- Crane, R. K. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.* **24**, 1000.
- Crane, R. K. (1967). In *Protides of the Biological Fluids*, vol. 15, p. 227. Ed. by Peeters, H. Amsterdam: Elsevier Publishing Co.
- Eddy, A. A. (1968). *Biochem. J.* **108**, 489.
- Fox, M., Thier, S. O., Rosenberg, L. & Segal, S. (1964). *Biochim. biophys. Acta*, **79**, 167.
- Heinz, E. & Walsh, P. M. (1958). *J. biol. Chem.* **233**, 1488.
- Hertz, L. (1968). *J. Neurochem.* **15**, 1.
- Huxley, A. F. (1960). In *Mineral Metabolism*, vol. 1, p. 163. Ed. by Comar, C. L. & Bronner, F. New York: Academic Press Inc.
- Jones, C. T. & Banks, P. (1970). *Biochem. J.* **118**, 791.
- Johnstone, R. M. & Scholefield, P. G. (1965). *Biochim. biophys. Acta*, **94**, 130.
- Kuchler, R. J. (1967). *Biochim. biophys. Acta*, **136**, 473.
- Lahiri, S. & Lajtha, A. (1964). *J. Neurochem.* **11**, 17.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Margolis, R. K. & Lajtha, A. (1968). *Biochim. biophys. Acta*, **163**, 374.
- Nakazawa, S. & Quastel, J. H. (1968). *Can. J. Biochem.* **46**, 355.
- Riggs, T. R. & Walker, L. M. (1963). *J. biol. Chem.* **238**, 2663.
- Riggs, T. R., Walker, L. M. & Christensen, H. N. (1958). *J. biol. Chem.* **233**, 1479.
- Schafer, J. A. & Jacquez, J. A. (1967). *Biochim. biophys. Acta*, **135**, 1081.
- Schoffeniels, E., Hagemeijer, F. & Rorive, G. (1966). *Archs int. Physiol. Biochim.* **74**, 845.
- Schwartzman, L., Blair, A. & Segal, S. (1967a). *Biochim. biophys. Acta*, **135**, 120.
- Schwartzman, L., Blair, A. & Segal, S. (1967b). *Biochim. biophys. Acta*, **135**, 136.
- Swain, G. S. (1944). *J. Am. chem. Soc.* **66**, 1696.
- Takagaki, G., Hirano, S. & Nagata, Y. (1959). *J. Neurochem.* **4**, 125.
- Tasaki, I. (1962). In *Neural Physiopathology*, p. 266. Ed. by Grennell, R. G. New York: Hoeber.
- Treherne, J. E. (1966). *The Neurochemistry of Arthropods*, p. 24. Cambridge University Press.
- Varon, S. & McIlwain, H. (1961). *J. Neurochem.* **8**, 262.
- Vidaver, G. A. (1964a). *Biochemistry, Easton*, **3**, 795.
- Vidaver, G. A. (1964b). *Biochemistry, Easton*, **3**, 803.
- Winegrad, S. & Shanes, A. M. (1962). *J. gen. Physiol.* **45**, 371.

APPENDIX

Determination of the Kinetic Constants of Two Consecutive First-Order Reactions

By C. T. JONES

Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

The efflux of [^{14}C]valine from the chopped brain tissue was first considered as a sequence of two consecutive irreversible first-order processes such that any label lost from the intracellular compartment, A , would have to enter the extracellular compartment, B , before entering the bulk phase, C . This can be represented thus:

with kinetic constants k_1 and k_2 respectively. Therefore:

$$dA/dt = -k_1A \quad (1)$$

$$dB/dt = k_1A - k_2B \quad (2)$$

$$dC/dt = k_2B \quad (3)$$

Eqn. (1) is simply solved:

$$A = A_0 e^{-k_1 t} \quad (4)$$

and eqn. (2) becomes:

$$dB/dt = k_1 A_0 e^{-k_1 t} - k_2 B \quad (5)$$

The solution to this equation was first obtained by Esson (1866):

$$B = B_0 e^{-k_2 t} + \left(\frac{A_0}{1 - k_2/k_1} \right) (e^{-k_2 t} - e^{-k_1 t}) \quad (6)$$

[Note: the changes taking place in A and B may be represented in a relative manner as their 'reduced concentrations' A/A_0 and B/B_0 , which, from eqns. (4) and (6), are:

$$A/A_0 = e^{-k_1 t}$$

$$B/B_0 = e^{-k_2 t} + \left(\frac{A_0/B_0}{1 - k_2/k_1} \right) (e^{-k_2 t} - e^{-k_1 t})]$$

Since at any time:

$$A + B + C = A_0 + B_0 + C_0$$

combining eqns. (3), (4) and (6) gives:

$$C = C_0 + A_0(1 - e^{-k_1 t}) + B_0 \left[(1 - e^{-k_2 t}) - \left(\frac{A_0/B_0}{1 - k_2/k_1} \right) (e^{-k_2 t} - e^{-k_1 t}) \right] \quad (7)$$