Output of [^{14}C]Adenine Nucleotides and their Derivatives from Cerebral Tissues

TETRODOTOXIN-RESISTANT AND CALCIUM ION-REQUIRING COMPONENTS

By IAN PULL and HENRY McILWAIN

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), De Crespigny Park, London SE5 8AF, U.K.

(Received 12 June 1973)

1. Neocortical tissues, exposed briefly to [^{14}C]adenine and containing over 98% of their ^{14}C as adenine nucleotides, when superfused with glucose–bicarbonate salines released about 0.1% of their ^{14}C content/min to the superfusate. 2. Addition of unlabelled adenosine to the superfusing fluid increased the ^{14}C output three- to four-fold; half-maximal increase was given by about $40\mu\text{M}$ -adenosine, and reasons are adduced for considering the activity of adenosine kinase to be a major factor in conditioning the ^{14}C output. Adenosine similarly increased the enhanced ^{14}C output caused by electrical excitation of the superfused tissue; it brought about only a small increase in tissue glycolysis. 3. Output of ^{14}C from the [^{14}C]adenine-labelled tissues was increased when Ca^{2+} was omitted from the superfusing fluids, but electrical stimulation did not then liberate more ^{14}C . Nevertheless, such tissues still responded to electrical stimulation by increased glycolysis, and their ^{14}C output again became susceptible to increase by electrical stimulation when Ca^{2+} was restored. 4. The six-fold increase in tissue glycolysis caused by electrical excitation was almost completely inhibited by tetrodotoxin at $0.1\mu\text{M}$ and above, but this was associated with about 50% inhibition only in the output of ^{14}C from tissues preincubated with [^{14}C]adenine. The ^{14}C -labelled compounds of which output was most inhibited by tetrodotoxin were adenosine, inosine and hypoxanthine whereas output in a nucleotide fraction was little affected.

From cerebral tissues prelabelled by incubation with [^{14}C]adenine, electrical excitation releases several ^{14}C -labelled derivatives (Pull & McIlwain, 1972*a,b*). We have now studied factors that affect their release, in order to appraise its significance. Thus the compounds that appear extracellularly include adenine nucleotides, which normally do not readily leave animal tissues; but extracellular release of ATP on stimulation can occur as, or in association with, a neurotransmitter (Burnstock, 1972; Douglas & Poisner, 1966; Geffen & Livett, 1971). The ^{14}C output has therefore been measured on stimulation in the presence and absence of Ca^{2+} ions, which are frequently necessary for secretory processes and neurotransmitter release (Rasmussen, 1970; Reuter, 1973), and also in the presence and absence of tetrodotoxin, which inhibits neural conduction dependent on Na^+ entry (Kao, 1966).

The non-nucleotide [^{14}C]adenine derivatives adenosine, inosine and hypoxanthine, however, preponderate among those released by excitation of cerebral tissues under normal conditions. We report below that their output increases when unlabelled adenosine is added to fluids superfusing the tissues, indicating that exchange diffusion of the nucleosides

occurs at the cerebral cells as concluded also by Shimizu *et al.* (1972) from study of the uptake of adenosine. Dephosphorylation of adenine nucleotides intracellularly on excitation could contribute to output of adenosine and its derivatives in superfusates. As an indication of such dephosphorylation, we have measured the glycolysis concomitant with ^{14}C output, as tissue glycolysis depends on the degree of phosphorylation of adenine nucleotides and on the P_i formed by their breakdown (Lowry *et al.*, 1964).

Experimental

Tissue preparation, superfusion and stimulation

Tissue samples comprised the outer 0.35 mm from the guinea-pig neocortex and weighed 60–80 mg, the posterior portion being discarded. They were prepared, weighed and incubated in 5 ml of medium containing $1\mu\text{Ci}$ of [^{14}C]adenine ($3.4\mu\text{M}$) as previously described (Pull & McIlwain, 1972*a*). Superfusion was started after 40 min incubation by using the superfusion systems of Pull *et al.* (1972) with a variable-speed peristaltic pump set to give a flow of 3.2 ml/min in each of the four vessels which were simultaneously

incubated. Collection of effluent medium for analysis commenced 20 min after starting superfusion. The standard glucose-bicarbonate saline used for incubation and superfusion contained: NaCl, 120 mM; KCl, 3.5 mM; KH_2PO_4 , 1.2 mM; MgCl_2 , 1.2 mM; CaCl_2 , 0.75 mM; NaHCO_3 , 25 mM; glucose, 10 mM, in equilibrium with $\text{O}_2 + \text{CO}_2$ (95:5).

At appropriate times, the fluid superfusing some of the tissues was changed to medium modified by the addition of adenosine or tetrodotoxin at specified concentrations or by the omission of CaCl_2 . These modified superfusion media were prepared immediately before use and were also maintained in equilibrium with $\text{O}_2 + \text{CO}_2$ (95:5) in a suitable reservoir. Tissues to be treated with Ca^{2+} -free medium were also transiently exposed to EDTA by rapidly injecting 0.5 ml of 100 mM-EDTA, dissolved in Ca^{2+} -free medium and adjusted to pH 7.4 with NaOH, into the inflow tube immediately before the vessel.

The tissues were electrically stimulated, normally for only one or two 2 min periods during an experiment, by applying pulses of exponential time-voltage relationship to silver-grid electrodes surrounding the tissue (McIlwain & Rodnight, 1962). Characteristics of the stimuli applied were monitored with an oscilloscope during these periods and were adjusted to a peak potential of 10 V, time-constant 0.4 ms and a frequency of 20 Hz.

Analytical methods

Tissues were removed from their holders at the end of superfusion, promptly homogenized in 2.5 ml of 6% (w/v) trichloroacetic acid and centrifuged. The deposits were extracted with a further 2 ml of the trichloroacetic acid solution and the combined supernatants were made to 5 ml before a sample (0.1 ml) was taken for the determination of radioactivity and a sample (0.5 ml) for dilution with water and determination of K^+ . For measurement of creatine phosphate the tissues were homogenized in 2.5 ml of 0.3 M- HClO_4 and centrifuged. Samples of the supernatant were then taken for the determination of K^+ and radioactivity. Of the remainder, 2 ml was neutralized with 5 M- K_2CO_3 and, after removal of KClO_4 precipitated during standing in ice for 20 min, was used for the determination of creatine phosphate by a fluorimetric enzyme analysis (Lowry *et al.*, 1964).

Lactate in samples of the collected superfusate media was determined by an automated fluorimetric method with lactate dehydrogenase (Pull *et al.*, 1972) and 1 ml samples were also taken for the determination of radioactivity. A further 4 ml of effluent medium from each of four collection periods was pooled, supplemented with non-radioactive carrier compounds, de-salted, and chromatographed to determine the distribution of ^{14}C between the released adenine derivatives as previously described (Pull &

McIlwain, 1972a), except that the pyridine eluates from charcoal were freeze-dried rather than evaporated to dryness. Radioactivity in the various samples was also measured as previously described (Pull & McIlwain, 1972a), by using a Nuclear Enterprises liquid-scintillation counter model 8312.

Expression of results

The radioactivity in tissue extracts, effluent media samples and medium remaining in the beakers at the end of superfusion was first expressed as nCi/g of tissue. The sum of these values was taken as the total amount of radioactivity in the tissue at the start of sample collection. This initial total minus the successive amounts of radioactivity collected during succeeding collection intervals gave the time-course of the decrease in tissue radioactivity during superfusion. For each collection period a rate coefficient for ^{14}C output was obtained as the amount of ^{14}C collected/min expressed as a percentage of the average amount of ^{14}C calculated to be remaining in the tissue during the period of collection (Shanes & Bianchi, 1959). Since the collection periods were all of 2 min duration the calculation becomes:

$$\begin{aligned} \text{Rate coefficient for each collection (\%} \cdot \text{min}^{-1}) \\ = (^{14}\text{C collected} \times 100) / (\text{tissue } ^{14}\text{C at beginning} \\ + \text{tissue } ^{14}\text{C at end of collection}) \end{aligned}$$

It was assumed for these calculations that any ^{14}C which had not emerged into the sample collection tubes was wholly contained within the tissue, although a variable fraction (less than 1%) of the tissue ^{14}C was actually contained within the outflow tubes and beaker of the superfusion system. Lactate in superfusate media was expressed as $\mu\text{mol/h}$ per g of tissue for each collection period. The average rate coefficient and rate of lactate production observed in the four periods immediately preceding electrical stimulation and the maximum values of these parameters attained in response to electrical stimulation were used for comparing the actions of electrical stimulation and of the modified media.

Materials

Tetrodotoxin was obtained from the Sankyo Co., Tokyo, Japan as a mixture of pure crystalline compound with sodium citrate buffer and was dissolved in water and dispensed into ampoules which were sealed and stored at -20°C after freeze-drying. The ampoules were then opened as required for each experiment. The Radiochemical Centre, Amersham, Bucks., U.K. supplied $[8\text{-}^{14}\text{C}]\text{adenine}$ (59 Ci/mol). Other materials were from sources described previously (Pull & McIlwain, 1972a).

Results

Output of [^{14}C]adenine derivatives into adenosine-containing media

Tissues preincubated with [^{14}C]adenine, and then superfused with standard medium to remove unassimilated ^{14}C , on continued superfusion released some 0.06% of their $^{14}\text{C}/\text{min}$ (Fig. 1). This could be increased about threefold, but not further, by adding adenosine to the standard superfusion medium. The increased rate was reached about 10 min after superfusion with adenosine began, and required not more than 0.1 mM-adenosine; half-maximal increase was reached at about 40 μM -adenosine (Fig. 2). Increasing

the adenosine concentration to 1.5 mM increased neither the augmented ^{14}C output, nor its rate of augmentation, beyond the values reached at 0.1 mM.

At each adenosine concentration examined, from 0 to 1.5 mM, electrical excitation gave greater output of [^{14}C]adenine derivatives. The electrical characteristics of the stimuli used were just greater than the minimum values needed for maximal change in the Na^+ , K^+ and respiration of the tissues (McIlwain & Joanny, 1963). The ^{14}C output could thus be increased, by electrical stimulation in the presence of added adenosine, to 12 times its resting value.

Tissue glycolysis, as measured by lactate output, by contrast, was much less affected by added adenosine (Figs. 1 and 2). Increase by a factor of 1.3 was found on superfusion with 0.03–0.5 mM-adenosine, and by 1.6 with 1.5 mM-adenosine. Electrical excitation, again, caused a much greater increase: glycolysis rose to 6 times its resting value.

Fig. 1. Augmented output (a) of [^{14}C]adenine derivatives and (b) of lactate, caused by adenosine and by electrical excitation

Four neocortical tissues from two guinea pigs were separately incubated and superfused as described in the Experimental section. Points give mean values of duplicate tissues without ($\circ$) and with ($\bullet$) 0.1 mM-adenosine. Superfusion was initially with the standard bicarbonate–glucose medium, and at the time indicated by vertical arrows this was replaced in two vessels by the same medium with adenosine. For the 2 min period indicated at S, all four tissues were electrically stimulated. Superfusion fluids were collected for analysis each 2 min. The tissues at the end of superfusion, with and without adenosine, contained 75.5 ± 0.6 (S.D. of four values) $\mu\text{equiv.}$ of K^+/g . Points A and B give the plateau and peak values quoted in Fig. 2.

Fig. 2. Concentrations of adenosine required for augmented output of (a) ^{14}C and (b) lactate from guinea-pig neocortex

Summarized results are given from eight experiments of the type described in Fig. 1 in which superfusion was carried out with adenosine concentrations between 0 and 1.5 mM. Plateau values ($\circ$) before stimulation and peak values during electrical stimulation ($\bullet$) are shown and correspond to regions lettered A and B in Fig. 1. Mean values from four tissues are shown and their S.D. is indicated by vertical lines when these exceed the size of the symbols used.

Dependence on calcium salts

The incubating fluids used for superfusing the cerebral tissues normally contained 0.75 mM-Ca^{2+} (cf. McIlwain & Bachelard, 1971*a,b*). When tissues that had assimilated [^{14}C]adenine were superfused with similar fluids but lacking Ca^{2+} , output of ^{14}C increased but now responded less to electrical excitation. These effects were both accentuated by a small injection of EDTA to the superfusing solution; this addition gave momentarily a solution of 10 mM-

Fig. 3. Ca^{2+} ions and the response of guinea-pig neocortical tissues to electrical excitation (a) in output of ^{14}C from [^{14}C]adenine and (b) in output of lactate

○, Without Ca^{2+} ; ●, with Ca^{2+} . Cortical samples were preincubated with [^{14}C]adenine in normal glucose-bicarbonate saline with 0.75 mM-Ca^{2+} . After 40 min superfusion with adenine-free solutions was commenced in vessels (1) with the normal medium and in (2) with the same medium lacking Ca^{2+} . After 10 min superfusion the medium flowing to vessels (2) received an injection of 0.5 ml of 100 mM-EDTA ; 10 min later (zero time in the Figure) 2 min collections of the superfusate were made and measurements made of their content of ^{14}C and lactate. Tissues were all stimulated at 20 Hz for the 2 min periods shown (S). Tissues extracted at the end of the experiment (40 min on Figure) contained: from vessels (1) K^+ , $70\mu\text{equiv./g}$; creatine phosphate, $2.9\mu\text{mol/g}$. From vessels (2), K^+ , $51\mu\text{equiv./g}$; creatine phosphate, $1.8\mu\text{mol/g}$.

EDTA in the incubating fluid, which was immediately diluted by the continuously flowing superfusion fluid. Tissue output of ^{14}C was markedly increased by this treatment (Fig. 3*a*); injection of the same quantity of EDTA to a normal, Ca^{2+} -containing fluid did not act similarly.

Output of ^{14}C from the Ca^{2+} -deprived tissues now responded little to electrical excitation (Fig. 3*a*). This was not because the tissues were inherently incapable of responding to excitation, as was shown by measurement of their concomitant rate of lactate output (Fig. 3*b*), which was also high in the Ca^{2+} -free solutions, but nevertheless doubled on excitation. The K^+ and creatine phosphate contents of the Ca^{2+} -depleted tissues were much below those of normal tissues (Fig. 1). The chemical nature of the ^{14}C -labelled compounds which appeared in the superfusates with Ca^{2+} -free solutions was examined by adsorption and t.l.c. and was similar to that of the compounds yielded to normal media (Table 1). The ^{14}C output from tissues to Ca^{2+} -free media before stimulation increased threefold in these experiments and most of the increase was in adenosine, inosine and hypoxanthine, that is, in the compounds which preponderated on superfusion with normal media.

Effects of Ca^{2+} lack on ^{14}C output were reversible. The Ca^{2+} -free conditions of sample (2) (Fig. 3) caused the tissue to lose ^{14}C at some 0.5% of the tissue content/min in the absence of excitation. When Ca^{2+} was restored to such tissues, the resting rate of ^{14}C loss

Fig. 4. Restoration of tissue response by addition of Ca^{2+} salts

Experiments were commenced as with tissues (2) (Fig. 3), but after the superfusion with Ca^{2+} -free solutions and brief treatment with 0.75 mM-EDTA Ca^{2+} was restored, first in the solution to vessels (3) and then to vessels (4). Stimulation periods and measurements were the same as in Fig. 3. ○, No Ca^{2+} in superfusion fluids; ●, with 0.75 mM-Ca^{2+} . Tissues extracted at the end of the experiment contained: from vessels (3), K^+ , $64\mu\text{equiv./g}$; creatine phosphate, $2.8\mu\text{mol/g}$. From vessels (4), K^+ , $60\mu\text{equiv./g}$; creatine phosphate, $2.2\mu\text{mol/g}$.

Table 1. ¹⁴C-labelled components recovered from superfusates of tissues stimulated in the presence and absence of tetrodotoxin

Of the effluent superfusion fluids from experiments carried out as described in Fig. 5, the samples A collected during the 8min immediately before stimulation and samples B collected in 8min during and after stimulation were taken for analysis by the methods described in the Experimental section. The volumes flowing during periods A and B were equal and were approx. 25ml/tissue sample. Mean values are quoted ±s.d. when of four samples and ±0.5 (difference) when of two.

¹⁴ C-Labelled derivatives measured	Four tissues in normal media (nCi/g of tissue)			Two tissues in media with 0.1 μM-tetrodotoxin (nCi/g of tissue)		
	Period A	Period B	(B-A)	Period A	Period B	(B-A)
ATP, ADP, 5'-AMP	1.12 ± 0.47	2.56 ± 1.50	1.44	0.94 ± 0.11	2.34 ± 0.21	1.40
Inosine	1.15 ± 0.11	5.25 ± 1.02	4.10	1.24 ± 0.01	1.96 ± 0.19	0.72
Cyclic AMP	0.24 ± 0.09	0.86 ± 0.44	0.62	0.22 ± 0.03	0.36 ± 0.02	0.14
Hypoxanthine	2.80 ± 0.74	8.61 ± 0.28	5.81	2.73 ± 0.48	4.07 ± 0.20	1.34
Adenosine	2.71 ± 0.69	13.07 ± 3.89	10.36	1.46 ± 0.11	3.75 ± 0.18	2.29

became about 0.2%/min, close to its value in normal media. Moreover, the ¹⁴C output responded to excitation even before the lower rates were restored, as shown by tissues (3) (Fig. 4). Tissues (4) (Fig. 4), when compared with tissues (2) (Fig. 3), showed that excitability could be restored after 45min of Ca²⁺ depletion. Under these conditions the K⁺ and creatine phosphate contents of the tissues were also partly recovered (Fig. 4). Lactate formation from these tissues before and after restoration of Ca²⁺ was similar to that found for tissues (2) (Fig. 3).

Action of tetrodotoxin, and the ¹⁴C compounds released

Tetrodotoxin also acted differentially on the output of lactate and of [¹⁴C]adenine derivatives which occurred in response to excitation (Fig. 5). The tetrodotoxin concentration giving half-maximal inhibition of glycolysis under these conditions was about 40nM (Fig. 6, and see McIlwain *et al.*, 1969), and above 0.1 μM inhibition of the output of lactate response was almost complete. By contrast, 1 μM-tetrodotoxin inhibited only partially the output of [¹⁴C]adenine derivatives (Figs. 5 and 6). However, of the ¹⁴C output that was susceptible to tetrodotoxin, about 50% inhibition was given at 40nM-tetrodotoxin (Fig. 6). The ¹⁴C output thus appears to exhibit tetrodotoxin-insensitive and -insensitive components.

The chemical nature of the compounds found in superfusates on excitation in the presence of tetrodotoxin are given in Table 1. In the absence of tetrodotoxin, as reported previously (Pull & McIlwain, 1972a), the preponderating products were adenosine, hypoxanthine and inosine, and excitation increased the release of ¹⁴C in each of these fractions. Tetrodotoxin at 0.1 μM, a minimum concentration needed for almost complete inhibition of the output of ¹⁴C compounds collectively after electrical stimulation (Fig.

Fig. 5. Differential inhibition by tetrodotoxin of the output (a) of ¹⁴C and (b) of lactate on electrical stimulation of guinea-pig neocortical tissues

Initial treatment of the tissues was as described in Fig. 1, all being superfused with standard bicarbonate-glucose saline. At the time indicated by vertical arrows, this saline was replaced in two vessels by one containing 1 μM-tetrodotoxin (●) and for the 2min period marked S all tissues were electrically stimulated. Effluent medium collected during periods A and B were pooled for chromatography (see Table 1).

Fig. 6. Concentration of tetrodotoxin causing inhibition of output (a) of ^{14}C and (b) of lactate from guinea-pig neocortex

Collected results are from six experiments of the type reported in Fig. 5, in which superfusion was carried out with tetrodotoxin concentrations of the values indicated. Plateau values (○) obtained before stimulation and peak values reached during electrical stimulation (●) are shown. Mean values from four tissues each are shown with their s.d. indicated by vertical lines when these exceed the size of the symbols used.

6), diminished ^{14}C output in each of the fractions named except the nucleotide fraction carrying ATP, ADP and 5'-AMP (Table 1). It was notable that the proportion of ^{14}C in these nucleotides in effluents from normal tissues diminished on excitation (from 13.7 ± 3.9 to $8.0 \pm 2.9\%$), whereas on stimulation in the presence of tetrodotoxin their proportion increased (from 14.4 ± 2.5 to $18.7 \pm 0.6\%$; mean $\pm$ s.d. of four samples in each case).

Discussion

We first consider routes from the [^{14}C]adenine administered to the [^{14}C]purines found in effluent fluids, with minimal consideration of factors related

to tissue heterogeneity (see, however, McIlwain, 1972).

Incorporation and output of ^{14}C from [^{14}C]adenine

Cerebral tissues incubated with [^{14}C]adenine, as during the present experiments, contain most of their total ^{14}C as adenine nucleotides (Santos *et al.*, 1968) of which ATP preponderates under normal aerobic conditions. Incorporation proceeds (Santos *et al.*, 1968) by reaction between [^{14}C]adenine and 5-phosphoribosyl 1-pyrophosphate according to reaction (1), catalysed by adenine phosphoribosyltransferase (EC 2.4.2.7), followed by further phosphorylation of AMP to ADP (reaction 2: adenylate kinase, EC 2.7.4.3.):

Reaction (2) can proceed in cerebral preparations at a maximum rate of about $300 \mu\text{mol}/\text{h}$ per g of tissue, and oxidative and other phosphorylation reactions also lead to the formation of [^{14}C]ATP from [^{14}C]ADP.

The amount of tissue ^{14}C found in adenosine, inosine and hypoxanthine in the present experiments totals less than 2% of the incorporated radioactivity, as has been observed also for similarly incubated mouse cerebrum (Wong & Henderson, 1972). Since the ^{14}C collected in superfusates during the present and previous experiments totals some 2–15% of the initial tissue content and is mainly in the form of adenosine, inosine and hypoxanthine, these effluent compounds must have been derived at least in part from the adenine nucleotides of the tissue (Pull & McIlwain, 1972b).

Formation of adenosine from the nucleotides requires the action of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5; reaction 3) on AMP, and this mononucleotide is formed from ADP by adenylate kinase. Deamination of adenosine by adenosine deaminase (EC 3.5.4.4; reaction 4):

leads to inosine. Although AMP is the preferred substrate for 5'-nucleotidase (Kluge *et al.*, 1972), inosine may be formed also by deamination of AMP (adenylate deaminase, EC 3.5.4.6) followed by 5'-nucleotidase action on the resulting IMP. The concentrations of AMP and IMP are normally low relative to the K_m of 0.2 mM for 5'-nucleotidase acting on these substrates (Kluge *et al.*, 1972), so that the formation of adenosine and inosine may well be limited by the concentrations of these nucleotides available to 5'-nucleotidase. The intracellular concentration of 5'-

AMP maintained by guinea-pig neocortical tissues in oxygenated media is 0.07mM (Rolleston & Newsholme, 1967) and the concentration of IMP in whole brain is similar to that of AMP (Mandel & Edel-Harth, 1966). Situations which favour dephosphorylation of ATP in cerebral tissues thus also favour the conversion of adenine nucleotides collectively into inosine and its derivatives.

Output on electrical excitation

Electrical excitation increases dephosphorylation of ATP mainly because it increases the intracellular Na^+ concentration; the enhanced rates of active cation transport which result are mediated by an Na^+ - K^+ -stimulated ATPase*, reaction (5):

which can proceed maximally at some 2mmol/h per g of tissue (Schwartz *et al.*, 1962).

Increased Na^+ entry on excitation was shown by Keesey & Wallgren (1965) and is considered to be responsible for increases observed concomitantly in tissue respiration and lactate formation (McIlwain & Bachelard, 1971a). Lactate formation in cerebral tissue is also increased by ADP and P_i (Lowry *et al.*, 1964). Respiration was not measured during the present experiments but the increase in lactate production on electrical stimulation (Figs. 1–5) suggests that acceleration of reaction (5) has occurred.

Reactions regenerating ATP are slower in their maximal speed than reaction (5) (McIlwain & Bachelard, 1971b), so that on maximal stimulation $[^{14}\text{C}]\text{-ADP}$, and hence $[^{14}\text{C}]\text{-AMP}$ by adenylate kinase action, accumulate in the tissue and become available in increased amounts to 5'-nucleotidase and adenylate deaminase. The cerebral content of 5'-AMP in rat brain increases approximately 10-fold after an applied shock of 1 s duration (Minard & Davis, 1962). The increased efflux of $[^{14}\text{C}]\text{-adenosine}$ and $[^{14}\text{C}]\text{-inosine}$ occurring during excitation can then be attributed in part to the increased substrate concentrations available for 5'-nucleotidase. This enzyme has a maximum activity at saturating substrate concentrations of about 200 $\mu\text{mol/h}$ per g of tissue in the rat brain (Banik & Davison, 1969) and could possibly become rate-limiting for the efflux of ^{14}C compounds during electrical excitation.

Output in the presence of adenosine

Formation of 5'-AMP from adenosine in cerebral tissue is metabolically distinct from its formation from adenine and occurs by passive diffusion of adenosine into the tissue and phosphorylation by

adenosine kinase (EC. 2.7.1.20) according to reaction 6 (Shimizu *et al.*, 1972):

Adenylate kinase, reaction (7), brings about exchange of the ^{14}C among the intracellular nucleotides so that incubation of labelled tissue with unlabelled adenosine would be expected to lead to an increased $[^{14}\text{C}]\text{-AMP}$ content in the cell and so also (see above) to an increased ^{14}C efflux. This efflux was found to occur (Fig. 2) and the concentration of extracellular adenosine giving half-maximum output was about 40 μM . This value is of the same order of magnitude as the K_m for adenosine kinase of 20 μM found by Shimizu *et al.* (1972), who found close quantitative agreement with an apparent K_m for adenosine uptake by the tissue (also of 20 μM). It is accordingly suggested that the stimulation of ^{14}C efflux by added adenosine is conditioned largely by the activity of adenosine kinase, though other factors may make some contribution and account for the higher apparent K_m found in the present experiments.

When tissues were electrically stimulated in the presence of various concentrations of adenosine their ^{14}C output also increased with increasing adenosine concentrations below 100 μM (Fig. 2). Adenosine had, with or without excitation, a stable maximum effect between 0.1 and 1.5mM. A further regularity displayed in Fig. 2 is that electrical excitation increased ^{14}C output in similar proportion whether or not adenosine was added. This suggests that the limiting factor in the modification of ^{14}C by adenosine is not directly affected by the excitation. This criterion could probably be met by the adenosine kinase system, for excitation does not greatly diminish the tissue concentration of ATP (McIlwain & Bachelard, 1971b), which is the other component of the kinase reaction, and which is normally present in the tissue at 2–3 $\mu\text{mol/g}$. When purified from liver adenosine kinase showed a K_m (ATP) of about 0.5mM (Lindberg *et al.*, 1967).

Modified responses to excitation

Evidence for at least two components in the ^{14}C output on electrical excitation was obtained with tetrodotoxin, which could suppress about 50% only of the ^{14}C output (Figs. 5 and 6). The tetrodotoxin concentration needed for this partial inhibition was close to that needed for inhibition of the Na^+ entry caused by excitation (see the Results section). Inhibition of the Na^+ entry would prevent the increase in reaction (5), the Na^+ -requiring ATPase, normally associated with excitation and, indeed, the associated increase in lactate production was almost completely prevented by 0.1 μM -tetrodotoxin. Nevertheless, considerable increase in ^{14}C output occurred on

* ATPase, adenosine triphosphatase.

excitation in the presence of 1 and 2 μM -tetrodotoxin, whereas lactate production in response to excitation was inhibited completely (Fig. 6).

Though major components of neural excitation are Na^+ -dependent and inhibited by approx. 0.1 μM -tetrodotoxin, tetrodotoxin-resistant processes unaffected by 100 or more times this concentration are also well established (Kao, 1966). In particular, miniature end-plate potentials, Ca^{2+} -dependent components and transmitter-induced changes of conductance are reported in the presence of tetrodotoxin (Hagiwara & Nakajima, 1966; Elmqvist & Feldman, 1965; Ozeki *et al.*, 1966). That fraction of the release of [^{14}C]adenine derivatives now found to be tetrodotoxin-resistant is presumed to be a chemical accompaniment of one such process, and in its further characterization the particular ^{14}C derivatives released are especially significant (Table 1).

The compounds of which release was minimally affected by tetrodotoxin were the nucleotides ATP, ADP and 5'-AMP. Also, about 20–30% of the output of adenosine, inosine and hypoxanthine persisted in the presence of tetrodotoxin. Here it should be recalled that superfusion of tissues with [^{14}C]ATP under conditions similar to those of the present experiments yielded [^{14}C]adenosine, [^{14}C]inosine and [^{14}C]hypoxanthine without appreciable entry of ^{14}C into the tissue (Pull & McIlwain, 1972a). Thus a simple interpretation of the present results is that only one ^{14}C compound is released on excitation in the presence of tetrodotoxin, namely [^{14}C]ATP.

Changes in ^{14}C output brought about by depleting tissue Ca^{2+} are complex, as shown by Figs. 3 and 4; the augmented output in the absence of excitation appears likely to follow K^+ loss, Na^+ entry and increase in reaction (5), with consequent increases in reactions (7), (3) and (4). It is noteworthy therefore that the specific aspect now being investigated, namely response to excitation, shows a reverse relationship to Ca^{2+} depletion. In particular, added Ca^{2+} restores to depleted tissues the output of ^{14}C compounds induced by excitation, although having little or no action on the tissue's glycolytic response to excitation. Known Ca^{2+} -dependent output processes include those of neurosecretion and transmitter release and certain of these processes also afford occasions for the extracellular appearance of ATP (see the introduction). Release of catecholamines and their associated proteins from sympathetic nerves and from the adrenal medulla are the systems most extensively studied (Smith & Winkler, 1972) and they have been shown to involve Ca^{2+} -dependent processes of exocytosis. The granules undergoing exocytosis also contain adenine nucleotides and in an instance in which this has been investigated, nucleotide output was found also to be Ca^{2+} -dependent (Douglas & Poisner, 1966).

Present evidence thus suggests that the 5'-adeno-

sine nucleotides observed to appear extracellularly on excitation of the cerebral systems are released by processes akin to neurosecretion, but leaves open the question of whether the processes are primarily purinergic (Burnstock, 1972) or whether the nucleotides accompany other biologically active neurohumoral agents.

We are indebted to Mr. A. McNeil and Mr. W. Chivers for assistance during these experiments, to the Research Fund of the Bethlem Royal Hospital and the Maudsley Hospital for their support, and to the Medical Research Council for providing the scintillation counter used.

References

- Banik, N. L. & Davison, A. N. (1969) *Biochem. J.* **115**, 1051–1062
- Burnstock, G. (1972) *Pharmacol. Rev.* **24**, 509–581
- Douglas, W. W. & Poisner, A. M. (1966) *J. Physiol. (London)* **183**, 249–256
- Elmqvist, D. & Feldman, D. S. (1965) *Acta Physiol. Scand.* **64**, 475–476
- Geffen, L. B. & Livett, B. G. (1971) *Physiol. Rev.* **51**, 98–157
- Hagiwara, S. & Nakajima, S. (1966) *J. Gen. Physiol.* **49**, 793–806
- Kao, C. Y. (1966) *Pharmacol. Rev.* **18**, 997–1049
- Keesey, J. C. & Wallgren, H. (1965) *Biochem. J.* **95**, 301–310
- Kluge, H., Hartmann, W., Wiczorek, V. & Zahlten, W. (1972) *J. Neurochem.* **19**, 1409–1411
- Lindberg, B., Klenow, H. & Hansen, K. (1967) *J. Biol. Chem.* **242**, 350–356
- Lowry, O., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) *J. Biol. Chem.* **239**, 18–30
- Mandel, P. & Edel-Harth, S. (1966) *J. Neurochem.* **13**, 591–595
- McIlwain, H. (1972) *Biochem. Soc. Symp.* **36**, 69–85
- McIlwain, H. & Bachelard, H. S. (1971a) *Biochemistry and the Central Nervous System*, pp. 67–73, Churchill-Livingston, London
- McIlwain, H. & Bachelard, H. S. (1971b) *Biochemistry and the Central Nervous System*, pp. 81–82, Churchill-Livingston, London
- McIlwain, H. & Joanny, P. (1963) *J. Neurochem.* **10**, 313–323
- McIlwain, H. & Rodnight, R. (1962) *Practical Neurochemistry*, p. 166, Churchill, London
- McIlwain, H., Harvey, J. A. & Rodriguez, G. (1969) *J. Neurochem.* **16**, 363–370
- Minard, F. N. & Davis, R. V. (1962) *J. Biol. Chem.* **237**, 1283–1289
- Ozeki, M., Freeman, A. R. & Grundfest, H. (1966) *J. Gen. Physiol.* **49**, 1319–1334
- Pull, I. & McIlwain, H. (1972a) *Biochem. J.* **126**, 965–973
- Pull, I. & McIlwain, H. (1972b) *Biochem. J.* **130**, 975–981
- Pull, I., Jones, D. A. & McIlwain, H. (1972) *J. Neurobiol.* **3**, 311–323

- Rasmussen, H. (1970) *Science* **170**, 404–412
- Reuter, H. (1973) *Progr. Biophys.* **26**, 3–43
- Rolleston, F. S. & Newsholme, E. A. (1967) *Biochem. J.* **104**, 524–533
- Santos, J. N., Hempstead, K. W., Koppe, L. E. & Miech, R. P. (1968) *J. Neurochem.* **15**, 367–376
- Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962) *Biochem. J.* **84**, 626–637
- Shanes, A. M. & Bianchi, G. P. (1959) *J. Gen. Physiol.* **42**, 1123–1137
- Shimizu, H., Tanaka, S. & Kodama, T. (1972) *J. Neurochem.* **19**, 687–698
- Smith, A. D. & Winkler, H. (1972) *Handb. Exp. Pharmacol.* **33**, 538–605
- Wong, P. C. L. & Henderson, J. F. (1972) *Biochem. J.* **129**, 1085–1094