Studies on Brain-Cortex Slices

THE INFLUENCE OF VARIOUS INHIBITORS ON THE RETENTION OF POTASSIUM IONS AND AMINO ACIDS WITH GLUCOSE OR PYRUVATE AS SUBSTRATE

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(Received 17 October 1966)

1. The K^+ content of brain-cortex slices incubated in a Krebs–Ringer phosphate medium was the same whether the substrate was glucose (11mM) or pyruvate (11mM). 2. 2,4-Dinitrophenol (30 μ M) and ouabain (83 μ M) lowered the K^+ content of brain-cortex slices by approx. 40% with either glucose or pyruvate as the substrate. Triethyltin (1.3 μ M) lowered the K^+ content by 73% with glucose as substrate, but by only 18% when the substrate was pyruvate. 3. Quantitative estimations of six individual amino acids were made on brain-cortex slices and on the incubation medium after 75 min. at 37°. Some differences were found depending on whether the substrate was glucose or pyruvate. The effects of 2,4-dinitrophenol, oligomycin, triethyltin and ouabain were studied. Triethyltin caused a marked increase in the medium of each amino acid when the substrate was glucose, but not when it was pyruvate. Ouabain caused a similar increase with either glucose or pyruvate as substrate. 4. The uptake of triethyltin by brain-cortex slices was measured by using radioactive triethyl[^{113}Sn]tin chloride. Various experimental conditions did not alter the uptake; these included adding glucose or pyruvate as a substrate. 5. From the author's own results and those of others it is suggested that pyruvate is an unsuitable substrate to use for the study of regulatory factors of endogenous pyruvate metabolism in brain-cortex slices. Glucose is preferable.

In the preceding paper (Cremer, 1967) data were given to show that brain-cortex slices metabolized exogenous pyruvate differently from pyruvate formed endogenously from glucose. A far greater portion of endogenous pyruvate was reduced to lactic acid. The addition of triethyltin or oligomycin to brain-cortex slices with glucose as substrate caused a marked increase in the formation of lactic acid. Neither substance increased lactic acid formation from exogenous pyruvate.

The present paper is concerned with two other aspects of brain-cortex slices, namely their content of K^+ and free amino acids. The influence of either glucose or pyruvate as the added substrate has been studied together with the effects of several agents known to influence brain metabolism.

Some of the data for free amino acids in brain-cortex slices with glucose have already been published (Cremer, 1964).

METHODS

Tissues. Male albino rats of the Porton strain were used. The general procedure for the cutting and incubation of tissue slices in a Krebs–Ringer phosphate medium was as described previously (Cremer, 1967).

Determination of K^+ . Potassium was estimated by flame photometry as described by Pappius & Elliott (1956). The results are expressed as μ equiv. of K^+ /g. original wet wt. of tissue slice. A correction was made for the increase in wet weight at the end of the incubation as indicated by Pappius & Elliott (1956).

Determination of free amino acids. The procedure used for the separation of the amino acids was essentially that of Gaitonde (1962) as previously described for tissue slices and incubation medium (Cremer, 1964).

The results are expressed as μ moles of amino acid/g. original wet wt. of tissue slice.

Uptake of radioactive triethyltin by tissue slices. Triethyltin chloride labelled with ^{113}Sn was prepared by The Radiochemical Centre, Amersham, Bucks. The specific activity of the original material was 0.63 μ C/mg. of triethyltin chloride. Portions of a stock solution in ethanol were diluted with an appropriate Krebs–Ringer phosphate medium to give a final solution of 10 μ g./ml.

Standard solutions of triethyl[^{113}Sn]tin chloride were always counted with each experiment and corrections for decay were made. The half-life of ^{113}Sn is 118 days.

The following general procedure for determining the uptake of triethyl[^{113}Sn]tin chloride by tissue slices was used. Vials containing 2 ml. of an oxygenated Krebs–Ringer phosphate were warmed to 37° in a Dubnoff shaker. Tissue slices were cut and placed in the vials, and exactly 15 min. later 1.0 ml. of triethyl[^{113}Sn]tin chloride solution

was added. At selected times, slices were removed, rapidly rinsed in water and placed in counting tubes. Samples (1ml.) of the incubation medium were also taken for counting. Samples had to be left for a minimum of 16hr. before counting to allow equilibrium between ^{113}Sn and its indium daughter isotope ^{113}In (Heath, 1963). The total ^{113}Sn was estimated by scintillation counting by using a Packard automatic γ -scintillation spectrometer.

Where appropriate, values are expressed as means $\pm$ s.d. and were examined statistically by Student's t test as modified by Fisher (1934) for small samples. The assumption was made that the variances of the control and experimental groups were the same, so that the degrees of freedom equal to $n_1 + n_2 - 2$ were used.

RESULTS

K⁺ content of brain-cortex slices. For K^+ to be maintained at normal concentrations in brain-cortex slices at least two main processes must be functional. One is involved in the generation of ATP, or a form of utilizable energy, and the other is involved in the utilization of this energy for cation transport. An inhibitor of either of these processes could be expected to decrease the K^+ concentration in a tissue slice.

Triethyltin and 2,4-dinitrophenol have been shown to be inhibitors of ATP synthesis in isolated mitochondrial preparations. They are therefore examples of inhibitors of the first process mentioned above. Ouabain has been shown to inhibit the Na^+ -plus- K^+ -stimulated adenosine triphosphatase thought to be connected with active cation transport.

The effects of these three agents on K^+ concentrations in brain-cortex slices were determined. The results reported below are concerned with the maintenance of the K^+ concentration in brain-cortex slices. This is rather different from measuring the uptake of K^+ by brain-cortex slices previously depleted of their K^+ content, as has been studied by Bilodeau & Elliott (1963).

Table 1 (column *a*) gives the K^+ concentrations

in brain-cortex slices incubated with glucose as the added substrate. All three agents lowered the K^+ concentration, but by far the greatest effect was seen with triethyltin.

Similar experiments were carried out with pyruvate as the added substrate (Table 1, column *b*). There was no significant difference between the K^+ content of control brain-cortex slices with either glucose or pyruvate as substrate. Both 2,4-dinitrophenol and ouabain lowered the K^+ concentration in brain-cortex slices incubated with pyruvate as substrate. With 2,4-dinitrophenol the decrease was significantly greater with pyruvate than with glucose. With triethyltin, although there was a significant decrease in K^+ with pyruvate as substrate, this was very much less than when the substrate was glucose, being 18% compared with 73%.

Free amino acids in brain-cortex slices. Previous work has shown that the moderate efflux of free amino acids from the tissue into the incubation medium that occurs with normal brain-cortex slices can be increased in the presence of metabolic inhibitors (Ernsting, Kafae, Nauta, Oosterhuis & de Waart, 1960; Cremer, 1964).

The effects of oligomycin, an inhibitor of mitochondrial ATP formation, and of ouabain were measured. The results for brain-cortex slices with glucose as substrate are given in Table 2 together with those previously reported for triethyltin and 2,4-dinitrophenol.

The four compounds tested could be divided into two groups. Triethyltin and ouabain lowered the amounts of free amino acids in the slice and greatly increased the concentrations in the incubation medium. The exceptions were the amount of γ -aminobutyric acid in the brain-cortex slices, which increased in the presence of triethyltin, and the amount of glutamine in the incubation medium in the presence of ouabain, which was the same as the control value.

Table 1. K^+ concentration in brain-cortex slices with either glucose or pyruvate as added substrate

Each flask contained 3.0ml. of Krebs-Ringer phosphate with either glucose (11mM) or sodium pyruvate (11mM). The K^+ content of the slices was determined at the end of 75 min. incubation as described in the Methods section. The probabilities that values are significantly different from the control means are: * $P < 0.001$; ** $P < 0.02$. The probabilities that values in columns (*a*) and (*b*) are significantly different are: controls, not significant; triethyltin, $P < 0.001$; 2,4-dinitrophenol, $P < 0.01$; ouabain, not significant.

Compound	Substrate ...	Concn. of K^+ ($\mu\text{equiv./g. wet wt.}$)	
		Glucose (<i>a</i>)	Pyruvate (<i>b</i>)
None		51.4 $\pm$ 6.8 (12)	46.3 $\pm$ 6.4 (8)
Triethyltin sulphate (1.3 μM)		13.8 $\pm$ 2.4 (7)*	38.1 $\pm$ 5.1 (8)**
2,4-Dinitrophenol (30 μM)		34.2 $\pm$ 5.1 (12)*	26.9 $\pm$ 4 (6)*
Ouabain (83 μM)		30.8 $\pm$ 4.6 (6)*	24.3 $\pm$ 6.1 (6)*

Table 2. *Amino acid concentrations in brain-cortex slices with glucose as added substrate*

Each flask contained 3.0 ml. of Krebs-Ringer phosphate with glucose (11 mM) and two slices of tissue to give approx. 100 mg. total wet wt. Incubation was for 75 min. For the control slices mean values $\pm$ S.D. are given with the numbers of determinations in parentheses. All other values are the means of two experiments.

Compound	Concn. of amino acids (μ moles/g. wet wt.)									
	γ -Aminobutyric acid		Alanine		Glutamine		Glycine		Glutamic acid	
	Slice	Medium	Slice	Medium	Slice	Medium	Slice	Medium	Slice	Medium
None	1.02 $\pm$ 0.21 (13)	0.0 (7)	0.6 $\pm$ 0.16 (14)	0.39 $\pm$ 0.16 (5)	2.66 $\pm$ 0.4 (11)	2.38 $\pm$ 0.7 (6)	1.24 $\pm$ 0.36 (13)	0.93 $\pm$ 0.08 (4)	6.9 $\pm$ 0.92 (13)	1.93 $\pm$ 0.47 (5)
Triethyltin sulphate (1.3 μ M)	1.23	1.02	0.42	1.04	1.65	4.05	0.69	2.66	2.31	7.2
2,4-Dinitrophenol (30 μ M)	1.93	0.0	0.43	0.40	1.58	2.90	1.16	0.89	5.65	1.02
Oligomycin (6 μ g./ml.)	1.52	0.0	0.40	0.92	1.20	3.62	0.58	0.87	5.44	2.16
Ouabain (200 μ M)	0.34	2.0	0.0	2.20	0.14	2.30	0.49	1.70	2.63	7.5
									1.54	0.72
									1.09	0.78
									0.79	2.13

Table 3. *Amino acid concentrations in brain-cortex slices with pyruvate as added substrate*

The conditions were as described in the legend to Table 2, except that sodium pyruvate (11 mM) was present in place of glucose. The numbers of determinations are given in parentheses. The probabilities that values are significantly different from control means are: * $P < 0.02$; † not significant.

Compound	Concn. of amino acids (μ moles/g. wet wt.)									
	γ -Aminobutyric acid		Alanine		Glutamine		Glycine		Glutamic acid	
	Slice	Medium	Slice	Medium	Slice	Medium	Slice	Medium	Slice	Medium
None	0.89 $\pm$ 0.37 (4)	0.0 (6)	0.63 $\pm$ 0.10 (4)	1.25 $\pm$ 0.38 (4)	1.15 $\pm$ 0.28 (5)	2.60 $\pm$ 0.43 (6)	0.74 $\pm$ 0.10 (5)	0.75 $\pm$ 0.30 (6)	7.78 $\pm$ 0.76 (6)	0.52 $\pm$ 0.28 (6)
Triethyltin sulphate (1.3 μ M)	1.03 $\pm$ 0.48 (3)†	0.0 (6)†	0.70 $\pm$ 0.02 (3)†	0.94 $\pm$ 0.06 (4)†	0.93 $\pm$ 0.30 (4)†	2.28 $\pm$ 0.20 (6)†	0.88 $\pm$ 0.06 (5)†	0.83 $\pm$ 0.22 (5)†	5.97 $\pm$ 1.1 (5)*	0.57 $\pm$ 0.42 (5)†
Ouabain (200 μ M)	0.23 (2)	1.39 (2)	0.19 (2)	2.01 (2)	0 (2)	0.99 (2)	0.40 (2)	1.50 (2)	1.30 (2)	10.30 (2)
									4.00 $\pm$ 0.58 (6)	0.55 $\pm$ 0.3 (6)
									3.95 $\pm$ 0.57 (5)†	0.56 $\pm$ 0.39 (5)†
									0.66 (2)	4.70 (2)

With oligomycin and 2,4-dinitrophenol there was no apparent increase in the leakage of free amino acids from the brain-cortex slices into the incubation medium. There was an increase in the amount of γ -aminobutyric acid inside brain-cortex slices and a decrease of glutamine and aspartic acid.

Some similar experiments were done with pyruvate as the added substrate. The concentrations and distribution of the amino acids in the control slices were, for the most part, very similar to those observed with glucose as the substrate (Table 3). The exceptions were the amount of glutamine in the brain-cortex slice, which was lower, and the amount of alanine in the medium, which was higher with pyruvate than with glucose as substrate.

Ouabain caused a very marked leakage of amino acids from the slice into the medium, so that its effect was similar with either pyruvate or glucose as the substrate.

In the presence of triethyltin the only significant change in the concentrations of free amino acids with pyruvate as substrate was a 20% decrease in the amount of glutamic acid in the brain-cortex slice (Table 3). This lack of effect with triethyltin was in complete contrast with the results obtained with glucose as the substrate (Table 2).

Uptake of triethyl[^{113}Sn]tin. In view of the very different findings of the effects of triethyltin on the metabolism of the brain-cortex slice, depending on whether the substrate was pyruvate or glucose, it was essential to establish whether there was any difference in the uptake of triethyltin under the two conditions.

Triethyl[^{113}Sn]tin chloride was used. The main procedure adopted is described in the Methods section. Brain-cortex slices were incubated for

15 min. with an added substrate before the addition of triethyl[^{113}Sn]tin. This preincubation period was considered sufficient for the tissue to readjust from the trauma of isolation and sectioning, during which it is known that concentrations of metabolites such as ATP and creatine phosphate are decreased to zero.

The final concentration of triethyltin in the vials was $13.7\mu\text{M}$. This was tenfold greater than that most often used in the biochemical studies on brain-cortex slices. This high concentration was necessary to obtain reliable radioactivity measurements.

Determinations of the amount of ^{113}Sn in brain-cortex slices at times of incubation later than 25 min. gave extremely variable results. During the first 25 min., however, the rate of uptake was linear and the reported values have been confined to this period.

A comparison of Figs. 1(a) and 1(b) shows that the uptakes of triethyltin by brain-cortex slices in the presence of either glucose or pyruvate were very similar.

Certain modifications to the procedure were made in an attempt to gain some knowledge of the factors controlling the uptake of triethyltin. Brain-cortex slices incubated without substrates are depleted of ATP and creatine phosphate. Such treatment did not prevent the uptake of triethyltin (Fig. 1d). The presence of $30\mu\text{M}$ -2,4-dinitrophenol, which would also prevent ATP formation, did not prevent triethyltin uptake (Fig. 1c). These results make it unlikely that active transport was involved.

Figs. 1(a)–(d) show that in none of the systems of incubation used do the lines plotted for the uptake of triethyltin go through the origin. If

Fig. 1. Uptake of triethyl[^{113}Sn]tin by brain-cortex slices. (a) Brain-cortex slices were preincubated for 15 min. with glucose (11 mM) before the addition of triethyl[^{113}Sn]tin. (b) Brain-cortex slices were preincubated for 15 min. with pyruvate (11 mM). (c) Brain-cortex slices were preincubated with glucose (11 mM) and 2,4-dinitrophenol ($30\mu\text{M}$). (d) Brain-cortex slices were preincubated without a substrate. The full procedure is described in the Methods section.

Fig. 2. Uptake of triethyl[¹¹³Sn]tin by kidney slices. Slices were preincubated for 15 min. with glucose (11 mM) before the addition of triethyl[¹¹³Sn]tin (see the Methods section).

triethyltin became evenly distributed between the incubation medium and the tissue fluid of the slice then, assuming the latter to be 80% of the wet weight, the amount present in the slice would be 0.145 μ g. This would be equivalent to 0.58 count/min./mg. wet wt. of tissue slice. It is apparent from Fig. 1(a) that this value was attained virtually instantaneously. It is also apparent that the value of 10.6 counts/min./mg. wet wt. reached at 25 min. was almost 20-fold greater than the value calculated for equal distribution between the incubation and tissue fluids.

The rate of uptake of triethyl[¹¹³Sn]tin by kidney slices was determined (Fig. 2). The results show that there was a large concentration gradient in favour of the tissue with kidney slices as well as with brain-cortex slices.

DISCUSSION

In the preceding paper (Cremer, 1967), experiments were reported which showed that in brain-cortex slices the fate of pyruvate added as an exogenous substrate was not the same as pyruvate formed endogenously from glucose. The experiments of the present paper are concerned with the retention of cellular K⁺ and free amino acids by brain-cortex slices given either pyruvate or glucose as oxidizable substrate.

Abadom & Scholefield (1962) compared glucose

and pyruvate as substrates for brain-cortex slices. These authors found that the ATP concentration (measured as 7 min.-hydrolyssable nucleotide pyrophosphate) was the same with either substrate. However, only half as much glycine was transported from the medium into the slice with pyruvate as the substrate as was transported with glucose. They expressed their results in terms of a 'transport quotient'. This was defined as the ratio of μ moles of amino acid taken up/ μ moles of nucleotide pyrophosphate (ATP) per g. wet wt. of slice. The transport quotient was 7.9 with glucose and 4.2 with pyruvate.

Cremer (1962) found that the concentration of creatine phosphate in incubated brain-cortex slices was the same with either glucose or pyruvate as added substrate.

K⁺ in brain-cortex slices. The results recorded in Table 1 show that the concentration of K⁺ in incubated brain-cortex slices was the same whether glucose or pyruvate was the substrate. However, changes in the K⁺ concentration in response to the addition of several metabolic inhibitors depended on which of the two substrates was present. The most outstanding difference was observed after the addition of triethyltin, the loss of K⁺ being far greater with glucose than with pyruvate.

It is known that K⁺ is essential for metabolism. In nervous tissue there are at least three sites of action of K⁺ in stimulating glucose oxidation via pyruvate oxidation: (1) the conversion of pyruvate into acetyl-CoA; (2) the passage of pyruvate across the mitochondrial membrane; (3) the active transport of K⁺ across the cell membrane, giving rise to ADP and P_i. Sites (1) and (3) have been suggested by Kini & Quastel (1959), Elliott & Bilodeau (1962) and Bilodeau & Elliott (1963). Evidence for site (2) has been reported by Krall, Wagner & Gozensky (1964).

Triethyltin inhibits the oxidation of glucose by brain-cortex slices in the presence of 5 mM- or 105 mM-K⁺ in the incubation medium. It does not prevent the conversion of glucose into lactic acid. Not all drugs that inhibit respiration of brain-cortex slices affect the K⁺ concentration. Bilodeau & Elliott (1963) have suggested that, since the same drugs stimulate glycolysis, then the ATP formed during it can promote the accumulation of K⁺. A similar explanation is thought to be true for 2,4-dinitrophenol to account for the partial maintenance of K⁺ concentrations when ATP production by oxidative phosphorylation is inhibited. This is not true for triethyltin. At a time when the rate of glycolysis is high the K⁺ concentration is low.

If triethyltin altered the permeability of cell membranes or inhibited the active transport of Na⁺ and K⁺, then it would be expected to cause a leakage of K⁺ from brain-cortex tissue with

pyruvate as well as with glucose as substrate. For example, the decrease caused by ouabain was similar with either substrate. It is therefore unlikely that triethyltin acts directly on cell membranes.

Although triethyltin is concentrated within a brain-cortex slice, the amount present that effectively inhibits glucose oxidation is several orders less than the normal tissue K^+ concentration. A direct replacement of K^+ by $(C_2H_5)_3Sn^+$ is not possible. Whether triethyltin has a higher affinity than K^+ for one or more of the three sites mentioned has yet to be established.

Free amino acids in brain-cortex slices. Of the six amino acids measured in normal brain-cortex slices, five were present in the same amount with either glucose or pyruvate as substrate. The exception was glutamine, which was lower with pyruvate.

In the presence of 2,4-dinitrophenol or oligomycin, two compounds that are known to inhibit mitochondrial ATP synthesis, there were only small changes in the concentrations of amino acids in brain-cortex slices with glucose as the substrate. The argument that the ATP produced during glycolysis might be sufficient for the maintenance of amino acids at near normal concentrations cannot be extended to the situation where triethyltin is present. In the latter instance the rate of glycolysis is high, but an extensive leakage of all amino acids occurs. When the substrate was pyruvate the only change in the presence of triethyltin was a lowering of the glutamic acid concentration. It has been shown that creatine phosphate is decreased to zero amount under these conditions (Cremer, 1962). From these results it can be implied that the presence of ATP or creatine phosphate is not necessary for the maintenance of free amino acid concentrations in brain-cortex slices. On the other hand, respiratory activity of 70% or more of the control value seems essential. This does not mean that when the respiratory activity is at this level the amino acids will necessarily be unaffected. In the presence of $80\mu M$ -ouabain, for example, there is only a 20% decrease in respiration, but there is a very marked decrease in amino acid concentrations in the slice. The effects of ouabain are the same whether glucose or pyruvate is the substrate.

In summary, when the concentrations of free amino acids in brain-cortex slices were studied in relation to the addition of either glucose or pyruvate as an oxidizable substrate a marked distinction was observed when triethyltin was present. The same

was true for studies on the K^+ concentration in brain-cortex slices.

Pyruvate is a natural metabolic intermediate in glucose oxidation by nervous tissue. Pyruvate does not enter the brain from the blood *in vivo*, but is first converted into glucose, probably by the liver (Koeppe & Hahn, 1962; McMillan & Mortensen, 1963). The replacement of glucose with pyruvate as a substrate *in vitro* leads to some differences in the metabolic behaviour of brain-cortex slices. Certain comparative aspects between glucose and pyruvate as substrates have been reported by Abadom & Scholefield (1962) and Bilodeau & Elliott (1963). The present work, together with that of the preceding paper (Cremer, 1967), extends such a comparison. The joint results raise the question of the desirability of using non-physiological concentrations of metabolic intermediates to assess the physiological fate of such substances. The addition of pyruvate to brain-cortex slices is not considered satisfactory for the study of regulatory factors of endogenous pyruvate metabolism in this tissue.

I thank Mr P. A. Matthews for his valuable technical assistance.

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