Studies on Brain-Cortex Slices

DIFFERENCES IN THE OXIDATION OF ^{14}C -LABELLED GLUCOSE AND PYRUVATE REVEALED BY THE ACTION OF TRIETHYLtin AND OTHER TOXIC AGENTS

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1. The rate of appearance of $^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]\text{glucose}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ was measured. Pyruvate is oxidized to carbon dioxide twice as fast as glucose, although the oxygen uptake is almost the same with each substrate. 2. The presence of $30\text{ }\mu\text{M}$ -2,4-dinitrophenol increases the output of $^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]\text{glucose}$ sixfold whereas the oxygen uptake is not quite doubled. Similar results are obtained with $0\cdot1\text{ M}$ -potassium chloride. The stimulating action of these two agents on the output of $^{14}\text{CO}_2$ from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ is much less than on that from $[6\text{-}^{14}\text{C}]\text{glucose}$. 3. The effects of oligomycin, ouabain and triethyltin on the respiration of control and stimulated brain-cortex slices were studied. Triethyltin ($1\text{-}3\text{ }\mu\text{M}$) inhibited the oxidation of $[6\text{-}^{14}\text{C}]\text{glucose}$ more than 70%, but did not inhibit the oxidation of $[3\text{-}^{14}\text{C}]\text{pyruvate}$. 4. The production of lactic acid by brain-cortex slices incubated with glucose is twice as great as that with pyruvate. Lactic acid increases two and a half times in the presence of either triethyltin or oligomycin when the substrate is glucose, but is no different from the control when the substrate is pyruvate. 5. With kidney slices the production of lactic acid from glucose is very low. It is increased by oligomycin but not by triethyltin. 6. The results are discussed in terms of the oxidation of the extramitochondrial NADH_2 produced during glycolysis.

In a previous paper (Cremer, 1962) some unusual features in the action of triethyltin and triethyllead on tissue-slice metabolism were described. Both compounds inhibited glucose oxidation by brain-cortex slices, but did not inhibit respiration when pyruvate completely replaced glucose as the substrate. The oxidation of glucose by the brain is almost entirely via the Embden-Meyerhof pathway of glycolysis. The possibility that the site of inhibition of glucose oxidation was at some stage in glycolysis was eliminated by the demonstration that the formation of lactic acid was greatly increased in the presence of either of the two trialkylmetal compounds (Cremer, 1957, 1959).

The purpose of the present study was to investigate further the factors concerned in the oxidation of glucose and of pyruvate when either compound is added to brain-cortex slices as the sole substrate. Differences between the oxidation of these two substrates have become apparent by the use of a variety of agents known to affect carbohydrate metabolism. These include 2,4-dinitrophenol, ouabain and oligomycin as well as triethyltin.

In certain instances some comparative experiments were made with kidney slices, for as previ-

ously shown the oxidation of glucose by kidney slices is not inhibited by either triethyltin or triethyllead (Cremer, 1962).

In the following paper (Cremer, 1967) parameters other than respiratory ones associated with the metabolism of either glucose or pyruvate by brain-cortex slices are described.

METHODS

Tissues. The tissues were from male albino rats of the Porton strain, of 190–210 g. body wt. The preparation and use of tissue slices were essentially as previously described (Cremer, 1957). Brain-cortex slices were from the grey matter of the cerebral cortex. One outer slice was cut from each cerebral hemisphere. Each kidney was cut through its plane of symmetry and the cut surface placed downwards. The outermost slice was discarded. The next two slices were used. These contained material from the renal cortex and medulla.

Each flask contained 3 ml. of Krebs-Ringer phosphate solution [Umbreit, Burris & Stauffer (1951), with the modification that the concentration of CaCl_2 was $1\cdot5\text{ mM}$] with either glucose (11 mM) or sodium pyruvate (11 mM) and tissue slice of 50–60 mg. wet wt. The centre well contained either 0.2 ml. of 20% (w/v) KOH or 0.1 ml. of 10% (w/v) NaOH (see below) and folded filter paper for the

absorption of CO_2 . The manometers and flasks were placed in a water bath at 37° , gassed for 5 min. with 100% O_2 and equilibrated for a further 10 min., and O_2 uptake was measured at 10 min. intervals. The O_2 uptake is expressed as $\mu\text{l./mg. dry wt. of tissue slice}$.

Variations in this general procedure are given in the description of the particular experiments.

Lactic acid was estimated either by the technique of Barker & Summerson (1941) or enzymically (Bergmeyer, 1963). Samples of the incubation medium only were taken for the estimation of lactic acid.

Determination of radioactivity. [$\text{U-}^{14}\text{C}$]Glucose, [$6\text{-}^{14}\text{C}$]glucose and [$3\text{-}^{14}\text{C}$]pyruvate were obtained from The Radiochemical Centre, Amersham, Bucks.

In most experiments the following procedure was used. The main compartment of each flask contained 2.8 ml. of Krebs-Ringer phosphate solution with unlabelled substrate (11 mm) and tissue slice. The ^{14}C -labelled substrate, usually 0.5–5 μC in 0.2 ml. of 0.9% NaCl , was placed in the side arm and tipped into the main compartment at the end of the period of gassing and equilibration (15 min. after placing the flasks in the water bath). This was taken as zero time. The radioactivity of the expired $^{14}\text{CO}_2$ trapped on the filter paper in the centre well was determined by either of two procedures.

Procedure (1). The centre well contained 0.2 ml. of 20% (w/v) NaOH and a 2 cm.-square folded piece of filter paper. At the end of the incubation the contents of the centre well were transferred to 10 ml. of deionized water and left for several hours with intermittent swirling of the tubes to release the trapped CO_2 from the filter paper. The filter paper was removed, and 3.0 ml. of carrier Na_2CO_3 (0.2 M Na_2CO_3 in 0.1 N- NaOH) and 5.0 ml. of BaCl_2 (0.4 M) were added. The mixture was boiled and neutralized. The BaCO_3 precipitate was filtered on to sintered-glass disks, washed, dried and counted at infinite thickness with a Nuclear-Chicago gas-flow counter. The weight of BaCO_3 formed from the CO_2 evolved by the tissue slices was so small that the final weight of the BaCO_3 filtered after addition of carrier Na_2CO_3 was almost constant.

Procedure (2). The centre well contained 0.1 ml. of 10% (w/v) NaOH and a 2 cm.-square folded piece of filter paper. At the end of the incubation the filter paper was dropped into a glass vial containing 10 ml. of dioxan scintillation fluid. Radioactivity was measured on a Packard Tri-Carb model 3000 liquid-scintillation counter before and after the addition of an internal standard of [^{14}C]toluene. All values were corrected to absolute radioactivity (disintegrations/min.). This procedure was essentially that described by Buhler (1962), who showed the satisfactory recovery of ^{14}C from standard $\text{Na}_2^{14}\text{CO}_3$ solutions.

The total radioactivity added to each flask was determined by the complete oxidation of the ^{14}C -labelled substrate by the method of Katz, Abraham & Baker (1954). The oxidation was carried out in Warburg flasks and the radioactivity of the $^{14}\text{CO}_2$ was determined by either procedure (1) or (2) as described above.

The amount of $^{14}\text{CO}_2$ evolved by the tissue slices is expressed as either counts/min. or disintegrations/min./mg. of dry wt. of tissue.

The relative specific activity of the expired CO_2 was calculated from the following equation:

$$\frac{\mu\text{moles of substrate equivalent to } ^{14}\text{CO}_2 \text{ produced}}{\mu\text{moles of substrate equivalent to } \text{O}_2 \text{ taken up}} \times 100$$

The following is an example for [$\text{U-}^{14}\text{C}$]glucose added as substrate. Let the added [$\text{U-}^{14}\text{C}$]glucose be 32 μmoles of glucose and 16000 counts/min. after conversion into BaCO_3 . The specific activity of the added glucose is $16000/32 = 500$ counts/min./ μmole of glucose. If the O_2 uptake is 13.3 $\mu\text{l./mg. dry wt./hr.}$ then this is equivalent to 0.1 μmole of glucose. If the expired $^{14}\text{CO}_2$ counted as BaCO_3 gives 21.8 counts/min./mg. dry wt./hr. then this is equivalent to $21.8/500 = 0.043$ μmole of glucose. Then the relative specific activity of the CO_2 expired during 1 hr. is $(0.043/0.1) \times 100 = 43\%$. The assumption that the respiratory quotient (rate of moles of CO_2 evolved/rate of moles of O_2 consumed) was 1.0 was made throughout.

RESULTS

The main fuel of the brain is glucose. The oxygen consumption of the brain *in vivo* is almost equivalent to the amount required for the complete oxidation to carbon dioxide and water of the glucose taken up from the blood. From this it follows that if the glucose supplied to the brain is uniformly labelled with ^{14}C then the carbon dioxide expired by the brain will attain the same specific activity, per atom of carbon, as the glucose. Gainer, Allweiss & Chaikoff (1963) have shown that such an equilibrium was reached within 1 hr. of intravenously infusing the brain of an intact dog with [$\text{U-}^{14}\text{C}$]glucose.

With brain-cortex slices incubated in a Krebs-Ringer medium *in vitro* the proportion of glucose going to lactic acid is very much greater than occurs in the intact brain *in vivo*. Under the experimental conditions used in the present study, of the glucose used by rat brain-cortex slices 50% can be accounted for as lactic acid and 50% is equivalent to the consumption of oxygen (Cremer, 1957). From values reported in the literature where brain-cortex slices of several different animal species have been used, it would appear that the rate at which glucose carbon is converted into carbon dioxide is considerably lower *in vitro* than *in vivo* (Sutherland, Burbridge & Elliott, 1955; Beloff-Chain *et al.* 1955; Gainer, Allweiss & Chaikoff, 1962).

The rate of labelling of the carbon dioxide by normal rat brain-cortex slices incubated with [$\text{U-}^{14}\text{C}$]glucose was determined (Table 1). The experimental conditions were such that the slices oxidized unlabelled glucose for a period of 15 min. before the addition of tracer amounts of radioactive glucose. At the end of 2 hr. incubation the specific activity of the expired carbon dioxide was 41.5% of that of the added glucose. From the example given in the Methods section for the calculation of the relative specific activity of the carbon dioxide evolved during a stated period of time it is apparent that an average value is obtained. The specific activity of the expired carbon dioxide will increase with time, but the method of sampling includes the

Table 1. *Rate of oxidation of [U-¹⁴C]glucose by normal brain-cortex slices*

The contents of the flasks and general procedure were as described in the Methods section. The substrate was glucose (11mM) and at zero time 3 μ C of [U-¹⁴C]glucose (equivalent to 48 000 counts/min. as BaCO₃) was added from the side arm. Determination of the radioactivity in the centre well was by procedure (1) (see the Methods section). The relative specific activity (R.S.A.) of the expired CO₂ was calculated as in the example given in the Methods section.

Time of incubation (min.)	O ₂ uptake (μ l./100 mg. wet wt.)	¹⁴ CO ₂ output (counts/min./100 mg. wet wt.)	R.S.A. of expired CO ₂
0-15	53	51	8.5
0-30	120	234	17.4
0-60	257	787	27.2
0-90	381	1510	35.4
0-120	518	2410	41.5
15-30	67	183	24.2
30-60	137	553	36.0
60-90	124	723	52.0
90-120	137	900	59.0

Table 2. *Stability of brain-cortex slices in their rate of oxidation of [U-¹⁴C]glucose*

The flask contents were the same as described in Table 1. Zero time was taken from the placing of the flasks in the water bath. Incubation was continued for 15 min. after the addition of [U-¹⁴C]glucose.

Time of adding [U- ¹⁴ C]glucose (min.)	¹⁴ CO ₂ output (counts/min./mg. wet wt.)
0	127
15	51
40	48
60	51

total amount of carbon dioxide (¹⁴CO₂ plus unlabelled carbon dioxide) evolved during a given period. When the results given in Table 1 are expressed as difference values for each consecutive 30 min. period of incubation they show there was a fairly steady increase in the relative specific activity of the expired carbon dioxide.

The stability of the brain-cortex slices in their rate of oxidation of glucose was checked by adding the [U-¹⁴C]glucose from the side arm at different times and then continuing the incubation for a subsequent 15 min. Zero time in this instance was taken as the moment the flasks were placed in the water bath. The results given in Table 2 show that the output of ¹⁴CO₂ for a period of 15 min. remained constant when slices had been previously incubated with unlabelled glucose for various times up to 1 hr. The greater output of ¹⁴CO₂ by brain-cortex slices that came into contact immediately with [¹⁴C]-glucose probably reflects a surge of activity within the slice to restore those metabolic intermediates lost during the preparative procedures.

Effect of inhibitors on the oxidation of [6-¹⁴C]-glucose. Since the main aim of this work was to compare the oxidation of glucose with that of pyruvate by brain-cortex slices, [6-¹⁴C]glucose and [3-¹⁴C]pyruvate were used. This eliminated the possibility of ¹⁴CO₂ being produced by (a) decarboxylation of pyruvate and (b) direct oxidation of glucose. It was assumed that with either substrate the ¹⁴CO₂ evolved could only have arisen by the functioning of the tricarboxylic acid cycle.

Three inhibitors were selected whose mechanisms of action on subcellular particles have been studied extensively. They were ouabain, which is known to inhibit microsomal Na⁺-plus-K⁺-stimulated adenosine triphosphatase, oligomycin, an inhibitor of the final stages of ATP synthesis during mitochondrial oxidative phosphorylation, and triethyltin, which inhibits mitochondrial oxidative phosphorylation and blocks the stimulatory action of 2,4-dinitrophenol. Their effects on [6-¹⁴C]glucose oxidation by brain-cortex slices are shown in Table 3. Ouabain (final concn. 80 μ M) had a slight inhibitory effect on oxygen consumption, but less effect on ¹⁴CO₂ output, so that the relative specific activity of the expired carbon dioxide was slightly higher than the controls. Oligomycin (final concn. 3 μ g./ml.) inhibited equally oxygen uptake and ¹⁴CO₂ output, so that the relative specific activity of the expired carbon dioxide was the same as the controls. Triethyltin (final concn. 1.3 μ M) inhibited markedly oxygen uptake and ¹⁴CO₂ output, and the relative specific activity of the expired ¹⁴CO₂ was greatly diminished.

Effect of inhibitors on the oxidation of [6-¹⁴C]-glucose stimulated by K⁺. The addition of potassium chloride (final concn. 0.1M) to the incubation medium increased the output of ¹⁴CO₂ from [6-¹⁴C]-glucose by brain-cortex slices to a greater extent

Table 3. *Effect of inhibitors on the oxidation of [6-14C]glucose by brain-cortex slices*

The main compartment of each flask contained 2.8 ml. of Krebs-Ringer phosphate with glucose (11 mM), brain-cortex slice and, as indicated, triethyltin sulphate (1.3 μM), ouabain (80 μM) or oligomycin (3 μg./ml.). The side arm contained 0.2 ml. of 11 mM-glucose solution containing 0.5 μC of [6-14C]glucose. The flasks were warmed to 37°, gassed for 5 min. and equilibrated a further 10 min., and then the contents of the side arm were added to the main compartment. The addition of the side-arm contents was taken as zero time. The O₂ uptake is expressed as μl./mg. dry wt. of brain slice; ¹⁴CO₂ output is expressed as disintegrations/min. of the expired CO₂/mg. dry wt. of brain slice; the relative specific activity (R.S.A.) of expired CO₂ is as defined in the Methods section. Liquid-scintillation counting was used (procedure 2; see the Methods section). All values are the means of two experiments.

Time of incubation (min.)	Control				Ouabain				Oligomycin				Triethyltin			
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	
0-30	7.2	50	2.8		6.2	46	2.9		5.3	32	2.4		3.7	21	2.3	
0-60	13.4	238	7.6		10.9	233	8.5		9.9	196	7.9		4.5	30	2.7	
0-90	17.6	415	9.4		15.3	420	10.9		10.8	258	9.5		5.2	33	2.5	
0-120	24.5	829	13.5		19.4	795	16.3		13.7	416	12.1		6.6	48	2.9	

Table 4. *Effect of inhibitors on the oxidation of [6-14C]glucose stimulated by 0.1 M-potassium chloride*

Conditions were as described in the legend to Table 3 except that side arms of the flasks contained 0.2 ml. of KCl (1.5 M) in addition to [6-14C]glucose.

Time of incubation (min.)	Control				Ouabain				Oligomycin				Triethyltin			
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂		O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	
0-30	7.9	178	9.0		10.3	113	4.4		6.6	108	6.5		2.2	5	0.9	
0-60	15.3	583	15.2		18.6	748	16.0		11.8	277	9.3		3.6	30	3.3	
0-90	24.7	2140	34.5		24.8	1730	27.8		15.1	467	12.3		4.9	29	2.3	
0-120	30.0	2680	35.6		29.3	2605	35.4		—	—	—		4.4	28	2.5	

Table 5. *Effect of inhibitors on the oxidation of [6-¹⁴C]glucose stimulated by 2,4-dinitrophenol*

Conditions were as described in the legend to Table 3 except that the side arms contained 0.2 ml. of 2,4-dinitrophenol (450 μ M; final concn. 30 μ M) in addition to [6-¹⁴C]glucose.

Time of incubation (min.)	Control			Ouabain			Oligomycin			Triethyltin		
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂
0-30	12.5	296	9.4	11.0	113	4.1	14.4	437	12.1	4.7	19	1.6
0-60	28.2	1900	26.8	18.3	496	10.8	16.6	492	11.8	5.9	49	3.3
0-90	35.6	2860	32.0	22.3	824	14.7	19.2	974	20.2	5.2	29	2.2
0-120	54.9	4850	35.1	24.7	1410	22.7	24.6	1389	22.5	5.6	32	2.3

than it increased the oxygen uptake. Consequently the relative specific activities of the expired carbon dioxide were two to three times the control values (Table 4). Bilodeau & Elliott (1963) have suggested that there are two main sites at which high concentrations of K⁺ exert an effect. One, in agreement with Kini & Quastel (1959), is the conversion of pyruvate into acetyl-CoA, and the other is its involvement with the Na⁺-K⁺-pump mechanism that uses ATP and so continuously supplies ADP and P_i for oxidative phosphorylation. Since ouabain is known to block the Na⁺-plus-K⁺-stimulated adenosine-triphosphatase transport mechanism of cells it was expected that it would decrease the stimulating action of 0.1M-potassium chloride. The results given in Table 4 show that, contrary to expectation, ouabain did not prevent the stimulation. Oligomycin, on the other hand, did decrease the stimulating effect of 0.1M-potassium chloride on both oxygen uptake and ¹⁴CO₂ output. There was, however, a strong indication that this inhibitory action of oligomycin increased with time. Triethyltin not only prevented the stimulating action of 0.1M-potassium chloride, but inhibited the oxidation of glucose to the same marked extent as it did when added alone.

Effect of inhibitors on the oxidation of [6-¹⁴C]-glucose stimulated by 2,4-dinitrophenol. The stimulating action of 2,4-dinitrophenol on the oxidation of [6-¹⁴C]glucose by brain-cortex slices was quantitatively greater but qualitatively similar to that of 0.1M-potassium chloride (Table 5), i.e. the output of ¹⁴CO₂ was increased six- to seven-fold whereas the oxygen uptake was not quite doubled. The relative specific activities of the expired carbon dioxide were considerably greater than the control values and were similar to those obtained with 0.1M-potassium chloride stimulation. That the effect of 2,4-dinitrophenol was an increase in the release of ¹⁴CO₂ from [6-¹⁴C]glucose can be seen if the 60 min. values (Table 5) are compared with 120 min. control values (Table 3). In each instance the amount of oxygen taken up is almost the same, but the output of ¹⁴CO₂ in the presence of 2,4-dinitrophenol is twice as great as without.

Ouabain definitely diminished the stimulating action of 2,4-dinitrophenol, particularly on the output of ¹⁴CO₂ (Table 5). The overall effect of oligomycin was very similar to that of ouabain, apart from the first 30 min. period of incubation. During this early period oligomycin did not prevent the stimulating action of 2,4-dinitrophenol, either on oxygen uptake or on ¹⁴CO₂ output. This finding is extended in experiments described below. Triethyltin again caused a marked inhibition of glucose oxidation. The degree of inhibition was the same as in the absence of 2,4-dinitrophenol.

Effect of adding 2,4-dinitrophenol to brain-cortex

Table 6. *Effect of the addition of 2,4-dinitrophenol to brain-cortex slices incubated with various drugs*

The main compartment of each flask contained 2.8 ml. of Krebs-Ringer phosphate with glucose (11 mM), tissue slice and drug as indicated. At 45 min. 0.2 ml. of either 2,4-dinitrophenol (450 μ M; final concn. 30 μ M) or Krebs-Ringer phosphate was added from the side arm.

Drug	O ₂ uptake (μ l./mg. dry wt.)		
	Without 2,4-dinitrophenol (15-45 min.)	Without 2,4-dinitrophenol (45-75 min.)	With 2,4-dinitrophenol (45-75 min.)
None	6.8	6.6	13.5
Triethyltin sulphate (1.3 μ M)	3.4	1.3	1.6
Oligomycin (3 μ g./ml.)	5.8	3.6	8.8
Oligomycin (6 μ g./ml.)	5.3	3.6	7.0
Ouabain (400 μ M)	7.7	3.3	3.8

Table 7. *Effect of inhibitors on the oxidation of [3-¹⁴C]pyruvate by brain-cortex slices*

Conditions were similar to those described in the legend to Table 3. The substrate was 11 mM-sodium pyruvate. The side arm contained 0.5 μ C of [3-¹⁴C]pyruvate. Triethyltin sulphate, where present, was 5.2 μ M, and ouabain 80 μ M. The radioactivity of the expired CO₂ was counted as BaCO₃ and is expressed as counts/min./mg. dry wt. of brain slice (procedure 1; see the Methods section). Complete oxidation of the total flask content of 28 μ moles of pyruvate gave 27 000 counts/min. as BaCO₃.

Time of incubation (min.)	Control			Ouabain			Triethyltin		
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂
0-30	8.0	10	8	8.8	9	6	7.5	9	7
0-60	15.9	38	15	12.0	25	13	17.0	58	21
0-90	23.1	92	24	20.0	56	17	21.1	88	25
0-120	30.4	137	27	25.4	92	22	28.4	153	33

slices incubated in the presence of various inhibitors. It has been established that oligomycin does not prevent the stimulation by 2,4-dinitrophenol of mitochondrial respiration (Huijing & Slater, 1961). Since it was found that oligomycin did diminish the stimulating action of 2,4-dinitrophenol on glucose oxidation by brain-cortex slices (Table 5), some further experiments were carried out. The time-scales used in the experiments with isolated mitochondria and brain-cortex slices were very different. To test the more immediate effect of oligomycin on 2,4-dinitrophenol stimulation of brain-cortex slices the design of the experiments was modified from that previously used. Pairs of flasks were set up containing glucose, brain-cortex slice and drug as indicated in Table 6. The oxygen uptake was recorded during the 15-45 min. period of incubation. Then to one of each pair of flasks was added 2,4-dinitrophenol from the side arm. The oxygen uptake of each flask was recorded for the subsequent 30 min. of incubation.

The results in Table 6 show very clearly that, in the presence of oligomycin, on the addition of 2,4-dinitrophenol there was an increased rate of

oxygen uptake. However, the oxygen uptake after the addition of 2,4-dinitrophenol was not as high as when 2,4-dinitrophenol was added to normal slices. It is not possible to decide whether 2,4-dinitrophenol stimulated that portion of the oxygen uptake not inhibited by oligomycin or whether it released respiration from inhibition by oligomycin without stimulating it. Nevertheless, the results were different from those obtained with either ouabain or triethyltin with 2,4-dinitrophenol (Table 6). Both these compounds completely blocked any stimulation by 2,4-dinitrophenol. The prevention by triethyltin of respiratory stimulation by 2,4-dinitrophenol agreed well with the findings of Aldridge (1958) and Aldridge & Threlfall (1961) with isolated mitochondria.

Effect of inhibitors on the oxidation of [3-¹⁴C]-pyruvate. Control brain-cortex slices oxidized pyruvate to carbon dioxide and water at a faster rate than they oxidized glucose (Table 7). The rate of oxygen uptake was slightly higher with pyruvate as the added substrate, and the output of ¹⁴CO₂ was considerably greater, so that the relative specific activity of the expired carbon dioxide was

Table 8. *Effect of inhibitors on the oxidation of [3-¹⁴C]pyruvate stimulated by 0.1 M-potassium chloride*

Conditions were the same as those described in the legend to Table 7 except that the side arm contained 0.2 ml. of KCl (1.5 M) in addition to [3-¹⁴C]pyruvate.

Time of incubation (min.)	Control			Ouabain			Triethyltin		
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂
0-30	11.2	14	8	10.1	10	6	5.8	5	5
0-60	23.2	97	25	15.7	35	13	14.0	58	25
0-90	35.7	211	36	23.4	64	17	19.4	106	33
0-120	44.4	302	41	25.8	69	16	27.6	162	35

Table 9. *Effect of inhibitors on the oxidation of [3-¹⁴C]pyruvate stimulated by 2,4-dinitrophenol*

Conditions were the same as those described in the legend to Table 7 except that the side arm contained 0.2 ml. of 2,4-dinitrophenol (450 μ M; final concn. 30 μ M) in addition to [3-¹⁴C]pyruvate.

Time of incubation (min.)	Control			Ouabain			Triethyltin		
	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂	O ₂ uptake	¹⁴ CO ₂ output	R.S.A. of expired CO ₂
0-30	12.0	21	11	10.3	8	5	7.6	10	8
0-60	21.9	84	23	14.0	22	9	14.7	50	21
0-90	33.2	192	35	21.0	46	13	20.0	84	25
0-120	42.3	310	44	26.6	61	14	21.0	125	36

at least twice as high as with glucose at each time-interval. (The method of counting ¹⁴CO₂ in the series of experiments with [3-¹⁴C]pyruvate was not the same as the series with [6-¹⁴C]glucose. The true disintegrations/min. values for the ¹⁴CO₂ from [3-¹⁴C]pyruvate are not known, but an approximate value can be obtained by multiplying the given counts/min. by 38.) The reason for the high output of ¹⁴CO₂ from [3-¹⁴C]pyruvate compared with [6-¹⁴C]glucose is not known. Similar results were obtained with brain-cortex slices incubated with either [U-¹⁴C]glucose or [U-¹⁴C]pyruvate. The relative specific activities of the expired carbon dioxide after 60 min. incubation were 27 and 60 respectively.

Ouabain showed some inhibition of pyruvate oxidation, both of oxygen uptake and ¹⁴CO₂ output, particularly the latter (Table 7). Triethyltin, on the other hand, did not inhibit the oxidation of pyruvate (Table 7). This lack of inhibition of [3-¹⁴C]pyruvate oxidation confirmed earlier results with either [1-¹⁴C]- or [2-¹⁴C]-pyruvate (Cremer, 1962).

Effect of inhibitors on the oxidation of [3-¹⁴C]-pyruvate stimulated by 0.1 M-potassium chloride. The response of control brain-cortex slices with [3-¹⁴C]pyruvate as the added substrate to the

presence of 0.1 M-potassium chloride differed quantitatively from that of slices with glucose as the substrate (Table 8). The percentage stimulation of oxygen uptake was about the same with either substrate, but the output of ¹⁴CO₂ with [3-¹⁴C]-pyruvate was less stimulated than with [6-¹⁴C]-glucose.

Ouabain completely prevented any stimulating action of K⁺ on [3-¹⁴C]pyruvate oxidation.

Triethyltin also completely prevented the stimulating effect of K⁺ on pyruvate oxidation. It did not lower the rate below that of control slices without a high concentration of K⁺.

Effect of inhibitors on the oxidation of [3-¹⁴C]-pyruvate stimulated by 2,4-dinitrophenol. The stimulation of [3-¹⁴C]pyruvate oxidation by 2,4-dinitrophenol was very similar, quantitatively, to that by 0.1 M-potassium chloride (Table 9). Its effect on the output of ¹⁴CO₂ from [3-¹⁴C]pyruvate was less than on that from [6-¹⁴C]glucose (see Table 5). Both ouabain and triethyltin prevented the stimulating action of 2,4-dinitrophenol on [3-¹⁴C]pyruvate oxidation.

Lactic acid production by brain-cortex slices with either glucose or pyruvate as substrate. Previous work had shown that the addition of triethyltin to brain-cortex slices with glucose as the added

substrate brought about a three- to four-fold increase in the concentration of lactic acid at the end of 1 hr. incubation (Cremer, 1957). The rate of production of lactic acid had not previously been determined. Fig. 1 shows the production of lactic acid by brain-cortex slices, with and without triethyltin, at various times after incubation in a medium containing glucose as substrate. The results not only confirmed that triethyltin caused

Fig. 1. Production of lactic acid by brain-cortex slices. ○, Control; ●, with triethyltin (0.66 μM); ▲, with triethyltin (1.3 μM).

a marked increase in lactic acid, but showed that the increase was continuous with time, whereas in the control slices the amount of lactic acid remained constant after the first 15 min. Further, the increase in lactic acid was greater at the lower concentration of triethyltin. At this concentration (0.66 μM) the oxygen uptake was only inhibited by 30%, whereas at 1.3 μM-triethyltin it was inhibited by 70%.

In control slices when the substrate was pyruvate the concentration of lactic acid was only half that when glucose was the substrate (Table 10).

A comparison was made of the effect of several agents on the amount of lactic acid produced by brain-cortex slices at the end of 75 min. incubation with either glucose or pyruvate as the added substrate. None of the compounds tested increased the amount of lactic acid when the substrate was pyruvate, although each of them did increase it when the substrate was glucose. The most striking differences between the two substrates were seen in the presence of triethyltin and oligomycin (Table 10).

A set of experiments was carried out in which pyruvate and glucose were added together as substrates, each at a concentration of 5 mM. The effects of triethyltin and oligomycin were tested (Table 10). The amount of lactic acid in control slices was significantly greater ($P < 0.001$) than for glucose as the only substrate, and was very close to the sum of the values obtained with either glucose or pyruvate. In the presence of triethyltin or oligomycin the amount of lactic acid increased to values similar to those attained when glucose was the only substrate.

Lactic acid concentrations in kidney slices with either glucose or pyruvate as substrate. In a previous study (Cremer, 1962) on the oxidation of ^{14}C -labelled substrates a comparison was made between

Table 10. Production of lactic acid by brain-cortex slices with glucose, pyruvate or glucose plus pyruvate as added substrates

Each flask contained 3.0 ml. of Krebs-Ringer phosphate with glucose (11 mM) or sodium pyruvate (11 mM) or glucose (5 mM) plus sodium pyruvate (5 mM). Lactic acid was determined in samples of the medium at the end of 75 min. incubation at 37° by using the enzymic method. Mean values $\pm$ S.D. are given with the numbers of determinations in parentheses. The probabilities that values are significantly different from the control means are: * $P < 0.001$; ** $P < 0.01$; *** $P = 0.02$; † not significant.

Compound	Substrate ...	Lactic acid (μmoles/g. wet wt.)		
		Glucose	Pyruvate	Glucose + pyruvate
None		42.9 $\pm$ 3.8 (6)	24.6 $\pm$ 2.4 (4)	64.8 $\pm$ 7.0 (6)
Triethyltin sulphate (1.3 μM)		106.2 $\pm$ 12.8 (6)*	22.2 $\pm$ 1.3 (4)†	122.0 $\pm$ 13.6 (6)*
Oligomycin (6 μg./ml.)		122.2 $\pm$ 12.1 (4)*	—	—
Oligomycin (3 μg./ml.)		—	25.0 $\pm$ 1.9 (4)†	134.0 $\pm$ 13.4 (4)*
Oligomycin (0.67 μg./ml.)		107.0 $\pm$ 15.3 (4)*	—	—
2,4-Dinitrophenol (30 μM)		74.2 $\pm$ 14.6 (4)*	26.1 $\pm$ 0 (4)†	—
Ouabain (200 μM)		62.4 $\pm$ 8.9 (4)**	19.4 $\pm$ 1.9 (4)***	—
Ouabain (80 μM)		48.6 $\pm$ 8.6 (4)†	—	—

Table 11. *Production of lactic acid by kidney slices with either glucose or pyruvate as added substrate*

Conditions were as described in the legend to Table 10. The probabilities that values are significantly different from the control means are: * $P < 0.001$; † not significant.

Compound	Substrate ...	Lactic acid (μ moles/g. wet wt.)	
		Glucose	Pyruvate
None		5.9 ± 1.0 (6)	33.8 ± 4.9 (4)
Triethyltin sulphate ($1.3 \mu\text{M}$)		4.4 (2)	29.5 (2)
Oligomycin ($6 \mu\text{g./ml.}$)		20.8 ± 4.6 (4)*	—
Oligomycin ($3 \mu\text{g./ml.}$)		—	39.0 ± 2.7 (4)†
Oligomycin ($0.67 \mu\text{g./ml.}$)		16.8 ± 3.1 (4)*	—

brain-cortex and kidney slices. It was shown that the rate of oxidation of $[\text{U-}^{14}\text{C}]\text{glucose}$ to $^{14}\text{CO}_2$ was the same in both tissues. Pyruvate was oxidized faster in kidney slices than in brain-cortex slices. In the present study the amount of lactic acid produced by kidney slices at the end of a 75 min. period of incubation was determined (Table 11).

The value for control slices with glucose as added substrate was very low. It was only 14% of that of brain-cortex slices. The concentration of lactic acid was not increased by the addition of triethyltin, but it was increased by the addition of oligomycin.

When pyruvate was the substrate the amount of lactic acid in kidney slices was more than five times as great as that with glucose as substrate and was higher than in brain-cortex slices. Triethyltin and oligomycin were without effect (Table 11).

DISCUSSION

Observations made several years ago (Cremer, 1962) on the action of certain organometal compounds on the respiratory activity of rat tissue slices indicated that factors controlling the oxidation of glucose by brain-cortex slices were different from those controlling the oxidation of pyruvate.

Since in brain-cortex slices virtually all of the glucose utilized passes through pyruvate via the Embden-Meyerhof pathway, it might be supposed (a) that the fate of pyruvate added exogenously would be the same as that formed endogenously from glucose and (b) that the behaviour of brain-cortex slices would be the same with either glucose or pyruvate as the added substrate. That these two suppositions are only partially correct can be seen from the results given in the present paper. Further evidence is given in the following paper (Cremer, 1967) from both experimental findings and reports in the literature.

Oxidation of glucose and pyruvate. The glycolysis of 1 mole of $[\text{6-}^{14}\text{C}]\text{glucose}$ will yield 1 mole of $[\text{3-}^{14}\text{C}]\text{pyruvate}$. For the complete oxidation of 1 mole of glucose or pyruvate 6 or 2.5 moles of

oxygen will be required respectively. ^{14}C will not appear in the expired carbon dioxide until a molecule of pyruvate has passed at least two turns round the tricarboxylic acid cycle. From the way in which the relative specific activity of the expired carbon dioxide of brain-cortex slices was calculated (see the Methods section) there should be no difference between the values obtained with either $[\text{6-}^{14}\text{C}]\text{glucose}$ or $[\text{3-}^{14}\text{C}]\text{pyruvate}$ if the rate of oxidation of each substrate were the same. However, the values given for control slices in Tables 3 and 7 show that the rate at which ^{14}C appeared in the expired carbon dioxide was twice as great for $[\text{3-}^{14}\text{C}]\text{pyruvate}$ as for $[\text{6-}^{14}\text{C}]\text{glucose}$. These results suggest that when glucose is the substrate the production of pyruvate is rate-limiting, but that when pyruvate is the added substrate the rate-limiting factor is the oxidation pathway.

Since the original studies of Ashford & Dixon (1935) and Dickens & Greville (1935) many other people have examined the stimulating action of high concentrations of K^+ in the incubation medium on the respiration of brain-cortex slices. It is well established that when the K^+ concentration is raised from the usual 5 mM to 100 mM there is an increase of one-and-a-half- to two-fold in the rate of oxygen uptake with either glucose or pyruvate as the added substrate. In the present experiments the increase in oxygen uptake by the addition of 100 mM- K^+ was not very great. This may have been due to a lower concentration of Ca^{2+} present in the incubation medium than that originally proposed by Krebs (see Umbreit *et al.* 1951). There is a definite relationship between the oxygen uptake of brain-cortex slices and the $\text{Ca}^{2+}/\text{K}^+$ ratio in the incubation medium (Rose, 1965).

With $[\text{6-}^{14}\text{C}]\text{glucose}$ as the added substrate the presence of 100 mM- K^+ caused a three- to four-fold increase in the rate of appearance of ^{14}C in the expired carbon dioxide. A similar result was obtained when 30 μM -2,4-dinitrophenol was added, but the stimulation of oxygen uptake and $^{14}\text{CO}_2$ output was even greater than with 100 mM- K^+ .

Gainer *et al.* (1962) did not observe an increase in the relative specific activity of the expired carbon dioxide in the presence of 2,4-dinitrophenol. Hoskin (1960) did observe such an increase both with 2,4-dinitrophenol and with a high K^+ concentration. The same phenomenon was reported by Cremer (1964) for brain-cortex slices incubated with $[U-^{14}C]$ glucose and 2,4-dinitrophenol. The present results with $[6-^{14}C]$ glucose show that the effect must involve an increase in the functioning of the tricarboxylic acid cycle. This would agree with the increased specific activity of glutamic acid, aspartic acid and γ -aminobutyric acid previously reported (Cremer, 1964). Since ATP or creatine phosphate concentrations are greatly decreased in the presence of 2,4-dinitrophenol or 100mM- K^+ (McIlwain, 1952), any reactions requiring ATP for the incorporation of glucose carbon will be decreased and more of the glucose could be oxidized to carbon dioxide. Reactions of this type could include a decrease in the formation of glutamine (Gonda & Quastel, 1963) and decreased incorporation of amino acids, such as alanine, into protein (Cremer, 1964).

The results for control slices given in Tables 3–9 showed there were quantitative differences between the response to K^+ or 2,4-dinitrophenol by brain-cortex slices, depending on whether the substrate was glucose or pyruvate. Greater differences were revealed by the addition of either ouabain or triethyltin. Ouabain (80 μ M) slightly inhibited the oxidation of $[6-^{14}C]$ glucose and $[3-^{14}C]$ pyruvate. It did not prevent the stimulating action of 100mM-potassium chloride on glucose oxidation, but it did prevent the stimulation of pyruvate oxidation. Ouabain prevented the stimulating action of 2,4-dinitrophenol on either glucose or pyruvate oxidation. These results are not easy to reconcile with the generally proposed sites of action for ouabain, 2,4-dinitrophenol and K^+ , and no attempt will be made to do so here. Other authors have commented on some of these interrelationships more fully (see Rose, 1965). The results showed that a difference could be observed in the response of brain-cortex slices to glucose or pyruvate as a substrate. They also showed that the action of ouabain was different from that of triethyltin. At a low concentration of triethyltin the oxidation of $[6-^{14}C]$ glucose was greatly inhibited, but at the same or higher concentrations the oxidation of $[3-^{14}C]$ pyruvate was slightly stimulated. These findings were an extension of those reported by Cremer (1962). When glucose was the substrate neither 100mM-potassium chloride nor 2,4-dinitrophenol increased the oxidation above the very low rate observed with triethyltin only. When pyruvate was the substrate and triethyltin was present with either of the stimulating agents the rate of oxida-

tion was the same as that of slices to which no agent had been added.

Lactic acid production. Isolated cerebral-tissue preparations have a very high rate of lactic acid formation under optimum conditions, indicating the intrinsic capacity of this tissue to convert pyruvate into lactate. The K_m for pyruvate is 10 μ M for L-lactate-NAD oxidoreductase (EC 1.1.1.27) (McIlwain, 1959). The amount of endogenous pyruvate present in a brain-cortex slice oxidizing glucose is about 60 μ M (Cremer, 1957). When added as exogenous substrate the pyruvate concentration is 200 times that present endogenously. Significantly less lactic acid was formed by brain-cortex slices with pyruvate as substrate than with glucose (Table 10).

Each of the agents tested increased the concentration of lactic acid when glucose was the substrate. The greatest increases were with triethyltin and oligomycin. With each of these agents lactic acid was increased at least threefold at a time when the oxygen uptake was only slightly inhibited. In complete contrast, none of the compounds tested brought about an increase in lactic acid when pyruvate was the added substrate. These results seem pertinent to a consideration of the factors controlling glucose metabolism in brain.

Conclusions. Glycolysis takes place extramitochondrially. A consideration has to be given as to how, in an integrated cellular structure, the pyruvate formed from glucose extramitochondrially is normally channelled into being oxidized by the mitochondria rather than being reduced to lactic acid.

Many studies have been made on the control factors in glycolysis. The systems most used are unable to oxidize pyruvate, so that the end product is lactic acid. In systems of this type it has been shown that two of the most important rate-limiting reactions are catalysed by the enzymes ATP-D-hexose 6-phosphotransferase (EC 2.7.1.1) and ATP-D-fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.11) (see Lowry & Passonneau, 1964). However, from the present results there is no evidence that triethyltin, or any of the drugs used, prevented either the entry of glucose into brain-cortex slices or its conversion into lactic acid. The present discussion need not be concerned with the control of glycolysis if by this is meant the phosphorylation of glucose and its conversion into pyruvate. The point under consideration is the fate of the pyruvate as it is formed from glucose.

During glycolysis equimolar amounts of pyruvate and $NADH_2$ are produced. Theoretically, all the pyruvate could be reduced to lactic acid. Since this is not so, and at least 50% of the pyruvate is oxidized, a mechanism must exist for the oxidation of the extramitochondrial $NADH_2$ produced during

glycolysis other than by the action of L-lactate-NAD oxidoreductase. Whatever this mechanism may be it appears to be sensitive to the action of triethyltin and oligomycin. Triethyltin does not inhibit the oxidation of the pyruvate added as a substrate, but it does prevent the oxidation of the pyruvate formed by glycolysis. It is difficult to reconcile the known inhibition by triethyltin of pyruvate oxidation by isolated brain mitochondria (W. N. Aldridge, personal communication) with the lack of inhibition of the oxidation of pyruvate added to brain-cortex slices.

The present results show that concentrations of triethyltin and oligomycin that only slightly inhibit the respiration of brain-cortex slices will cause a two- to three-fold increase in the production of lactic acid from glucose. Tobin & Slater (1965) suggested that the effect of oligomycin on lactic acid was due to its inhibition of oxidative phosphorylation, which prevented the aerobic suppression of glycolysis. This explanation does not seem entirely satisfactory for low concentrations of oligomycin.

When pyruvate is the added substrate presumably NADH_2 is only generated within the mitochondria. The reduction of exogenous pyruvate to lactic acid was found to be limited and could not be increased by the addition of triethyltin or oligomycin. When both glucose and pyruvate were added together as exogenous substrates triethyltin and oligomycin could cause an increase in lactic acid. The findings outlined above indicate that NADH_2 generated in brain mitochondria is not readily accessible to the enzyme L-lactate-NAD oxidoreductase. The availability of extramitochondrially generated NADH_2 for this enzyme is increased by the addition of triethyltin or oligomycin to brain-cortex slices.

Although kidney slices oxidized glucose at about the same rate as brain-cortex slices the amount of lactic acid formed was very low. This would imply that the oxidation of extramitochondrially generated NADH_2 is highly efficient in this tissue. It is not prevented by the presence of triethyltin. However, in the presence of oligomycin lactic acid does increase (Wu, 1965; and Table 11).

Various mechanisms for the oxidation of extramitochondrial NADH_2 by different tissues have been considered (see Boxer & Devlin, 1961).

Among these the part played by the microsomal oxidizing enzymes seems worthy of further study.

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REFERENCES

- Aldridge, W. N. (1958). *Biochem. J.* **69**, 367.
 Aldridge, W. N. & Threlfall, C. J. (1961). *Biochem. J.* **79**, 214.
 Ashford, C. A. & Dixon, K. C. (1935). *Biochem. J.* **29**, 157.
 Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Beloff-Chain, A., Catanzaro, R., Chain, E. B., Masi, I., Pocchiar, F. & Rossi, C. (1955). *Proc. Roy. Soc. B*, **143**, 481.
 Bergmeyer, H.-U. (1963). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H.-U. New York and London: Academic Press Inc.
 Bilodeau, F. & Elliott, K. A. C. (1963). *Canad. J. Biochem.* **41**, 779.
 Boxer, G. E. & Devlin, T. M. (1961). *Science*, **134**, 1495.
 Buhler, D. R. (1962). *Analyt. Biochem.* **4**, 413.
 Cremer, J. E. (1957). *Biochem. J.* **67**, 87.
 Cremer, J. E. (1959). *Brit. J. industr. Med.* **16**, 191.
 Cremer, J. E. (1962). *J. Neurochem.* **9**, 289.
 Cremer, J. E. (1964). *J. Neurochem.* **11**, 165.
 Cremer, J. E. (1967). *Biochem. J.* **104**, 223.
 Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
 Gainer, H., Allweiss, C. & Chaikoff, I. L. (1962). *J. Neurochem.* **9**, 433.
 Gainer, H., Allweiss, C. & Chaikoff, I. L. (1963). *J. Neurochem.* **10**, 903.
 Gonda, O. & Quastel, J. H. (1962). *Biochem. J.* **84**, 394.
 Hoskin, F. C. G. (1960). *Arch. Biochem. Biophys.* **91**, 43.
 Huijing, F. & Slater, E. C. (1961). *J. Biochem., Tokyo*, **49**, 493.
 Katz, J., Abraham, S. & Baker, N. (1954). *Analyt. Chem.* **26**, 1503.
 Kini, N. N. & Quastel, J. H. (1959). *Nature, Lond.*, **184**, 252.
 Lowry, O. H. & Passonneau, J. V. (1964). *J. biol. Chem.* **239**, 31.
 McIlwain, H. (1952). *Biochem. J.* **52**, 289.
 McIlwain, H. (1959). *Biochemistry and the Control Nervous System*, 2nd ed., p. 79. London: J. and A. Churchill Ltd.
 Rose, S. P. R. (1965). *Biochem. Pharmacol.* **14**, 589.
 Sutherland, V. C., Burbridge, T. N. & Elliott, H. W. (1955). *Amer. J. Physiol.* **180**, 195.
 Tobin, R. B. & Slater, E. C. (1965). *Biochim. biophys. Acta*, **105**, 214.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1951). In *Manometric Techniques and Tissue Metabolism*, p. 119. Minneapolis: Burgess Publishing Co.
 Wu, R. (1965). *J. biol. Chem.* **240**, 2373.