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The Ability of some Carboxylic Acids to maintain Phosphate Levels and support Electrical Stimulation in Cerebral Tissues

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(*Received* 15 *November* 1952)

In normal circumstances the energy requirements of mammalian brain are provided by glucose which is metabolized either completely to carbon dioxide and water or partially to lactate and pyruvate (for references, see Himwich, 1951). Glucose also enables creatinephosphate to be maintained in separated cerebral tissues (McIlwain, Buchel & Cheshire, 1951) and supports the additional respiration induced in such tissues by applied electrical impulses (McIlwain, 1951*b*). The complete oxidation of glucose in cerebral tissues can proceed through the tricarboxylic acid cycle (Coxon & Peters, 1950; Coxon, 1952; for earlier work see Quastel, 1939) and the present investigations were carried out to determine whether carboxylic acids intermediate in glucose catabolism would maintain creatinephosphate or allow response to applied impulses. Glutamic acid has already been examined from this point of view (McIlwain, 1951*a*; 1952*a*).

METHODS

The saline contained 0·128 M-NaCl, 0·0051 M-KCl, 0·00128 M-KH_2PO_4, 0·00128 M-$MgSO_4$, 0·05 M-glycylglycine (brought to pH 7·4 with NaOH) and 0·00275 M-$CaCl_2$. In some experiments, specifically mentioned in the text, this concentration of $CaCl_2$ was reduced by half and in some omitted completely; a corresponding volume of NaCl was added. In a few cases, also specially noted in the text, the glycylglycine was replaced by an equal volume of Na_2HPO_4 (brought to pH 7·4 by HCl) to give a final concentration of 0·01 M. The substrates used were either glucose or a carboxylic acid (pyruvic, succinic, α-ketoglutaric, citric or fumaric). The latter were added to the medium as solutions of the Na salt prepared by neutralizing the free acids. Glucose was added to 0·0056 M; and the carboxylic acid to 0·0056 or 0·02 M. Media were oxygenated at 37°.

Guinea-pig cerebral cortex slices were cut parallel to the outer surface and weighed as described by McIlwain (1951*b*). Slices which were to be exposed to impulses were in the type *D* tissue-holding electrodes which were used in conjunction with the type A^1 electrode vessels described by McIlwain (1951*b*). Control slices were usually freely suspended in the medium.

In most cases alternating electrical impulses were applied from a condenser as described by McIlwain (1951*b*), with a pulse frequency 100/sec., time constant about 0·1 msec., peak potential 15–20 V. Some of the slices respiring in pyruvate were activated by an applied voltage of 2·5 V. supplied by a transformer run from the 50 cyc. a.c. mains. The results of experiments using this form of excitation have been included with those obtained with condenser pulses as the responses from the two impulses were similar quantitatively.

At the end of the experiment, the slices were rapidly removed from the electrodes or vessels in the manner described by McIlwain & Gore (1951). Phosphates were determined either by a chemical method involving Ca-ethanol separation (McIlwain *et al.* 1951), or by the enzymic method described by Kratzing & Narayanaswami (1953). In a few experiments, creatine was determined as creatinine (McIlwain *et al.* 1951).

Synthetic phosphopyruvate was prepared by the method of Baer & Fischer (1949). The variation in the amounts of

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creatinephosphate accumulated by pyruvate oxidation was greater than for glucose but no explanation has been found for this difference. Since there was a possibility that phosphopyruvate was being estimated as creatinephosphate, the phosphate liberated from synthetic phosphopyruvate in the course of Ca-ethanol separation was determined. This showed that only about 5% of the phosphopyruvate was measured as creatinephosphate by the Ca-ethanol procedure.

RESULTS

Respiration

Rates of unstimulated respiration of cerebral cortex slices during the first 20–30 min. after equilibration are given in Table 1. The rate of respiration with glucose as substrate was similar to that obtained earlier (Kratzing, 1951). The concentration of glucose used in these experiments gave rates of respiration comparable with that obtainable at the higher concentration of 0·013 M used by McIlwain & Gore (1951) and supported respiration without any fall in rate for the duration of the experiment.

Respiration rates with pyruvate were identical with those for glucose and were similar to values calculated from Long's (1938) results with pigeon-brain brei. With succinate, the rate of respiration was dependent on its initial concentration; 0·02 M gave decidedly higher rates than 0·0056 M. (An increase in the rate of oxygen uptake by rat-heart slices on increasing the concentration of succinate had been reported by Webb, Saunders & Thienes, 1949.) The other substrates were investigated only at 0·02 M. Of these, citrate was the only one to support a uniform rate of respiration for the duration of the experiment. Both succinate and α-ketoglutarate showed a slight drop in respiration after the first 30 min. Although slices in fumarate had an initial rate of respiration which was similar to slices with no added substrate, the former maintained a higher rate of respiration than the latter at the end of the experiment (see Table 2).

Table 1. *Mean initial respiration rates of cerebral cortex slices with various substrates*

(Slices of guinea-pig cortex were in glycylglycine saline containing 2·75 mM-Ca^{2+} at 37°.)

Substrate	Concentration (mM)	No. of determinations	Initial O_2 consumption (μmoles/g. wet wt./hr.)
Glucose	5·6	36	55·5±1·5*
Pyruvate	5·6	11	56·4±2·3
Succinate	5·6	7	78·0±3·1
Succinate	20	28	95·6±4·6
α-Ketoglutarate	20	16	67·9±1·2
Citrate	20	16	61·1±2·8
Fumarate	20	12	46·8±2·1
None	—	10	42·7±3·1

* Standard error of the mean.

Effect of electrical impulses. Respiration rates for cerebral slices with different substrates during the passage of electrical impulses are shown in Table 2. Since placing cerebral cortex slices in tissue-holding electrodes caused only minor changes in the respiratory rate of slices respiring with glucose (McIlwain, 1951*b*; Kratzing, 1951), all values for control

Table 2. *Oxygen consumption on exposure to electric current*

(Guinea-pig cerebral cortex slices were respiring in glycylglycine saline at 37°. Substrate concentration for glucose and pyruvate was 5·6 mM; other substrates were at 20 mM. The initial period was for 20–30 min. after equilibration and was followed by a period of 20–30 min. in which the 'exposed' tissue received impulses from a condenser as described in text. 'Control' tissue received no electric impulses.)

Substrate	Tissue	No. of determinations	Mean O_2 consumption (μmoles/g. wet wt./hr.) Initial period	Second period	Mean change and S.E.M (%)	Statistical significance of mean change in exposed tissue to mean change in control tissue
Glucose	Control	14	55·4	57·4	4·3±2·4	S ($P<0·001$)
	Exposed	14	53·8	94·1	74·6±8·1	
Pyruvate	Control	5	56·2	56·2	0·0±0·0	S ($P<0·001$)
	Exposed	5	56·8	92·8	76·7±6·8	
Succinate	Control	8	86·6	81·0	−6·1±2·3	NS
	Exposed	8	88·8	78·9	−10·2±4·2	
α-Ketoglutarate	Control	8	66·1	62·4	−5·5±1·8	NS
	Exposed	8	69·6	65·8	−9·2±3·2	
Citrate	Control	8	55·9	55·9	0·0±5·0	NS
	Exposed	8	66·3	61·3	−6·4±4·7	
Fumarate	Control	6	45·0	38·5	−14·2±3·7	NS
	Exposed	6	48·7	37·7	−16·9±3·5	
None	Control	4	37·0	25·7	−27·9±10·0	S ($P<0·05$)
	Exposed	4	43·5	26·5	−39·4±7·2	

S, significant; NS, not significant.

slices, whether held in electrodes or free, have been combined. The statistical significance of observed changes in exposed slices compared with those for control slices has been estimated by the t test.

Respiration of cerebral slices metabolizing glucose or pyruvate showed a significant increase ($P < 0.001$) over the control slices during exposure to impulses. No other substrate used was able to support the respiratory response to electrical impulses. Indeed it appeared that electrical impulses caused a slight depression of respiration. This was marked in the experiments with no added substrate.

Effect of Ca^{2+}. Since citrate combines with Ca^{2+}, experiments were undertaken to find the effect of low calcium concentrations on the rate of respiration of cerebral cortex slices during electrical stimulation. Dickens & Greville (1935) and Krebs & Eggleston (1938) have shown the inhibitory effect of Ca^{2+} on respiration, and recently Gore & McIlwain (1952) have shown that reduced Ca^{2+} concentrations in the medium caused an increased rate of respiration in cerebral cortex slices.

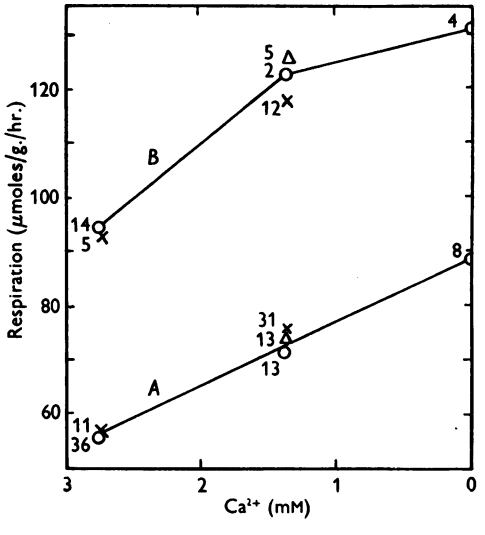

Fig. 1. Respiration of cerebral tissues and Ca^{2+} content of media. *A*, mean values in experiments with sections which did not receive electrical impulses; *B*, mean values in experiments with sections which received electrical impulses as described in text. ○, 5·6 mM-glucose with glycylglycine buffer; ×, 5·6 mM-pyruvate with glycylglycine buffer; △, 5·6 mM-pyruvate with phosphate buffer. The numbers adjacent to points denote the number of experiments in each group.

The respiratory rates of stimulated and unstimulated cerebral slices have been measured in glucose- or pyruvate-containing media in which the Ca^{2+} concentrations have been varied. Fig. 1 shows that the rate of respiration of unstimulated slices, with glucose as substrate, increased with lowered Ca^{2+} concentrations. The same results were obtained with pyruvate as substrate. On electrical stimulation the greatest response to the impulses was obtained with less Ca^{2+} than the 0·00275 M normally used.

Determination of phosphates

Inorganic. The mean values of the inorganic phosphate content of cerebral cortex slices, obtained by pooling the results of several experiments are shown in Table 3. The values obtained with glucose are within the range reported by McIlwain *et al.* (1951). Cerebral cortex slices respiring in pyruvate had a significantly greater inorganic phosphate content than slices in glucose. The highest inorganic phosphate concentrations were obtained with slices in succinate media.

Table 3. *Inorganic phosphate content of slices under various conditions*

(Pooled results from several experiments. The values for phosphates are adjusted for the inorganic phosphate content of adhering saline as described by McIlwain *et al.* (1951).)

Substrate (mM)	Ca^{2+} in medium (mM)	No. of determinations	Mean inorganic phosphate content, ±standard error (μmoles/g. wet wt.)
Glucose, 5·6	1·38	9	3·43±0·15
Glucose, 5·6	2·75	4	3·28±0·27
Pyruvate, 5·6	1·38	15	4·22±0·22
Pyruvate, 5·6	2·75	6	4·14±0·38
Succinate, 5·6	2·75	7	6·40±0·27
Succinate, 20	2·75	10	8·02±0·25

Energy-rich phosphates. The enzymic method of phosphate analysis described by Kratzing & Narayanaswami (1953) has been used to measure the ability of some carboxylic acids to maintain the energy-rich phosphate content of cerebral cortex slices as well as to study the effect of electrical impulses on the levels of this phosphate. In these studies the energy-rich phosphates determined were creatinephosphate (CP), adenosinetriphosphate (ATP), and adenosinediphosphate (ADP) since together these compounds account for most of the energy-rich phosphates of tissue. Results obtained by this method of analysis for the phosphate content of cerebral cortex slices respiring in glucose, citrate, succinate or fumarate media are given in Table 4. This method is not suitable for the estimation of phosphates under all circumstances (see Slater, 1953) and could not be used for experiments with pyruvate. It was also found unsatisfactory for the experiments with α-ketoglutarate.

Glucose maintained the greatest amount of energy-rich phosphate in the slices (3·01 ± 0·18 μmoles/g. wet weight tissue). Citrate was the most effective of

Table 4. *Effect of substrates and electrical impulses on phosphates of cerebral cortex slices*

(Guinea-pig cortex slices were respiring under conditions described in Table 2. The phosphates were determined by an enzymic method. The electrical impulses were condenser discharge, as described in text. The 'exposed' tissue received the electrical impulses.)

Substrate	Tissue	No. of determinations	Total energy-rich phosphate (±standard error) (μmoles/g. wet wt.)	Creatine-phosphate (μmoles/g. wet wt.)	Adenosine-triphosphate (μmoles/g. wet wt.)	Adenosine-diphosphate (μmoles/g. wet wt.)
Glucose	Control	6	3·01±0·18	1·11	0·89	0·02
	Exposed	6	1·96±0·13	0·63	0·63	0·11
Citrate	Control	4	1·68±0·15	0·87	0·32	0·17
	Exposed	4	1·02±0·11	0·69	0·06	0·08
Succinate	Control	3	0·87±0·02	0·45	0·07	0·25
	Exposed	3	0·72±0·05	0·42	0·05	0·21
Fumarate	Control	3	0·76±0·13	0·44	0·12	0·11
	Exposed	3	0·66±0·08	0·38	0·09	0·09
No substrate	Control	2	0·66	0·37	0·13	0·03
	Exposed	2	0·66	0·41	0·11	0·02

the carboxylic acids tested in maintaining the amount of energy-rich phosphate. Despite the high oxygen consumption of slices in a succinate medium, the energy-rich phosphate content of the slices was found to be 0·87 μmole/g. wet weight tissue, a value only slightly higher than the 0·66 μmole/g. wet weight tissue found in slices with no added substrate. (Cross, Taggart, Covo & Green (1949) have found that succinate oxidation supported a much lower esterification of phosphate than other substrates in washed particles of kidney and liver.) Slices respiring in fumarate showed little difference in their content of energy-rich phosphate from those with no added substrates.

Change in energy-rich phosphates. The total energy-rich phosphates of cortex slices respiring in glucose media showed a significant decrease ($P < 0·01$) from the control value after exposure to electrical impulses. The same result was obtained with slices respiring in citrate and, to a lesser extent, in succinate media. With fumarate and no added substrate, electrical impulses caused no change in the energy-rich phosphate content of the slices.

The CP content of slices respiring in glucose was 1·11 μmoles/g. wet weight tissue and electrical stimulation reduced this value to 0·63 μmole/g. tissue. These values are in good agreement with those obtained by McIlwain & Gore (1951). Citrate supported a CP content of 0·87 μmole/g. tissue. This value was reduced to 0·69 μmole/g. tissue by the passage of electrical impulses. The CP levels with succinate and fumarate were no higher than with no added substrate and were little affected by the electrical impulses.

Glucose produced a higher content of ATP than any of the other substrates, although this amount was only about half of the ATP content of rapidly frozen cerebral cortex (Kratzing & Narayanaswami, 1953). As with cortex *in vivo*, slices respiring in a glucose medium contained no ADP. Electrical stimulation caused lowered ATP content of the slice from 0·89 to 0·63 μmole/g. tissue and this was accompanied by the production of 0·11 μmole/g. tissue ADP.

Less ATP was maintained in the presence of the other substrates. The content with citrate was 0·32 μmole/g. tissue and this value was reduced by the passage of electrical impulses. With fumarate and also without added substrate, a content of approx. 0·1 μmole/g. tissue was maintained and this low value was reduced by half in slices respiring in succinate. The passage of electrical impulses produced no change in the content of ATP in these cases. All substrates except glucose caused the appearance of definite amounts of ADP. The ADP content of slices with succinate was 0·25 μmole/g. tissue and nearly double that found with citrate and fumarate. Electrical impulses caused no changes in the ADP content in these cases.

With the enzymic method of phosphate determination it was not possible to measure the energy-rich phosphates and the phosphorylated sugars in an extract prepared from 0·1 to 0·3 g. cerebral tissue. A value was obtained for the total of all the phosphorylated sugars, including triosephosphates, as well as for non-phosphorylated substances which interfere with the determination. Kratzing & Narayanaswami (1953) deal more fully with this determination called the 'blank', and with the probable range of phosphates measured. Glucose gave the greatest 'blank' value of 0·28 μmole/g. tissue which was nearly three times that for citrate, succinate and fumarate. With no added substrate the 'blank' was zero. The passage of electrical impulses did not alter the values of the 'blank'.

Some values for CP with pyruvate as substrate were obtained by a different method and are not reported in detail. They suggested that pyruvate

maintained a level of CP similar to that with glucose, and that this level fell on passage of electrical impulses.

Table 5. *Effect of succinate concentration on tissue creatinephosphate*

Substrate (M)	Creatinephosphate determined as: Phosphorus (μmoles/g. wet wt.)	Creatinine (μmoles/g. wet wt.)
0·0056	1·9, 1·6, 1·0 1·4, 1·5	—
0·02	0·3, 0·3 0·3, 0·3	0·3, 0·3 0·3, 0·2

During succinate oxidation, substrate concentration appeared important for the maintenance of the CP level as shown in Table 5. The higher concentration of succinate gave a markedly lower level of CP. Furchgott & Shorr (1948) had previously found that the amount of CP in slices of dog cardiac muscle or intestinal smooth muscle was lower when succinate was added to the medium than in its absence.

DISCUSSION

Respiratory rates observed in the absence of applied potentials in the present studies are comparable with those of previous reports (Elliott, Greig & Benoy, 1937; Jowett & Quastel, 1937; Krebs & Johnson, 1937; Lipsett & Crescitelli, 1950; Quastel & Wheatley, 1931, 1932).

The new findings with respect to respiratory rates with applied impulses, and the levels of phosphate derivatives, enable suggestions to be made on how the impulses affect metabolism. Because slices respiring in pyruvate exhibit respiratory response to impulses, the glycolytic breakdown of glucose is not essential to response. Other carboxylic acids which may not allow the full functioning of the tricarboxylic acid cycle, supported neither high levels of energy-rich phosphates nor respiratory response to impulses. With citrate, where an intermediate level of energy-rich phosphate was maintained, electrical impulses lowered these without altering the respiration rate of the slice. This would indicate that the change in phosphates on stimulation is a more fundamental change than the alteration in the rate of respiration. Many previous observations have connected the labile phosphates with respiratory response (McIlwain, 1952*b*).

From the results of Brody & Bain (1952) and Abood & Gerard (1952) it is apparent that pyruvate, succinate, citrate, fumarate and glutamate are all capable of being oxidized by particulate preparations from mammalian brain and this oxidation results in an esterification of inorganic phosphate which is similar quantitatively for all substrates except citrate and succinate. Thus the low values for labile phosphate now found in slices with fumarate as substrate must be due to factors not operating in mitochondria, possibly concerning transportation to site of reaction. Rate of production of available energy-rich phosphate after other such energy requirements have been satisfied appears to determine whether a substrate can support the metabolic response to electrical impulses.

In intact cerebral cortex cells, as distinct from mitochondrial preparations, there is a limited ability to oxidize malate or fumarate (Quastel & Wheatley, 1932; Elliott *et al.* 1937). This may be due to organization of the enzymes responsible for this oxidation in the case of cells, so that in the intact cell the tricarboxylic acid cycle functions slowly unless pyruvate is present to combine with oxaloacetate and drive the malate-fumarate equilibrium forward. In other words, the tricarboxylic acid cycle may proceed at its maximal speed only when pyruvate is present. This in turn would govern the rate of production of available energy-rich phosphate and thus determine the capacity to respond to electrical impulses.

SUMMARY

1. With pyruvate (as well as with glucose) as substrate, respiration of cerebral cortex slices increased on passage of electrical impulses.

2. Respiration of slices in glucose or pyruvate was dependent on the concentration of calcium ion in the media. The two substrates gave similar respiratory rates with two concentrations of calcium ion. Electrical stimulation produced rates of respiration which were also dependent on the calcium ion concentrations of the medium.

3. Respiration of slices metabolizing 0·02 M-succinate, α-ketoglutarate, citrate or fumarate was not increased by the application of electrical impulses.

4. There was a significant fall in the energy-rich phosphate content of slices respiring in glucose, citrate or succinate, during the passage of electrical impulses.

I wish to thank Dr H. McIlwain for his help and advice given during this work which was carried out during the tenure of a scholarship from the Australian National University.

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The Enzymic Determination of Energy-rich Phosphates in Brain

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(*Received* 15 *November* 1952)

Existing methods of measuring various phosphate esters in animal tissues are open to improvement in two ways. They are not sufficiently specific for certain constituents and they require relatively large amounts of tissue to carry out a complete analysis. In order to follow phosphate metabolism in preparations of cerebral tissues such as the slices used metabolically, it has been necessary to adapt methods of Kerr (1935) and Stone (1943) to deal with minimal quantities of material (see McIlwain, Buchel & Cheshire, 1951). Moreover, when such methods are used, even with the refinements which have been introduced to adapt them for minimal quantities of material, one can obtain values only for inorganic phosphate, creatinephosphate and total easily hydrolysable phosphate. Thus it is not possible to follow changes in adenosinetriphosphate or adenosinediphosphate specifically. Recent findings of Kornberg (1950) indicate that changes in inorganic pyrophosphate during metabolism are more extensive than previously realized. Changes in easily hydrolysable phosphate, therefore, may not necessarily be a true measure even of total nucleotide phosphate.

The introduction of spectrophotometric methods of phosphate analysis, based on the enzymic formation of dihydroxyacetonephosphate and its reduction of reduced diphosphopyridine nucleotide (Racker, 1947; Slater, 1951), offered a means of following the metabolism of phosphates in small pieces of tissue with the advantage of a high degree of specificity. Since we were mainly interested in the changes in individual energy-rich phosphates (adenosinetriphosphate, adenosinediphosphate and creatinephosphate) under various metabolic conditions, we have adapted Slater's (1953) methods of phosphate determination to our particular needs for cerebral tissues and have compared analyses by this method with that of Stone (1943). Previous publications from these laboratories have reported on the ability of different substrates and metabolic conditions to support the resynthesis of creatinephosphate and other labile phosphates in respiring slices of mammalian cerebral cortex (McIlwain *et al.* 1951; McIlwain & Gore, 1951; McIlwain, 1951, 1952*a*). It was of interest to know whether similar changes took place in the adenosinepolyphosphates of the slices, and whether there was an interrelation between changes in creatinephosphate and changes in the adenosinepolyphosphates. As the phosphocreatine and adenosinepolyphosphate content of

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