incubation, and suggested an autocatalytic activation of some part of the enzyme system as the cause. In the only previous case in which a time curve of oxaloacetate-pyruvate oxidation by plant mitochondria has been given, a lag phase is clearly apparent (Brummond & Burris, 1953); this was interpreted by the authors as an inhibition by the oxaloacetate, although the rate once established was as rapid as with malate, and it now seems to us that the alternative explanation outlined above is the more likely one.

Whatever may be the precise explanation of the spontaneous termination of the lag periods observed in the absence of one or more cofactors, the point that we wish to emphasize is that the marked stimulation of pyruvate oxidation by the full complement of cofactors, the initiation by oxaloacetate (or another system which would be expected to lead to the regeneration of coenzyme A) and the initial failure of the reaction to proceed at measurable rates in the absence of any one coenzyme provide strong grounds for believing that these substances are directly implicated in a reaction sequence analogous to that elucidated by recent work on isolated enzymes. This reaction sequence would appear to proceed both in the oxidation of an individual acid which was giving rise to an acetate acceptor as well as in the functioning of the cycle as an integrated unit, *in vivo*.

SUMMARY

1. The conditions under which pyruvate is oxidized by plant mitochondrial preparations, particularly those from the endosperm of germinating *Ricinus* seeds, have been investigated.

2. It has been established that maximal initial rates of oxygen uptake are not obtained unless coenzyme A, cocarboxylase and an acetyl-acceptor system, as well as adenosine triphosphate and diphosphopyridine nucleotide, are provided.

3. An induction period before the onset of oxygen uptake has been found to be a feature of pyruvate oxidation in certain circumstances. Possible explanations of this effect are discussed.

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Metabolic Effects of Azide on Electrically Stimulated Cerebral Cortex

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The metabolic rate of slices of cerebral tissue *in vitro* can be increased by the application of electrical pulses (McIlwain, 1951a; McIlwain, Anguiano & Cheshire, 1951). Heald (1953) demonstrated that metabolic inhibitors were able to depress the response to applied electrical pulses at concentrations which had negligible effects on the normally respiring tissue. A greater sensitivity to inhibitors had previously been reported by Gerard

& Doty (1950), who observed that azide could abolish the extra oxygen consumption of active nerve, while leaving the resting respiration virtually unaffected.

The present investigations were carried out to extend Gerard & Doty's (1950) observations and to determine the sensitivity of cerebral tissue to azide when metabolizing glucose as well as other substrates.

METHODS

The saline contained 0.137 M-NaCl, 0.0055 M-KCl, 0.00295 M-CaCl₂, 0.00137 M-KH₂PO₄, 0.00137 M-MgSO₄, 0.05 M tris-(hydroxymethyl)aminomethane (tris) (brought to pH 7.4 by HCl). The substrate used was glucose, sodium pyruvate or sodium glutamate. Glucose was added to 0.0056 M, pyruvate to 0.0112 M and glutamate to 0.02 M. Incubations were under O₂ at 37°.

Guinea-pig cerebral cortex slices were cut parallel to the outer surface and weighed as described by McIlwain (1951a). Slices were held in the type D tissue-holding electrodes which were used in conjunction with the type A'-manometric electrode vessels described by McIlwain (1951a). Alternating electrical pulses were generated by condenser discharge with a pulse frequency of 100/sec., time constant of 1 msec. and peak voltage 5-7 v, depending on the resistance of individual electrodes (McDonnell, unpublished work).

Aqueous solutions of sodium azide were prepared daily in such concentration that the addition of 0.2 ml. of azide to 7 ml. of saline (the volume in the vessels) gave the final concentration desired.

The manometers containing the slices were placed in a thermostat between 25 and 35 min. after the death of the

animal and equilibrated for 12 min., at which time the azide was tipped into the main compartment from the side arm. The rate of respiration was measured then for 1 hr., the electrical pulses being applied for the last half hour of this experimental period.

At the end of the experiment, the slices were rapidly removed from the electrodes in the manner described by McIlwain & Gore (1951). Inorganic phosphate was determined by the method of Ennor & Stocken (1950). Energy-rich phosphate (the sum of the creatine phosphate, adenosine triphosphate and adenosine diphosphate contents of the tissue) was determined by the enzymic method of Kratzing & Narayanaswami (1953). Lactate was measured by the method of Barker & Summerson (1941).

RESULTS

Oxygen uptake of cerebral cortex slices

Normal respiration. The rates of unstimulated respiration of cerebral cortex slices for the first 30 min. period after equilibration are included in Table 1. The rates of respiration with glucose or pyruvate as substrate, in the absence of azide, were similar to those obtained earlier (Kratzing, 1953).

Table 1. *Effect of sodium azide on respiration rates of cerebral cortex slices with various substrates*

Slices of guinea-pig cortex were respiring in tris-saline buffer at 37°. Substrate concentration for glucose was 5.6 mM, pyruvate 11.2 mM and glutamate 20 mM. The initial period was for 30 min. after equilibration and was followed by the second period of 30 min. in which the 'exposed' tissue received pulses from a condenser as described in text. 'Control' tissue received no electrical pulses. Sodium azide was tipped from vessel side arm just before commencement of manometric readings.

Substrate	Azide concn. (mM)	Treatment	No. of determinations	Mean O ₂ consumption and S.E.M. (μmoles/g. wet wt./hr.)	
				Initial period	Second period
Glucose	0	Control	27	55.3 ± 1.3	55.5 ± 0.2
		Exposed	27	53.7 ± 1.1	116.3 ± 2.1
	0.25	Control	18	58.6 ± 1.2	51.3 ± 1.9
		Exposed	18	61.2 ± 1.9	60.1 ± 2.8
	0.5	Control	14	52.8 ± 3.3	41.9 ± 5.6
		Exposed	14	56.4 ± 2.7	36.4 ± 3.9
Pyruvate	0	Control	15	56.5 ± 2.2	56.5 ± 2.2
		Exposed	16	58.4 ± 1.6	124.4 ± 4.7
	0.25	Control	8	59.4 ± 3.5	53.4 ± 3.6
		Exposed	7	62.1 ± 3.5	90.7 ± 3.5
	0.5	Control	7	58.0 ± 2.7	53.3 ± 3.1
		Exposed	8	59.2 ± 3.5	75.9 ± 6.0
	0.75	Control	5	60.6 ± 2.8	55.6 ± 3.2
		Exposed	5	66.8 ± 4.1	59.2 ± 1.6
Glutamate	0	Control	10	67.9 ± 1.8	67.7 ± 1.9
		Exposed	10	69.8 ± 1.6	81.6 ± 2.2
	0.25	Control	4	69.3 ± 2.5	66.0 ± 3.0
		Exposed	4	69.3 ± 1.3	73.0 ± 5.2
	0.5	Control	6	68.8 ± 2.0	63.0 ± 2.6
		Exposed	6	67.0 ± 2.9	64.8 ± 3.1
	0.75	Control	4	64.3 ± 2.5	53.0 ± 1.4
		Exposed	4	65.3 ± 1.7	51.0 ± 2.2
None	0	Control	4	42.7 ± 3.8	36.7 ± 2.5
		Exposed	4	44.0 ± 0.7	42.0 ± 2.2
	0.5	Control	4	42.3 ± 2.6	28.2 ± 1.2
		Exposed	4	45.3 ± 1.6	29.7 ± 2.6

The initial rate of respiration with glutamate was higher than that for glucose and agrees with McIlwain's (1952) observations with this substrate.

Effect of azide. The concentrations of azide used in these experiments had only a minor effect on the respiration rate of the tissue which did not receive electrical pulses. The respiration of slices metabolizing pyruvate was unaffected by concentrations of azide up to 0.75 mM. On the other hand, this concentration of azide caused a fall of 18% in the respiration rate during the second 30 min. period with glutamate as substrate. Tissues metabolizing glucose were more sensitive to azide than those metabolizing the other substrates studied, and at 0.5 mM azide caused an inhibition in respiration of over 20%.

Effect of electrical pulses. The respiration rate of slices metabolizing glucose or pyruvate in the absence of azide was increased markedly by the application of electrical pulses, as has been reported previously (McIlwain, 1951a, 1953; Kratzing, 1953). It was found that the application of electrical pulses to slices respiring in glutamate-saline caused an increase in respiration which amounted to 17%. This was contrary to the findings of McIlwain (1951b, 1953), who reported that glutamate was a substrate which did not support a respiratory response to electrical pulses with guinea-pig cerebral tissue.

Tissue slices respiring in glucose-saline and receiving electrical pulses were most sensitive to azide (see Table 1). The increased respiration produced by the pulses was nearly completely inhibited by 0.25 mM azide and completely inhibited by 0.5 mM azide. There was a lower degree of inhibition with pyruvate as substrate. 0.5 mM Azide in pyruvate-saline caused only a 70% inhibition of the increased respiration produced by the pulses; 0.75 mM azide was required for complete inhibition. The slight increase in respiration obtained by electrical pulses with glutamate as substrate was abolished by the presence of 0.25 mM azide.

Lactate production

The effects of azide on both the stimulated and unstimulated production of lactate are shown in Table 2. Over the range of azide concentration studied, the extra lactate produced in the presence of azide was approximately proportional to the azide concentration. This relationship also held with tissue receiving electrical pulses.

Changes in concentration of phosphates

Energy-rich phosphates. The mean values of the energy-rich phosphate content of cerebral cortex slices after respiration in glucose for 1 hr. are given

in Table 3. The values obtained for the energy-rich phosphate content of cerebral cortex slices in the absence of azide are comparable with those obtained earlier (Kratzing, 1953). In unstimulated slices, 0.5 mM azide caused a fall of 40% in the energy-rich phosphate content of the tissue, and in slices exposed to electrical pulses the same azide concentration caused a fall of 65%.

Table 2. *Effect of azide on lactate production*

Guinea-pig cerebral cortex slices were respiring in tris-saline buffer with 5.6 mM glucose under O_2 at 37° for 75 min. 'Exposed' tissue received alternating electrical pulses.

Azide concn. (mM)	Treatment	No. of determinations	Mean production of lactate and S.E.M. (μ moles/g. wet wt./experimental time)
0	Control	9	33.1 $\pm$ 3.2
	Exposed	9	50.8 $\pm$ 5.0
0.25	Control	8	50.4 $\pm$ 5.7
	Exposed	8	75.7 $\pm$ 4.6
0.5	Control	8	67.4 $\pm$ 8.2
	Exposed	8	96.3 $\pm$ 7.8

Table 3. *Effect of azide and electrical pulses on energy-rich phosphate content of cerebral cortex slices*

Guinea-pig cortex slices were metabolizing glucose under conditions described in Table 1. The 'exposed' tissue received the electrical pulses.

Azide concn. (mM)	Treatment	No. of determinations	Total energy-rich phosphate and S.E.M. (μ moles/g. wet wt.)
0	Control	8	3.05 $\pm$ 0.19
	Exposed	8	2.38 $\pm$ 0.24
0.25	Control	8	2.63 $\pm$ 0.12
	Exposed	8	1.75 $\pm$ 0.16
0.5	Control	8	1.81 $\pm$ 0.14
	Exposed	8	0.82 $\pm$ 0.05

Inorganic phosphate. The mean values of the inorganic phosphate content of cerebral cortex slices, obtained from slices respiring in glucose, pyruvate and glutamate salines, are given in Table 4. The values obtained with glucose and pyruvate with slices not receiving pulses and in the absence of azide are comparable with those obtained earlier (see McIlwain, Buchel & Cheshire, 1951; Kratzing, 1953). The values found for unstimulated tissue without azide and with glutamate as substrate were greater than those reported by McIlwain (1951b), but were comparable to those of Kratzing & Narayanaswami (1953). (See McIlwain, 1952, for discussion of metabolic changes with glutamate resulting in high inorganic phosphate.)

Increasing concentrations of azide increased the inorganic phosphate content of both unstimulated

slices and those exposed to electrical pulses. With all substrates tested, the application of pulses to the tissue produced a higher level of inorganic phosphate than that of the unstimulated tissue subjected to the same azide concentration. The difference in inorganic phosphate content between unstimulated slices and slices receiving pulses was greater with pyruvate than with the other substrates.

the extra oxygen consumption of caffeine-stimulated frog muscle. However, Stannard (1939) found that concentrations of azide of the order of 1.0 mM were required to attain maximum inhibition of stimulated respiration, which then coincided with the normal rates of respiration for resting muscle. This comparison of the effect of azide on muscular and nervous tissue suggests that nervous tissue is more sensitive to azide.

Table 4. *Effect of substrates and electrical pulses on inorganic phosphate of cerebral cortex slices*

Guinea-pig cerebral cortex slices were respiring in tris-saline under conditions described in Table 1. The 'exposed' tissue received the electrical pulses.

Substrate	Azide concn. (mM)	Treatment	No. of determinations	Mean inorganic phosphate and S.E.M. (μ moles/g. wet wt.)
Glucose	0	Control	11	3.4 ± 0.1
		Exposed	11	4.3 ± 0.1
	0.25	Control	7	5.6 ± 0.2
		Exposed	7	6.9 ± 0.1
	0.5	Control	4	5.8 ± 0.1
		Exposed	4	6.6 ± 0.3
Pyruvate	0	Control	10	4.3 ± 0.2
		Exposed	10	6.1 ± 0.4
	0.25	Control	5	4.9 ± 0.3
		Exposed	5	7.0 ± 0.4
	0.5	Control	5	4.7 ± 0.2
		Exposed	4	7.6 ± 0.7
	0.75	Control	4	5.8 ± 0.6
		Exposed	4	7.8 ± 0.7
	0	Control	10	6.7 ± 0.1
		Exposed	9	7.4 ± 0.4
Glutamate	0.25	Control	4	7.0 ± 0.3
		Exposed	4	7.5 ± 0.2
	0.5	Control	6	6.5 ± 0.3
		Exposed	5	7.4 ± 0.6
	0.75	Control	4	7.2 ± 0.7
		Exposed	4	8.3 ± 0.8
None	0	Control	4	6.6 ± 0.2
		Exposed	4	9.0 ± 0.4
	0.5	Control	4	7.9 ± 0.5
		Exposed	4	8.0 ± 0.2

DISCUSSION

In the present study, concentrations of azide were used which had only minor effects on the respiration rates of tissues which did not receive electrical pulses. However, these concentrations of azide were sufficient to inhibit strongly the respiratory response to pulses in the case of tissues utilizing glucose or pyruvate. Gerard & Doty (1950), using frog peripheral nerve, found that the extra oxygen consumption of stimulated nerve was inhibited by the addition of 0.1–0.3 mM azide; and previously Stannard (1939) has reported that azide inhibited

A new mode of action of azide inhibition has been elucidated by the work of Spiegelman, Kamen & Sussman (1948), Reiner (1948) and Loomis & Lipmann (1949). From the results of these workers it is apparent that azide inhibits oxidative phosphorylation in both microbial and mammalian tissues. The present results show that azide lowers the content of energy-rich phosphate in cerebral tissue. In slices receiving pulses, the fall in energy-rich phosphate is approximately the sum of the changes caused by the individual treatments (namely the application of pulses and the presence of azide). In slices utilizing glucose, the fall in

energy-rich phosphate is accompanied by an increase in the content of inorganic phosphate. This increase is much greater than would be expected by the lowering of energy-rich phosphate. Trevelyan, Gammon, Wiggins & Harrison (1952) have reported a marked rise in inorganic phosphate of yeast suspensions during fermentation in the presence of azide, and state that the rise in inorganic phosphate was due to increased decomposition of organically bound phosphate. It is possible that with cerebral tissue an analogous process of decomposition of a phosphorus-containing compound, other than those actually determined, may account for the increase in inorganic phosphate. In view of the low content of phosphorylated glycolytic intermediates in cerebral tissue (Kratzing, 1953), it is unlikely that they contribute significantly to the rise in inorganic phosphate in the presence of azide. The increase in inorganic phosphate of slices utilizing pyruvate was not as great as that for glucose at the same azide concentration. This, together with the data discussed above, could support the hypothesis for azide action proposed by Spiegelman *et al.* (1948) for yeast. They suggested that azide is able to form a complex with the acyl phosphate of diphosphoglycerate, while the latter is combined with glyceraldehyde 3-phosphate dehydrogenase. The resulting acyl azide would hydrolyse and the whole reaction would result in a liberation of inorganic phosphate. From the present results it is not possible to say at what stage azide affects the glycolytic cycle, but, since the sensitivity to azide of cerebral slices metabolizing glucose is greater than those utilizing pyruvate, it would seem possible that azide is an inhibitor of some enzymic process in the glycolytic pathway from glucose to pyruvate.

Heald (1953) found that with electrically stimulated cerebral tissue, iodoacetate and sodium fluoride inhibited the extra lactate produced as a result of stimulation whilst they caused only small changes in the respiration. On the other hand, malonate increased the lactate production and had only a slight effect on the respiration. In the present experiments with tissues utilizing glucose, azide increased both the lactate production of electrically stimulated tissues and the normal aerobic lactate production at very nearly the same rate. A feature of glucose metabolism in the presence of azide is that the degree of inhibition of the electrically stimulated respiration is greater than the accumulation of lactate resulting from the passage of the pulses. Handler (1945) has noted an accumulation of blood lactate in rabbits after injection of sodium azide.

In the present study, only the highest concentration of azide used had any effect on tissues metabolizing glutamate. At a concentration of 0.75 mM,

azide caused a fall in the respiration rate during the second half of the experiment. This fall in respiration was unaffected by the application of electrical pulses, although pulses caused a slight increase in respiration in slices without azide. Furthermore, increasing amounts of azide did not affect the content of inorganic phosphate in the slices not receiving pulses.

With slices utilizing only endogenous metabolites, azide was also without effect, whether or not the tissue was receiving pulses. Elliott & Sutherland (1952) also found that azide was without effect on the endogenous metabolism of human cerebral tissue.

SUMMARY

1. The effects of sodium azide on the oxygen uptake, lactate production, content of energy-rich phosphate and inorganic phosphate of electrically stimulated slices of guinea-pig cerebral cortex have been studied.

2. Azide depressed the stimulated oxygen uptake of tissue which was metabolizing glucose or pyruvate.

3. The lactate production of cerebral slices was increased by azide whether or not the tissue received electrical pulses.

4. The energy-rich phosphate content of tissue in glucose-saline was lowered by azide in both unstimulated and stimulated slices.

5. In unstimulated and stimulated slices, the inorganic phosphate content was increased by azide while the tissue metabolized glucose. With pyruvate or glutamate the increases in inorganic phosphate content were not so marked as those with glucose.

6. The respiration rate of cerebral cortex slices metabolizing glutamate was increased by 17% by the application of electrical pulses.

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The Metabolism of Glutamic Acid in Germinating Green Gram Seeds

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During germination of green gram seeds (*Phaseolus radiatus*), glutamic acid differs from the other amino acids by its rapid degradation (Sivaramakrishnan & Sarma, 1954a). It is converted into asparagine during germination (Sivaramakrishnan & Sarma, 1954b). This conversion takes place in Bengal gram (*Cicer arietinum*) and horse gram (*Dolichus biflorus*) seeds and may be presumed to be common to all legume seeds, supplying in part the energy requirements of their germination (Sivaramakrishnan, 1955). But a rough approximation revealed that, at the most, only a portion of the metabolized glutamic acid could have been converted into asparagine, suggesting the formation of other products. It has recently been shown that glutamic acid is converted into arginine and proline in *Escherichia coli* (Abelson, Bolton, Britten, Cowie & Roberts, 1953), and in rats (Salbach, Koeppe & Rose, 1951). The questions whether a similar conversion into arginine and proline takes place in germinating seeds also, and, if so, how far this conversion is quantitatively significant in the metabolism of glutamic acid, were deemed worth investigating. Hence a study of the metabolism of glutamic acid, using uniformly ^{14}C -labelled glutamic acid and involving the isolation of the four amino acids, glutamic acid, aspartic acid, arginine and proline, was conducted, and the details of the results obtained are presented here. These investigations on the catabolism of glutamic acid revealed that the rate of degradation of glutamic acid is far greater than was presumed earlier. They also suggested a simultaneous synthesis of glutamic acid during germination, and also that carbon dioxide is probably the major end-product of glutamic acid degradation.

EXPERIMENTAL

Materials

The green gram seeds were obtained from the Government Pulses Specialist, Coimbatore. They were carefully hand-picked, only the well-formed ones with a healthy green

colour being chosen, and the odd-sized or defective ones discarded. Seeds thus chosen invariably weighed 40 ± 5 mg. each; and at least 98–99% of these germinated.

L-Glutamic acid hydrochloride (4.6 mg., 0.1 mc), and D-glucose (1.3 mg., 0.1 mc), both uniformly labelled with ^{14}C , were obtained from the Radiochemical Centre, Amersham, England.

Methods

Catabolism of glutamic acid. All the apparatus and solutions used in germinations were previously sterilized by autoclaving at 15 lb./sq.in. pressure (120°) for 15 min.

Seeds (20 g.), divided into four equal portions, were used for the germination. The seeds were sterilized by soaking for 1–2 min. in 0.1% HgCl_2 , followed immediately by repeated washings with sterile distilled water. Under these conditions, no detrimental effects due to contact with HgCl_2 could be observed in the germination. Each portion was transferred aseptically into a sterile 11 cm. Petri dish containing a sterilized filter circle. The medium in each dish consisted of 0.23 mg. ^{14}C -labelled glutamic acid hydrochloride with an activity equal to 7.19×10^6 counts/min. in sterile water. The volume of the medium was adjusted with sterile water to 24 ml., which was just enough for 72 hr. germination. Germinations were carried out in diffuse light in a sterile chamber at room temp., which varied between 28 and 31°. Seedlings grown under these conditions, if crushed and plated out in a nutrient medium, were always found to be free from any bacterial contamination.

At the end of the germination, the seedlings and Petri dishes with filter-papers were washed separately, and the radioactivity in the collected washings was determined. This gave a measure of the maximum amount of the radioactive compound left behind.

The seedlings, containing approximately 17 g. dry solids and 85 ml. water, were ground to a fine paste, mixed with 125 ml. 10N-HCl (final vol. 210 ml. 6N-HCl) and refluxed for 22 hr., the solution being kept just boiling. Excess of HCl was then removed by vacuum distillation, the final concentrate was diluted with water and cooled in the ice chest to precipitate out the humin completely. The humin was filtered off and the hydrolysate made up to 200 ml. A sample of this solution (20 ml.) was removed for the estimation of the four amino acids, as well as the total radioactivity after suitable dilution. Arginine was estimated colorimetrically according to Macpherson (1946), glutamic acid manometrically by enzymic decarboxylation