Fluoroacetate and the Metabolism of Ammonia in Brain

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Much of the literature on the inhibition of cell respiration by fluoroacetate and on the accumulation of citrate in animal tissues after the administration of fluoroacetate has been summarized by Peters (1957). It is considered that the key reaction underlying the metabolic inhibitory effects of fluoroacetate is the formation of fluorocitrate, which suppresses citrate metabolism by its affinity for aconitase. Fluorocitrate is formed by the condensation of oxaloacetate and fluoroacetyl-CoA (Brady, 1955). However, pigeon-brain tissue, when used as a homogenate, apparently does not synthesize fluorocitrate from fluoroacetate, nor does the presence of fluorocitrate under these circumstances give rise to accumulation of citrate. When the brain tissue is more finely ground and re-inforced with ATP and Mg^{2+} ions, the presence of fluorocitrate causes accumulation of citrate in the presence of pyruvate (Peters & Wakelin, 1953). The convulsive effects caused by the administration of fluoroacetate seem not to be due to accumulation of citrate (Henderson & Chenoweth, 1954, 1955; Peters, 1957). Convulsions occur when there is little increase in the concentration of citrate in the brain, and the introduction of fluoroacetate and fluorocitrate directly into the cerebral cortex causes changes in the electrical patterns in various areas without increased citrate concentrations in these areas. Fluorocitrate convulsions in mice cannot be stopped by the intravenous administration of Ca^{2+} ions (Eeg-Larsen & Naeso, 1951). Nor is there evidence that convulsions are due to defective acetylcholine metabolism (Mehler & Chang, 1956; Peters, 1957), although bovine brain tissue can form fluoroacetylcholine from fluoroacetate (Wollemann & Feuer, 1957). Fluoroacetate has little or no effect on the respiration of mammalian brain tissue (Webb & Elliott, 1948, 1951). The respiration of pigeon-brain homogenate, in the presence of pyruvate, is unaffected by fluoroacetate (16.6 mM) (Liébecq & Peters, 1949). There seems to be no convincing evidence that fluoroacetate gives rise to fluorocitrate in brain (Peters, 1957; Wolfe & Elliott, 1962). It is, therefore, difficult to understand how cerebral effects of fluoroacetate can be due to significant inhibitions of the cerebral citric acid cycle. A relatively large increase in the ammonia concentration of dog brain is reported (Benitez, Pscheidt & Stone, 1951, 1954) to be associated with

the onset of convulsions in fluoroacetate metabolism, the increase in the concentration of ammonia preceding that in the concentration of citrate. It seems probable that the increased concentration of ammonia is one of the factors contributing to the seizures.

We have examined the action of fluoroacetate on the conversion of [^{14}C]glucose and of [^{14}C]glutamate into ^{14}C -labelled amino acids in rat brain cortex *in vitro*. We have found that at low concentrations (even below 1 mM), which have no effect on brain respiration, fluoroacetate suppresses glutamine biosynthesis and cerebral utilization of NH_4^+ ions. The present paper describes the results obtained and their possible interpretation.

MATERIALS AND METHODS

The method for the study of the conversion of [^{14}C]glucose into ^{14}C -labelled amino acids was that described by Gonda & Quastel (1962). The Warburg manometric apparatus was used for incubation of brain-cortex slices in oxygen at 37°. Brain-cortex slices, from adult hooded rats of about 150 g., were placed in ice-cold Krebs-Ringer phosphate medium of the following composition: NaCl, 128 mM; KCl, 5 mM; $CaCl_2$, 3.6 mM; $MgSO_4$, 1.3 mM; Na_2HPO_4 , 10 mM (brought to pH 7.4 with $N-HCl$). For study of the effects of high K^+ ion concentrations, the KCl concentration in the medium was increased to 105 mM, the concentrations of the other ions remaining the same. The slices were prepared with a Stadie-Riggs microtome, care being taken that the slices were not more than 0.3 mm. thick, but not so thin that they tended to disintegrate when shaken in a Warburg manometric apparatus. The slices (average wet wt. 60–70 mg.) were weighed at once on a torsion balance and suspended in the incubation medium in chilled manometric vessels. The dry wt. of the slices prepared under these conditions was about 20% of the wet wt. The final volume of medium in each Warburg manometric flask was 1 ml. and each flask contained uniformly labelled [^{14}C]glucose (5 mM). Corrections were not made for swelling of the tissue. This was variable and did not exceed 10%.

After aerobic incubation for 1 hr. at 37°, the flasks were chilled in crushed ice, and the slices were removed, rinsed with a small quantity of Krebs-Ringer phosphate medium, suspended in 5 ml. of 80% (v/v) ethanol and homogenized in a Potter-Elvehjem homogenizer. The homogenizer was washed with small portions of 80% ethanol and the washings were added to the original homogenate. The homogenate was kept at 5° for 2 hr. and then centrifuged. The ethanolic extract was collected and the insoluble residue was washed twice with 1.5 ml. portions of 80% ethanol and

finally with 1 ml. of water. The pooled ethanolic extract was evaporated to dryness at room temperature in a current of air, and the dried extract was dissolved in a small amount of 80% ethanol and 'spotted' with a micropipette on Whatman no. 1 filter paper for two-dimensional chromatography. The solvents used for chromatography were: phase I, butan-2-ol-90% (v/v) formic acid-water (70:11:17, by vol.); phase II, 90% (w/v) phenol-water-aq. ammonia (sp.gr. 0.88) (960:110:3, by vol.). The incorporation of ^{14}C into amino acids from [$^{14}\text{C}_6$]glucose was calculated as $\mu\text{mg. atoms}$ of carbon from glucose incorporated into amino acids/100 mg. wet wt. of tissue (Gonda & Quastel, 1962). The radioactivity, A , of the individual amino acids on the filter paper was corrected by a factor due to the decrease in the counting efficiency on the paper. This factor was determined by measuring the radioactivity, R_1 , of a sample (100 $\mu\text{l.}$) of the ethanolic tissue extract on an aluminium planchet and also that, R_2 , of another sample (100 $\mu\text{l.}$) 'spotted' on filter paper. The result for each labelled amino acid on the filter paper (measured as counts/min.) was multiplied by R_1/R_2 (which was about 3.4-3.6 and was determined after each experiment).

The corrected value (counts/min.) of amino acids, $A_{\text{corr.}}$, was transformed into $\mu\text{mg. atoms}$ of carbon by multiplying by a factor, F , given by: $[6 \times \text{amount of glucose } (\mu\text{m-moles}) \text{ in the incubation medium}] / [\text{radioactivity of glucose (counts/min.) in the incubation medium}]$. The amount of glucose in each experiment was 5000 $\mu\text{m-moles}$ (30 000 $\mu\text{mg-atoms}$) and the labelling was 200 000 counts/min. Therefore F equals 0.15. Finally, the term expressing $\mu\text{mg. atoms}$ of carbon was corrected for the weight of the tissue and expressed/100 mg. wet wt. of tissue. The $\mu\text{mg. atoms}$ of carbon derived from the glucose incorporated into amino acids in 100 mg. wet wt. of tissue are therefore given by: $(A_{\text{corr.}} \times F \times 100) / (\text{wet wt. of tissue})$.

After two-dimensional chromatography the paper was thoroughly dried and placed in contact with Kodak 'No Screen' medical X-ray film for 3 days. After the films had been developed the radioactive spots were located on the paper. The radioactivities of the spots on the paper were quantitatively measured with a Tracerlab counter with a mica window (diam. 28 mm., thickness 1.5-1.8 mg./cm.²). The spots were divided into sizes to suit the diameter of the counter window. Activities were corrected for background. All results were expressed as averages $\pm$ S.D. of at least four independent determinations.

Determination of ammonia. Ammonia determinations were carried out by the method of Braganca, Quastel & Schucher (1954).

After an initial incubation period of 1 hr. at 37°, the manometric vessels containing the respiring brain tissue were placed in crushed ice. The brain slices were removed, quickly rinsed in ice-cold water, and homogenized in 2 ml. of ice-cold water. The homogenate was added to Warburg flasks containing 1 ml. of the original incubation medium. The centre well contained a small roll of filter paper soaked with 0.2 ml. of $\text{N-H}_2\text{SO}_4$, and the side arm contained 0.3 ml. of saturated K_2CO_3 solution. The vessels were reattached to the manometers and after thermal equilibrium at 37° the stoppers were closed. The K_2CO_3 solution from the side arm was then tipped into the main compartment. This raised the pH of the contents to about 10.5 and stopped the enzymic reactions.

Under these conditions the ammonia present in the test

solution diffused into the centre well where it was absorbed by the acid on the filter paper. The vessels were shaken at 37° for 3 hr., which had been found sufficient to permit complete diffusion and absorption of ammonia. After 3 hr. of shaking, the vessels were removed from the bath and the rolls of filter paper were taken out from the centre wells and placed in tubes containing 2 ml. of water. The centre wells were washed three times with water and the washings were added to the tubes containing the filter paper. The tubes were stoppered tightly and allowed to stand overnight. Then the filter papers were taken out, washed with water and the washings added to the tubes. All the washings were transferred to Folin-Wu tubes for nesslerization.

Colour was developed by the addition of 1.0 ml. of Nessler solution and 2 ml. of 2N-NaOH. The intensity of colour produced was determined at 425 $\text{m}\mu$ with a Unicam colorimeter and the ammonia content of the sample was determined from the standard curve that was obtained concurrently with all experiments. The results are expressed as $\mu\text{g. of ammonia/100 mg. wet wt. of brain tissue}$.

Reagents. [$^{14}\text{C}_6$]Glucose and L-[$^{14}\text{C}_5$]glutamate were purchased from Merck and Co., Montreal. All other chemicals were of reagent grade and were used without further purification.

RESULTS

Effects of fluoroacetate on rat-brain-cortex respiration and on the formation of ^{14}C -labelled amino acids from [$^{14}\text{C}_6$]glucose. The results of the addition of sodium fluoroacetate (1 mM) to rat-brain-cortex slices incubated at 37° for 1 hr. in Krebs-Ringer phosphate medium containing glucose and with different concentrations of K^+ ions are shown in Table 1. The changes in the quantities of ^{14}C from [$^{14}\text{C}_6$]glucose incorporated into the amino acids due to the presence of fluoroacetate are given. The fluoroacetate was added to the tissue slices either before gassing with oxygen and temperature equilibration or after this procedure. In the former case the fluoroacetate was in contact with the tissue for about 15 min. longer than in the latter case.

The fluoroacetate had no effect, under the given experimental conditions, on the amount of oxygen consumed by the tissue (Table 1), and measurements of the $^{14}\text{CO}_2$ formed during the experiment showed no changes, within the experimental error, due to the addition of the fluoroacetate. Fluoroacetate had no suppressing action on the stimulation of oxygen uptake, or of $^{14}\text{CO}_2$ formation, brought about by addition of 100 m-equiv. of K^+ ions/l.

The most pronounced effect of the fluoroacetate was the considerable diminution in the yield of ^{14}C -labelled glutamine, this being greater in the presence of 105 m-equiv. of K^+ ions/l. than in that of the normal quantity of K^+ ions, i.e. 5 m-equiv./l. When the fluoroacetate was added to the tissue before gassing with oxygen and attainment of temperature equilibrium, the formation of ^{14}C -labelled glutamine was almost abolished. An addi-

tional effect was the increased yield of ^{14}C -labelled glutamate, this being greater in the presence of the higher concentration of K^+ ions. The increase in the quantity of ^{14}C -labelled glutamate was at least equal to the decrease in the quantity of ^{14}C -labelled glutamine. Relatively small changes took place in the yields of ^{14}C -labelled γ -aminobutyric acid and aspartic acid.

Results given in Table 2 show that sodium fluoroacetate, at a concentration as low as 0.03 mM, brings about a large diminution of the yield of ^{14}C -labelled glutamine, with a concomitant increase in that of ^{14}C -labelled glutamate, when incubations are carried out in the presence of 105 m-equiv. of K^+ ions/l. The yields of ^{14}C -labelled alanine also drop

under these conditions (Tables 1 and 2), though the absolute decreases are smaller than those of ^{14}C -labelled glutamine.

Further experiments (carried out in collaboration with Dr O. Gonda) showed that the presence of fluoroacetate, under the given experimental conditions, leads to no increase in the small amount of leakage of glutamine into the medium (Gonda & Quastel, 1962); thus fluoroacetate at low concentrations brings about a marked disturbance of that aspect of brain-cell metabolism concerned with the transformation of glutamate into glutamine.

Effects of fluoroacetate on the formation of amino acids from glucose in the presence of ammonium chloride. The addition of NH_4^+ ions (5 m-equiv./l.)

Table 1. *Effects of sodium fluoroacetate (1 mM) on the formation of ^{14}C -labelled amino acids from $[^{14}\text{C}_6]\text{glucose}$ in rat-brain-cortex slices*

Rat-brain-cortex slices from adult hooded rats of about 150 g. were placed in ice-cold Krebs-Ringer phosphate medium [of the following composition: NaCl , 128 mM; CaCl_2 , 3.6 mM; KCl , 5 mM; MgSO_4 , 1.3 mM; Na_2HPO_4 , 10 mM (brought to pH 7.4 with N-HCl)] containing glucose (5 mM; 2×10^5 counts/min., i.e. 4×10^4 counts/min./ μmole). Extra KCl was added, when required, to the main vessel at the start of the experiment. Fluoroacetate was tipped into the main vessel after gassing with O_2 and temperature equilibration, or added as indicated (*) to the main vessel with the other components before gassing. The average wet wt. of slices was 60–80 mg. The final volume of medium in each Warburg manometric flask was 1 ml. The temperature of incubation was 37° . Incubations were carried out in O_2 for 1 hr. The results are means $\pm$ S.D. of six independent determinations.

Additions (final concn.)	Q_{O_2}	Incorporation of C derived from glucose into amino acids ($\mu\text{mg.atoms of C/hr./100 mg. wet wt. of tissue}$)				
		Glutamic acid	Glutamine	γ -Aminobutyric acid	Aspartic acid	Alanine
K^+ ions (5 m-equiv./l.)	9.9 ± 0.5	828 ± 15	227 ± 12	238 ± 11	378 ± 18	109 ± 5
K^+ ions (5 m-equiv./l.) + fluoroacetate (1 mM)	9.6 ± 1.1	1090 ± 60	142 ± 16	291 ± 12	350 ± 20	196 ± 15
K^+ ions (5 m-equiv./l.) + fluoroacetate (1 mM)*	10.4 ± 0.2	1120 ± 75	20 ± 3	303 ± 11	365 ± 19	201 ± 14
K^+ ions (105 m-equiv./l.)	15.1 ± 1.1	918 ± 18	420 ± 14	380 ± 13	260 ± 17	176 ± 14
K^+ ions (105 m-equiv./l.) + fluoroacetate (1 mM)	16.6 ± 0.5	1473 ± 150	46 ± 5	376 ± 11	241 ± 12	102 ± 6
K^+ ions (105 m-equiv./l.) + fluoroacetate (1 mM)*	16.0 ± 1.0	1640 ± 100	0	381 ± 18	251 ± 13	106 ± 6

Table 2. *Effects of sodium fluoroacetate at various concentrations on the formation of ^{14}C -labelled amino acids from $[^{14}\text{C}_6]\text{glucose}$ in rat-brain-cortex slices*

The experimental conditions were as described in Table 1, the glucose concentration being 5 mM (2×10^5 counts/min., i.e. 4×10^4 counts/min./ μmole). Fluoroacetate was tipped in after gassing with O_2 and temperature equilibration. The results are means $\pm$ S.D. of at least four independent determinations.

Additions (final concn.)	Q_{O_2}	Incorporation of C derived from glucose into amino acids ($\mu\text{mg.atoms of C/hr./100 mg. wet wt. of tissue}$)				
		Glutamic acid	Glutamine	γ -Aminobutyric acid	Aspartic acid	Alanine
K^+ ions (105 m-equiv./l.)	16.0 ± 1.0	918 ± 21	439 ± 16	340 ± 18	250 ± 14	185 ± 17
K^+ ions (105 m-equiv./l.) + fluoroacetate (0.01 mM)	17.1 ± 1.2	792 ± 19	430 ± 20	328 ± 16	236 ± 11	147 ± 3
K^+ ions (105 m-equiv./l.) + fluoroacetate (0.03 mM)	16.5 ± 0.5	1084 ± 30	235 ± 14	262 ± 13	220 ± 16	98 ± 13
K^+ ions (105 m-equiv./l.) + fluoroacetate (0.1 mM)	16.7 ± 0.7	1590 ± 150	66 ± 7	298 ± 17	263 ± 12	68 ± 6
K^+ ions (105 m-equiv./l.) + fluoroacetate (1.0 mM)	16.0 ± 1.0	1538 ± 100	36 ± 8	381 ± 21	249 ± 18	84 ± 9

to rat-brain-cortex slices incubated in glucose media has a well-known twofold effect: (a) increase of the rate of oxygen consumption (Annau, 1934; Weil-Malherbe, 1938) and (b) increase of the rate of formation of glutamine (Weil-Malherbe, 1936; Tower, 1959). These two phenomena are shown in Table 3, which illustrates the effects of the addition of sodium fluoroacetate (1 mM), on the respiration and on the formation of amino acids from glucose, to brain tissue respiring in the presence and absence of NH_4^+ ions.

The most noteworthy effects of the addition of sodium fluoroacetate (1 mM) are: (a) abolition of the increased yields of ^{14}C -labelled glutamine, due to the addition of NH_4^+ ions, both with 5 m-equiv. of K^+ ions/l. and with 105 m-equiv. of K^+ ions/l., and (b) suppression of the increased rate of oxygen consumption brought about by the addition of NH_4^+ ions (Table 3). There is a smaller enhancement of the yield of ^{14}C -labelled glutamate than obtains in the absence of NH_4^+ ions (Table 1), but it is possible that this is due to a fall in the rate of respiration that does not occur in the latter condition.

These results would be explicable if the major effect of fluoroacetate, at concentrations of 1 mM or less, on brain-cortex metabolism is the suppression of the conversion of NH_4^+ ions into glutamine.

Effects of fluoroacetate on ammonia formation in rat-brain-cortex slices. The results of the incubation of rat-brain-cortex slices in Krebs-Ringer phos-

phate medium containing glucose, in the presence of sodium fluoroacetate (1 mM), on ammonia formation are shown in Table 4. The presence of fluoroacetate increases notably the amount of free ammonia in the rat-brain-cortex slices after incubation for 1 hr. at 37° . The amount found in the normal Krebs-Ringer phosphate medium containing glucose is $14\text{ }\mu\text{g.}$ of ammonia/100 mg. wet wt. of tissue, corresponding to $8\text{ }\mu\text{moles/g.}$ wet wt. In slices of rat brain cortex fixed about 20 min. after death it is usual to find about $5\text{ }\mu\text{moles/g.}$ (Weil-Malherbe & Green, 1955; Weil-Malherbe, 1962). The presence of fluoroacetate (1 mM) doubles the quantity of free ammonia found in the brain slices incubated in Krebs-Ringer phosphate medium containing glucose and with a high concentration (105 m-equiv./l.) of K^+ ions (Table 4), without appreciably affecting the respiratory rate. This result is consistent with the observation that fluoroacetate (1 mM) almost abolishes the formation of ^{14}C -labelled glutamine from $[^{14}\text{C}_6]\text{glucose}$ under the same experimental conditions (Table 2).

Effects of fluoroacetate on the formation of ^{14}C -labelled amino acids from L- $[^{14}\text{C}_5]\text{glutamate}$ (5 mM). Results given in Table 5 show the relative yields of ^{14}C -labelled glutamine, γ -aminobutyrate and aspartate derived from $[^{14}\text{C}_5]\text{glutamate}$ brought about by rat-brain-cortex slices in the presence of glucose and in the presence of either 5 m-equiv. of K^+ ions/l. or 105 m-equiv. of K^+ ions/l. under the given

Table 3. *Effects of sodium fluoroacetate (1 mM) on the formation of ^{14}C -labelled amino acids from $[^{14}\text{C}_6]\text{glucose}$ in the presence of ammonium chloride (5 mM) by rat-brain-cortex slices*

The experimental conditions were as described in Table 2. The results are means $\pm$ S.D. of at least four independent determinations.

Additions (final concn.)	Q_{O_2}	Incorporation of C derived from glucose into amino acids ($\mu\text{mg. atoms of C/hr./100 mg. wet wt. of tissue}$)				
		Glutamic acid	Glutamine	γ -Aminobutyric acid	Aspartic acid	Alanine
K^+ ions (5 m-equiv./l.)	11.0 ± 0.5	858 ± 20	232 ± 11	238 ± 10	345 ± 10	98 ± 8
K^+ ions (5 m-equiv./l.) + NH_4Cl (5 mM)	15.2 ± 0.7	894 ± 14	474 ± 17	222 ± 11	343 ± 11	175 ± 12
K^+ ions (5 m-equiv./l.) + NH_4Cl (5 mM) + fluoroacetate (1 mM)	11.5 ± 0.3	905 ± 15	187 ± 17	269 ± 18	182 ± 12	205 ± 16
K^+ ions (105 m-equiv./l.)	15.5 ± 1.2	937 ± 14	415 ± 6	316 ± 8	245 ± 15	182 ± 13
K^+ ions (105 m-equiv./l.) + NH_4Cl (5 mM)	16.4 ± 0.8	1034 ± 18	669 ± 19	258 ± 15	210 ± 10	213 ± 10
K^+ ions (105 m-equiv./l.) + NH_4Cl (5 mM) + fluoroacetate (1 mM)	13.2 ± 1.0	1121 ± 16	37 ± 4	281 ± 18	174 ± 9	77 ± 6

Table 4. *Effects of sodium fluoroacetate (1 mM) on the formation of ammonia by rat-brain-cortex slices*

The experimental conditions were as described in Table 2. The results are the means $\pm$ S.D. of at least four independent determinations.

Additions (final concn.)	Q_{O_2}	Ammonia formed in 1 hr. ($\mu\text{g./100 mg.}$ wet wt. of tissue)
K^+ ions (5 m-equiv./l.)	10.0 ± 0.4	14 ± 0.5
K^+ ions (5 m-equiv./l.) + fluoroacetate (1 mM)	9.6 ± 0.6	18 ± 1.5
K^+ ions (105 m-equiv./l.)	15.9 ± 0.5	11 ± 0.5
K^+ ions (105 m-equiv./l.) + fluoroacetate (1 mM)	15.2 ± 0.7	22 ± 2

Table 5. *Effects of sodium fluoroacetate (1 mM) on the formation of ^{14}C -labelled amino acids from sodium $\text{L-}^{14}\text{C}_6$ glutamate by rat-brain-cortex slices*

The experimental conditions were as described in Table 2. The concentration of glucose (unlabelled) was 5 mM and that of $\text{L-}^{14}\text{C}_6$ glutamate was 2 mM (10^5 counts/min., i.e. 5×10^4 counts/min./ μmole). The results are the means $\pm$ S.D. of at least four independent observations.

Additions (final concn.)	Q_{O_2}	Incorporation of C derived from glutamate into amino acids ($\mu\text{mg.atoms of C/hr./100 mg. wet wt. of tissue}$)		
		Glutamine	γ -Aminobutyric acid	Aspartic acid
K^+ ions (5 m-equiv./l.) + glutamate (2 mM)	12.1 ± 0.4	800 ± 91	358 ± 15	1760 ± 100
K^+ ions (5 m-equiv./l.) + glutamate (2 mM) + fluoroacetate (1 mM)	11.0 ± 0.7	500 ± 40	343 ± 25	1096 ± 16
K^+ ions (105 m-equiv./l.) + glutamate (2 mM)	15.0 ± 0.2	340 ± 22	354 ± 4	450 ± 15
K^+ ions (105 m-equiv./l.) + glutamate (2 mM) + fluoroacetate (1 mM)	15.0 ± 1.0	60 ± 10	365 ± 21	130 ± 12

experimental conditions. The yield of ^{14}C -labelled aspartate exceeds that of ^{14}C -labelled glutamine (see also Gonda & Quastel, 1962). The yields of ^{14}C -labelled glutamine and aspartate are lower in the presence of 105 m-equiv. of K^+ ions/l. than in the presence of 5 m-equiv. of K^+ ions/l. This may be due partly to increased oxidation of the $^{14}\text{C}_6$ -glutamate to $^{14}\text{CO}_2$ in the presence of increased concentrations of K^+ ions (S. S. Parmar & J. H. Quastel, cited by Quastel, 1959) and perhaps partly to increased isotopic dilution of radioactive α -oxoglutarate derived from the $^{14}\text{C}_6$ glutamate by non-radioactive α -oxoglutarate derived from glucose. The oxidation of the latter substance via the citric acid cycle is greatly increased, in brain-cortex slices, with increased concentrations of K^+ ions (Ghosh & Quastel, 1954; Kini & Quastel, 1959).

The effect of the addition of sodium fluoroacetate (1 mM) is to suppress the yields of ^{14}C -labelled glutamine and aspartate from ^{14}C glutamate in the presence of glucose and either 5 m-equiv. of K^+ ions/l. or 105 m-equiv. of K^+ ions/l. Suppression of the yield of ^{14}C -labelled glutamine would be expected from the results described above that show that fluoroacetate suppresses the conversion of ammonia into glutamine. The fall in the yield of ^{14}C -labelled aspartate may be due to a diminished rate of transamination of ^{14}C -labelled oxaloacetate brought about by the lowered concentration of glutamine, which is known to be an active transaminating agent (Meister & Tice, 1950).

Effects of Amytal on the formation of ^{14}C -labelled amino acids from $^{14}\text{C}_6$ glucose in the presence of fluoroacetate. It has already been shown that 0.5 mM-Amytal diminishes the rate of formation of ^{14}C -labelled glutamine from $^{14}\text{C}_6$ glucose in the presence of rat-brain-cortex slices (Kini & Quastel, 1959), the diminishing effect being greatly increased in the presence of 105 m-equiv. of K^+ ions/l. The effect of the barbiturate is attributed to its well-known inhibitory effect on the synthesis of ATP in

the cell, glutamine biosynthesis being an ATP-dependent reaction. Amytal abolishes the stimulatory effect of increased concentrations of K^+ ions on brain-cortex respiration and suppresses the increased rate of turnover of the citric acid cycle (Ghosh & Quastel, 1954). Malonate has a similar effect (Takagaki, Hirano & Tsukada, 1958). Fluoroacetate, however, at concentrations that are equally effective, in suppressing glutamine formation, as those of Amytal, has no such effect in abolishing K^+ ion-stimulated brain respiration. Typical results are shown in Table 6. Moreover, the considerably increased rate of formation of ^{14}C -labelled glutamate found in a medium with a high concentration of K^+ ions in the presence of 1 mM-sodium fluoroacetate is not seen with 0.5 mM-Amytal, doubtless because of the diminished rate of respiration brought about by the presence of the hypnotic. The addition of Amytal to the fluoroacetate medium suppresses the enhanced rate of formation of ^{14}C -labelled glutamate brought about by the fluoroacetate. These results, obtained with 1 mM-fluoroacetate, which stand in contrast with those obtained with 0.5 mM-Amytal, do not indicate any serious interference with the operation of the citric acid cycle in the stimulated brain tissue as shown either in the respiratory rate or in the formation of ^{14}C -labelled glutamate.

DISCUSSION

The results recorded above show that sodium fluoroacetate at low concentrations (1 mM or less), which do not affect the respiration of rat-brain-cortex slices incubated in Krebs-Ringer phosphate medium containing glucose, suppresses the rate of cerebral formation of glutamine derived from either glucose or glutamate. Such a suppression occurs in the presence of low concentrations (0.5 mM) of a barbiturate such as Amytal (Kini & Quastel, 1959), whose action is attributed to its inhibitory effect on

Table 6. *Effects of Amytal (0.5 mM) on the formation of ^{14}C -labelled amino acids from $[^{14}\text{C}_6]\text{glucose}$ by rat-brain-cortex slices in the presence of sodium fluoroacetate (1 mM)*

The experimental conditions were as described in Table 1. Amytal and fluoroacetate were tipped in after gassing with O_2 and temperature equilibration. The results are means $\pm$ S.D. of at least four independent observations.

Additions (final concn.)	Q_{O_2}	Incorporation of C derived from glucose into amino acids ($\mu\text{mg. atoms of C/hr./100 mg. wet wt. of tissue}$)				
		Glutamic acid	Glutamine	γ -Aminobutyric acid	Aspartic acid	Alanine
K^+ ions (5 m-equiv./l.)	10.0 ± 0.6	867 ± 18	250 ± 15	260 ± 21	378 ± 15	90 ± 8
K^+ ions (5 m-equiv./l.) + fluoroacetate (1 mM)	9.2 ± 0.7	1109 ± 50	150 ± 11	285 ± 11	365 ± 20	195 ± 15
K^+ ions (5 m-equiv./l.) + Amytal (0.5 mM)	7.8 ± 0.8	1122 ± 35	161 ± 12	329 ± 30	185 ± 13	261 ± 21
K^+ ions (5 m-equiv./l.) + Amytal (0.5 mM) + fluoroacetate (1 mM)	7.5 ± 0.5	960 ± 75	130 ± 15	290 ± 18	400 ± 29	146 ± 12
K^+ ions (105 m-equiv./l.)	14.9 ± 1.0	969 ± 20	404 ± 20	370 ± 30	228 ± 20	155 ± 10
K^+ ions (105 m-equiv./l.) + fluoroacetate (1 mM)	16.3 ± 0.1	1583 ± 75	50 ± 4	375 ± 28	261 ± 14	89 ± 7
K^+ ions (105 m-equiv./l.) + Amytal (0.5 mM)	9.4 ± 0.1	1027 ± 60	68 ± 5	242 ± 20	131 ± 10	85 ± 6
K^+ ions (105 m-equiv./l.) + Amytal (0.5 mM) + fluoroacetate (1 mM)	9.8 ± 0.1	703 ± 35	28 ± 6	312 ± 55	107 ± 8	94 ± 10

the oxidative synthesis of ATP. It also occurs in rat-brain-cortex slices in the presence of acetylsalicylate or 2,4-dinitrophenol (Gonda & Quastel, 1963), which are uncouplers of oxidative phosphorylation. There is no evidence that, at low concentrations, fluoroacetate (or fluorocitrate formed from it) suppresses, in the brain cell, to any marked extent, the formation of ATP. Abadom & Scholefield (1962*a-c*) reported an inhibition of 30% with 2 mM-sodium fluoroacetate in the presence of rat-brain-cortex slices respiring in a glucose medium. Such an inhibition seems insufficient to account for the marked suppression of glutamine formation brought about by concentrations of fluoroacetate less than 1 mM.

Fluoroacetate (1 mM) also suppresses the enhanced rate of cerebral formation of glutamine and the increased rate of respiration brought about by the addition of NH_4^+ ions to the brain tissue respiring in Krebs-Ringer phosphate medium containing glucose. In this respect the effect of fluoroacetate differs from that of ouabain, which also abolishes the cerebral formation of glutamine but whose action is neutralized by the addition of NH_4^+ ions (Gonda & Quastel, 1962). The evidence indicates that fluoroacetate at low concentrations suppresses the cerebral utilization of NH_4^+ ions. Indeed, the presence of fluoroacetate increases the yield of ammonia in brain-cortex slices incubated aerobically for 1 hr. in a glucose medium.

Enhanced glutaminase activity of the brain-cortex slices in the presence of fluoroacetate might account for the phenomenon, but such an explanation would not account for the fact that the fluoroacetate inhibition is considerably increased in

the presence of high concentrations of K^+ ions, which stimulate both respiration and the synthesis of glutamine. Experiments (carried out in collaboration with Dr O. Gonda) have shown that fluoroacetate does not affect the activity of a glutamine-synthetase system isolated from brain tissue, but it is not known whether fluorocitrate is inhibitory to this system. Results, given above, show that the effect of fluoroacetate is increased by preliminary incubation of the brain tissue in its presence, and the inhibitory effect may therefore be due not to fluoroacetate but to a product of its metabolism. Fluorocitrate seems to be the most likely product, and further clarification of the results obtained with fluoroacetate on the formation of both ammonia and glutamine in isolated brain awaits investigation with fluorocitrate.

SUMMARY

1. Sodium fluoroacetate, at concentrations of 1 mM or less, which have no effect on the respiration of rat-brain-cortex slices incubated in Krebs-Ringer phosphate medium containing glucose, suppresses the formation of ^{14}C -labelled glutamine from $[^{14}\text{C}_6]\text{glucose}$, the effect being greater in a medium containing 105 m-equiv. of K^+ ions/l. than in one containing 5 m-equiv. of K^+ ions/l. There is a concomitant increase in the amount of ^{14}C -labelled glutamate formed.

2. Sodium fluoroacetate (1 mM) suppresses the accelerating action of NH_4^+ ions on the rates of oxygen consumption and of the formation of ^{14}C -labelled glutamine (from $[^{14}\text{C}_6]\text{glucose}$) by the brain-cortex slices.

3. Sodium fluoroacetate (1 mM) increases the amount of free ammonia in the brain-cortex slices after incubation in the presence of glucose for 1 hr. at 37°.

4. The yields of ^{14}C -labelled glutamine and ^{14}C -labelled aspartate derived from [$^{14}\text{C}_5$]glutamate by rat-brain-cortex slices, incubated in Krebs-Ringer phosphate medium containing glucose, are diminished in the presence of sodium fluoroacetate (1 mM).

5. The addition of Amytal (0.5 mM) diminishes the enhanced rate of formation of ^{14}C -labelled glutamate from [$^{14}\text{C}_5$]glucose due to 1 mM-sodium fluoroacetate.

6. The results are consistent with the conclusion that sodium fluoroacetate, at concentrations of 1 mM or less, inhibits the utilization of NH_4^+ ions by rat-brain-cortex slices respiring in Krebs-Ringer phosphate medium containing glucose.

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The Chromatographic Behaviour of the Acetylcholine Activity of Brain Extracts

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There is abundant evidence, accumulated over the years, to indicate that the chemical entity acetylcholine acts as a transmitter of peripheral synaptic action and probably serves a similar function within the central nervous system. In this light we have thought it important to re-examine some recent reports which imply that, of the

material which can be extracted from the brain and which reacts as acetylcholine in biological assays, only a small proportion can be accounted for in terms of acetylcholine itself.

Hosein, Proulx & Ara (1962) have claimed that the largest part of the activity of brain extracts is due to the presence of certain betaine esters of