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EFFECT OF HYDRAZINE ACETIC ACID ETHYL ESTER AND ITS GLYCYL DERIVATIVE ON OXIDATION AND AMMONIA FORMATION IN RAT BRAIN TISSUES

S. R. GUHA

Central Drug Research Institute, Lucknow, India

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Abstract—The effects of hydrazine acetic acid ethyl ester and the glycyl-derivative were studied on the process of ammonia formation and oxidation in rat brain tissues *in vitro*. It was found that these substances increased the endogenous ammonia formation in brain homogenates and slices. When the hydrazine compounds were added to rat brain homogenates respiring in the presence of various oxidisable substrates, the rate of oxidation was greatly enhanced whereas the ammonia suppressing action of these substrates was strongly inhibited. Similar result was also obtained when brain cortex slices respiring in presence of glucose or glutamate were used. The possible mechanism of the action of these hydrazine compounds in inhibiting the process of ammonia suppressing action of these substrates has been discussed.

IN-VITRO experiments suggest that hydrazine derivatives effect carbohydrate metabolism. It has been observed that in rabbit brain tissues Iproniazid depressed oxidative reactions and stimulated aerobic glycolysis with an increased consumption of glucose, phosphate and hexose monophosphate.[1–4] Similarly, it was found that a marked stimulation of aerobic glycolysis occurred in rat brain and liver tissues, and in rabbit erythrocytes.[5–7] After oral application over a long period, Isoniazid caused a depression of oxidations of glucose, acids of the Krebs' cycle and acetic acid together with a decreased CO_2-production.[8, 9] Whether the effect of the hydrazine-derivatives on glycolysis can be explained on the basis of hydrazone formation with carbonyl groups of α-Keto acids is not clear.[10, 11]

The importance of glucose and other oxidizable substrates in cerebral tissues is well established. It has been observed by several workers that glucose and other oxidizable substrates are highly effective in supressing the endogenous formation of ammonia in cerebral tissues.[12–15] These findings also suggest that ammonia metabolism in brain tissue is in some way connected with the metabolic utilisation of these substrates. Ammonia formation in brain tissue is a complex process of which the exact mechanism is not well understood. However, various hypotheses about the origin of ammonia in brain have been proposed from time to time.[12, 13, 16]

It has been found that iso-nicotinic acid hydrazide inhibits the ammonia-suppressing action of glucose in rat brain slices and homogenates whereas glucose oxidation was decreased in brain homogenates.[15] However, it was also observed that iso-nicotinic acid hydrazide increases endogenous ammonia formation in rat brain homogenates.[17]

The present investigation was undertaken to study the effects of hydrazine acetic acid ethyl ester and its glycyl-derivative on the oxidation and corresponding

ammonia formation in brain tissues in the presence of various actively metabolised substrates.

The results presented in this communication show that these drugs which have been found to be potent inhibitors of transaminase reactions[18] increase the oxygen uptake of brain tissue with most of the substrates employed, but on the other hand strongly inhibit the ammonia suppressing action of these substrates, indicating that these substrates most possibly removes ammonia by the pathway of transamination reactions.

MATERIALS AND METHODS

General experimental procedure: adult male rats of 150–200 g were used. The animals were killed by decapitation and tissue preparations were incubated under conditions as described below. Krebs-Ringer phosphate buffer solution (pH 7·4) without calcium chloride was used in all experiments. In case of brain cortex slices, pure oxygen with 5% CO_2 was used as the gas phase, while air was the gas phase in all experiments using brain homogenates. All incubations were carried out in Warburg flasks at 37° for 1 hr with an equilibration period of 5 min. Approximately 100 mg wet weight of tissue preparation or its equivalent was taken in each flask.

Preparation of tissue: all the steps were carried out in ice-cold conditions. Tissue slices of rat brain cortex were made with a slicer of the Stadie-Rigg's type and care was taken to obtain intact slices which in each flask were generally 90–120 mg wet weight. Homogenate of the whole cerebral portions of brain was made in a glass homogeniser (Potter-Elvehjem type) with sufficient volumes of ice-cold 0·25 M sucrose solution so as to make a 10 per cent suspension.

Estimation of ammonia: this was carried out manometrically according to the method of Braganca *et al.*[19] and represents the free and labile ammonia. The centre well of the flask contained 0·2 ml of 1·0 N sulphuric acid with a filter-paper strip and the side-arm had 0·3 ml of saturated sodium carbonate solution. After an incubation period of one hour of the experimental system, alkali was tipped from the side-arm into the main compartment containing 3·0 ml of the reaction mixture and the evolved ammonia was absorbed by sulphuric acid placed in the centre well. Addition of alkali raised the pH to 10·5 and stopped enzymatic reactions. Under these conditions free ammonia in the main vessel diffuses in the centre well and is absorbed by the acid there. The vessels were shaken for a further period of 3 hr for the process of diffusion to be completed after which they were removed from the bath. Filter paper strips were taken out from the centre well and placed in graduated tubes. The contents of the centre well was then quantitatively removed by washing the centre well five times with distilled water with a micropipette and added to the graduated tubes. After making it up to a definite volume, aliquots were taken for assay by the usual method of nesslerization.

The colour intensity was read at 420 mμ in a spectrophotometer and the ammonia concentration was estimated from a calibration curve prepared by using known concentrations of ammonium sulphate solutions. The results are expressed as μg ammonia formed per 100 mg wet tissues per hour and corrected for all suitable blanks (zero hour values).

The oxygen uptake was measured by the conventional manometric procedure and the results are expressed as per cent of uptake of the control system containing the substrate only.*

RESULTS

Endogenous ammonia formation in brain is known to be effected by various metabolic inhibitors.[12, 17] The results presented in Table 1 indicate that hydrazine acetic

TABLE 1. EFFECTS OF HYDRAZINE ACETIC ACID ETHYL ESTER (HAE) AND GLYCYL-HYDRAZINOACETIC ACID ETHYL ESTER (GHAE) ON THE ENDOGENOUS AMMONIA FORMATION IN RAT BRAIN TISSUES

System	Ammonia formation, μg/hr/100 mg wet tissue
1. Slice only	5·5
2. Slice + HAE (10^{-4} *M*)	8·5
3. Slice + GHAE (10^{-4} *M*)	7·5
4. Homogenate only	8·7
5. Homogenate + HAE (10^{-4} *M*)	10·0
6. Homogenate + GHAE (10^{-4} *M*)	9·5

The hydrazine compounds were brought to pH 7·4 before addition to the flasks. Other details are given in the text.

acid ethyl ester and its glycyl derivative produced an increase of endogenous ammonia in rat brain homogenates and slices. Similar results were observed previously with isonicotinic acid hydrazide and desoxypyridoxine.[17]

Various oxidisable substrates tend to supress the endogenous ammonia formation of brain tissues[15] and the effect of hydrazine-acetic acid ethyl ester and the glycyl derivative on the ammonia formation and oxidation of brain homogenates respiring in the presence of such substrates are shown in Table 2. It is evident from these results that the hydrazine compounds used inhibited the ammonia-sparing action of most of these substrates. However, hydrazine-acetic acid ethyl ester produced a significant increase in the oxidation rate of oxaloacetate and α-Ketoglutarate, while the glycyl derivative showed a marked activation of the oxidations of glutamate and α-Ketoglutarate. In the case of glucose, pyruvate and succinate, hydrazine acetic acid ethyl ester produced a slight increase of oxidation whereas the effect produced by the glycyl derivative was almost negligible.

The effect of hydrazine acetic acid ethyl ester and the glycycl derivative on ammonia formation and oxidation of rat brain cortex slices in presence of added glucose and glutamate are shown in Table 3. The negative ammonia values observed when these substrates were used indicate that in the presence of these substrates the ammonia values are below the zero hour ammonia values due to utilisation of the preformed ammonia in the tissues. It was also observed that the hydrazine compounds inhibited the disappearance of ammonia when brain slices respired in the presence of glucose or glutamate whereas the rate of oxidations of these substrates was greatly enhanced, especially when glutamate was used. It is not clear whether the inhibition produced

* Hydrazine-acetic acid ethyl ester and the glycyl-derivative were prepared and kindly supplied by Knoblock and Niedrich.[25]

by these hydrazine compounds on the disappearance of ammonia when glutamate was used as the substrate reflects any interference with the glutamine–synthetase reactions, since Krebs[20] has shown that in slices of brain tissues glutamate binds free ammonia with the consequent formation of glutamine. It is also not known whether the inhibition of ammonia supressing action of glutamate is in some way related to the increased rate of glutamate oxidation induced by the hydrazine compounds.

TABLE 2. EFFECTS OF HYDRAZINE ACETIC ACID ETHYL ESTER (HAE) AND THE GLYCYL DERIVATIVE (GHAE) ON AMMONIA FORMATION AND OXYGEN UPTAKE BY RAT BRAIN HOMOGENATES IN PRESENCE OF VARIOUS SUBSTRATES.

System	Ammonia formation, μg/hr/100 mg wet tissue	Oxygen uptake (as % of uptake with substrate as control)
1. Endogenous only	8·7	—
2. Endogenous + Glucose (0·01 *M*)	0·3	100
3. Endogenous + Glucose + HAE	4·3	114
4. Endogenous + Glucose + GHAE	4·0	104
5. Endogenous + DL-glutamate (0·05 *M*)	8·5	100
6. Endogenous + DL-glutamate + HAE	8.3	120
7. Endogenous + DL-glutamate + GHAE	9·0	175
8. Endogenous + Pyruvate (0·02 *M*)	1·3	100
9. Endogenous + Pyruvate + HAE	3·0	117
10. Endogenous + Pyruvate + GHAE	3·5	113
11. Endogenous + α-Ketoglutarate (0·01 *M*)	1.1	100
12. Endogenous + α-Ketoglutarate + HAE	5·5	170
13. Endogenous + α-Ketoglutarate + GHAE	3·5	150
14. Endogenous + Succinate (0·05 *M*)	7·3	100
15. Endogenous + Succinate + HAE	7·5	123
16. Endogenous + Succinate + GHAE	8·0	108
17. Endogenous + Oxaloacetate (0·01 *M*)	0·5	100
18. Endogenous + Oxaloacetate + HAE	4·0	160
19. Endogenous + Oxaloacetate + GHAE	3·3	130

Final concentrations of the hydrazine compounds (pH 7·4) were 10^{-4} M. Other details are given in the text.

DISCUSSION

Although the question is still open whether ammonia formation in brain tissues *in vitro* takes place by the same mechanism as *in vivo*, it may be assumed that the ammonia level in brain tissue studied *in vitro* is regulated by certain complex biochemical reactions. It is not known how far the process of ammonia formation in brain is related to the deaminating systems present in brain,[12] or to the splitting of the protein-bound amide bonds[16] or to the oxidation of glutamic acid.[13] The process of endogenous ammonia formation in brain is a vital and complex process, the exact mechanism of which is not well understood. It has been previously observed that the process of endogenous ammonia formation is influenced by various substances,[17] and the fact that substances possessing widely different chemical and biological properties produce a similar resultant effect on the ammonia formation in brain tissues suggested that various factors may be involved in the general process of ammonia formation in brain. The results presented above show that hydrazine-acetic acid ethyl ester and the

glycyl derivative increased endogenous ammonia formation in brain homogenates and slices. However in this respect they are similar to isonicotinic acid hydrazide or desoxypyridoxine, both of which increased endogenous ammonia formation in brain homogenates.[17].

TABLE 3. EFFECTS OF HYDRAZINE ACETIC ACID ETHYL ESTER (HAE) AND THE GLYCYL DERIVATIVE (GHAE) ON AMMONIA FORMATION AND OXYGEN UPTAKE BY RAT BRAIN CORTEX SLICES IN THE PRESENCE OF GLUCOSE AND GLUTAMIC ACID.

System	Ammonia formation, μg/hr/100 mg wet tissue	Oxygen uptake, %
1. Slice only	5·5	—
2. Slice + Glucose (0·01 *M*)	−1·3	100
3. Slice + Glucose + HAE	4·0	134
4. Slice + Glucose + GHAE	4·5	128
5. Slice + DL-glutamate (0·05 *M*)	−3·1	100
6. Slice + DL-glutamate + HAE	3·5	166
7. Slice + DL-glutamate + GHAE	2·0	165

Final concentrations of the hydrazine derivative (pH 7·4) were 10^{-4} *M*. Other details are given in the text.

The ammonia supressing action of glucose and other substrates employed was greatly inhibited by both the hydrazine compounds. The fact that these compounds do not inhibit the ammonia-sparing action of the substrates by inhibiting their oxidation is well illustrated by the observation of increased oxidation of these substrates. Hence, it appears that some major reaction by which these substrates remove ammonia in brain is strongly inhibited. Such inhibition of ammonia supressing action of glucose was also observed with iso-nicotinic acid and desoxypyridoxine in rat brain homogenates.[15]

The mechanism for binding or removing ammonia in brain mainly operates through the reductive amination of α-ketoglutarate by glutamic dehydrogenase, or the formation of glutamine from glutamic acid by glutamine synthetase. Intimately associated with these ammonia removing reactions are also the transamination reactions whereby a rapid interchange between the α-ketoacids and amino-acids takes place, these reactions being dependant on pyridoxal phosphate and allied co-factors.[21, 22] It has been shown that carbon of isotopically labelled glucose appears in the amino-acids of brain proteins and such incorporation has also been shown to occur in suspensions of cerebral tissues in glucose-saline medium.[14, 23] Hence it appears that transamination reactions play a major role in removing ammonia by these substrates. The results reported by Oehme *et al.*[18] and by Guha[24] indicated that these hydrazine compounds are strong inhibitors of transamination reactions. The effect of these compounds on ammonia formation in brain in the presence of added substrates may be possibly due to their inhibition of the transamination reactions involved. Whether the effect of the hydrazine compounds on ammonia formation in brain as reported above is in any way related to the activation of oxidation of the added substrates is not known, nor is it known whether the increased

oxidation of these substrates involves a different focus of action of these hydrazine derivatives.

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