Biochemical Pharmacology, 1964, Vol. 13, pp. 1507–1511. Pergamon Press Ltd., Printed in Great Britain.

GLUTAMIC ACID METABOLISM *IN VIVO*: THE EFFECTS OF PRETREATMENT WITH MORPHINE SULPHATE*

LAURENCE S. MAYNARD and VICTOR J. SCHENKER

Biochemical Research Section, Department of Psychiatry, State University of New York, Brooklyn, N.Y., U.S.A.

(*Received* 12 *March* 1964; *accepted* 16 *June* 1964)

Abstract—The oxidative metabolism of glutamic acid was measured in living mice by injection with glutamate-1-^{14}C and also with glutamate-5-^{14}C, followed by continuous measurement of the $^{14}CO_2$ excreted in the expired air. Five minutes after the injection of morphine sulfate, a statistically significant degree of inhibition of glutamic acid breakdown was present. Three and a half hours later metabolic activity had returned to control levels.

GLUTAMIC acid is an amino acid of especial interest because of the number of other amino acids into which it may be converted, e.g. proline, hydroxyproline, ornithine, arginine; and also because of the several alternative routes by which it may be metabolized and the key position of some of these in CNS† function. It may be converted into α-oxoglutarate by glutamic dehydrogenase and NAD, or by transamination with oxaloacetate to form the α-oxoglutarate and aspartate. Alpha-oxoglutarate may serve as an amino group acceptor for several transaminases (forming glutamic acid once more), or it may enter directly into the tricarboxylic acid cycle. In the CNS another pathway of metabolism is available which is of especial interest to students of CNS function: decarboxylation by glutamic acid decarboxylase to form γ-aminobutyric acid.[1, 2]

In a comprehensive and detailed review article, Elliott and Jasper[3] have concluded that since GABA is present in the brain in large amounts, and exerts important effects on the physiological activity if the brain, there is little doubt that it or a closely related substance is concerned in some way with the regulation of transmission of nerve impulse in central gray matter. Some convulsant drugs, but not all, produce a fall in brain GABA, and one may protect against the action of these agents by intracerebral administration of GABA. It affords no protection from convulsants such as Metrazol (pentylenetetrazol), which do not produce a lowered level of brain GABA.

It would therefore seem logical in any study of the effects of CNS stimulants or depressants that an effect on the level of GABA in the CNS should be looked for, and that any change in the GABA level may be part of the mode of action of the drug affecting the CNS. This expectation has not been generally confirmed.[4]

* This work was supported by Grant U-1352 from the Health Research Council of the City of New York.

† Abbreviations used: CNS, central nervous system; GAD, glutamic acid decarboxylase; GABA, gamma-aminobutyric acid; MS, morphine sulfate.

The level of GABA present in the CNS at any given time is a function of its rate of synthesis by GAD, and the rate of breakdown by GABA–α-oxoglutaric acid transaminase. This equilibrium may be modified by the existence of GABA in the CNS in both free and bound forms, of which only the free is pharmacologically active and capable of being further metabolized.[5] Under normal conditions, GABA level is directly dependent on maximum potential GAD activity and appears to be unrelated to transaminase actvity.[6] However, the rise in brain GABA produced by carbonyl reagents, such as hydroxylamine, appears to be due to a selective inhibition of transaminase activity. It should be noted that hydroxylamine was ineffective in mice, although it did produce a rise in brain GABA in several other species.[7]

In addition to substances which affect the activity of GAD or transaminase, either directly or by action on the coenzyme pyridoxal phosphate, periods of hypoxia or monoamine oxidase inhibitors such as iproniazid may also change the level of brain GABA.[8]

In our laboratory we have been interested in the effects of potentially addicting drugs, specifically morphine sulfate and ethanol, on biogenic amine metabolism. We feel that it is particularly interesting to compare the effects of MS with those of ethanol, since the former causes true addiction in all subjects exposed to it for a sufficient number of doses whereas the latter effects addiction or habituation in only certain susceptible individuals. Our working hypothesis is that changes in mood and behavior that can be produced by these drugs may posses a meaningful correlation with changes in amine metabolism. We have demonstrated that both MS and ethanol significantly inhibit the activity of 5-hydroxytryptophan decarboxylase *in vivo* in mice, MS being the more potent inhibitor.[9] A new method for the assay of decarboxylase activity *in vivo* was developed during the course of this study, based on the continuous monitoring of $^{14}CO_2$ level in expired air, after the administration of a carboxyl-labelled amino acid.

We have applied this technique to the study of the metabolism of carboxyl-labeled (1-^{14}C and 5-^{14}C) glutamic acid in the living mouse, since we feel that such a study would be a useful preliminary to a more specific investigation of the effects of MS on glutamate and GABA metabolism in brain homogenates. Parenterally administered glutamate crosses the blood–brain barrier at a relatively slow rate,[10] so most of the $^{14}CO_2$ measured in our experiments is presumably the result of conversion of glutamate into α-oxoglutarate and subsequent metabolism in the tricarboxylic acid cycle. A significant inhibition of the rate of breakdown of carboxyl-labeled glutamate after pretreatment with MS is reported in this study.

METHODS

The apparatus and procedure used for the *in-vivo* assay of decarboxylase activity has been described in detail in a previous communication.[9] To summarize the essential features: an appropriate dose of glutamic acid labeled on the 1-carbon (carboxyl carbon) with ^{14}C is injected via the intraperitoneal route into a white mouse, which is placed in a respirometer flask. Enzymatic decarboxylation releases $^{14}CO_2$ in the expired air. Air is drawn through the respirometer flask at a constant rate, and then through an anthracene crystal flow cell, which is in the well of a liquid scintillation counter. The output of the counter is fed into a precision ratemeter, and its output in turn into an integrating recorder, so that a continuous record of the $^{14}CO_2$ level

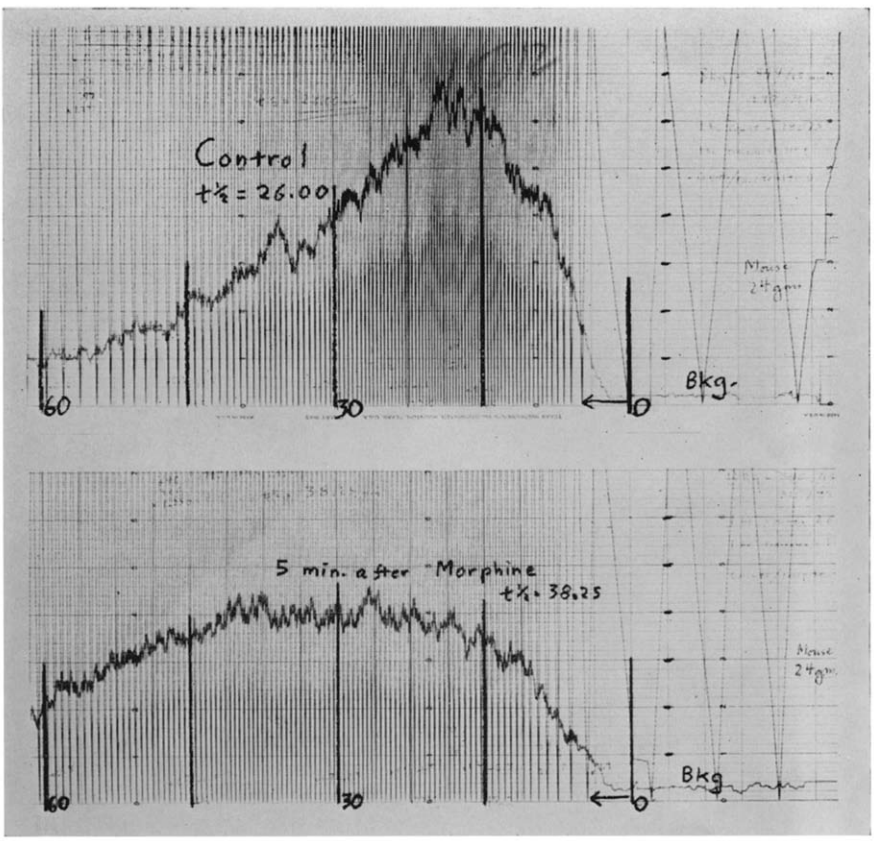

FIG. 1. Sample record of $^{14}CO_2$ activity of expired air after dose of glutamate-1-^{14}C. Read from right to left; 15-min background followed by 1-hr experimental run.

facing page 1508

in the expired air from the mouse is obtained. Background is measured for 15 min, the mouse injected with glutamate-1-^{14}C, and decarboxylation followed for 1 hr (see Fig. 1 for specimen curves obtained). The trace is divided into suitable time intervals, and the count is accumulated during each interval obtained from the integrator trace. By cumulative subtraction from the count representing the original dose of labeled material, making corrections for background and for the weight of the mouse (Table 1) a plot of per cent of dose retained vs. time is made. From this plot, the half-time ($t_{1/2}$) is determined, and is the parameter used to characterize the rate of decarboxylation.

The following modifications have been made in the method since our earlier publication. A new flow cell, made by the authors, containing about 20 ml of loosely packed fluorescence-grade anthracene crystals, was used, with a resulting increase of count/background ratio of about 15-fold, when compared to the previously used commercial cell. Further increase in count/background ratio (accepting some loss in total count rate) can be obtained by using the following discriminator settings on a model 701 Nuclear-Chicago liquid scintillation counter: with data voltage set at 1,150, and gate voltage at 1,150, the lower level of the discriminator was set at 2·3 V, and the upper level at 9·9 V, above the baseline. With air flow through the system maintained at 200 ml/min, background count is about 40 cpm, and a dose of 1·2 μc of glutamate-1-^{14}C in a 30-g mouse produces a peak count rate of about 12,00 cpm.

The count level representing 100% of the dose was determined with labeled bicarbonate, as previously described.[9]

The dosages of labeled amino acids were as follows: glutamate-1-^{14}C, 0·4 μc/10 g body weight; glutamate-5-^{14}C, 0·8 μc/10 g. The stock solutions were kept frozen until the day of use, and then diluted in an appropriate amount of isotonic saline so that 0·1 ml contained the dose per 10 g body weight. Labeled glutamate is relatively resistant to spontaneous breakdown; little or no $^{14}CO_2$ is produced even after 1 hr in a boiling water bath and the addition of acid or alkali.

Two groups of mice were pretreated with MS i.p. at a dosage of 0·15 mg/10 g body weight, one group 5 min before the labeled glutamate, the other 3·5 hr before the glutamate. Mice treated with this dose of MS carry the tail arched over the back, but appear alert and active. At 3·5 hr the tail phenomenon is beginning to disappear.

Mice were fed a diet of Purina lab chow without vitamin supplement. They were not fasted prior to experimental runs. Injection of pyridoxal phosphate, 0·1 mg. had no effect on control values, so this step was omitted from later experiments.

RESULTS

The effects of pretreatment with MS, 0·15 mg/10 g body weight, on the decarboxylation of glutamate-1-^{14}C and of glutamate-5-^{14}C are summarized in Table 2.

Glutamate-1-^{14}C

Results with this labeled amino acid represent metabolic rate as influenced by MS, immediately after the dose and during the recovery phase. Prolongation of the $t_{1/2}$ is indicative of decreased or inhibited rate of metabolism; shortening of the $t_{1/2}$ indicates increased or accelerated metabolic rate. Immediately after the dose of MS a fairly marked inhibition is apparent, which is significant ($P = < 0{\cdot}001$); 3·5 hr later, a slight shortening of the $t_{1/2}$ is observed, but this is not statistically significant.

Table 1. LEVELS OF $^{14}CO_2$ IN EXPIRED AIR AFTER THE ADMINISTRATION OF CARBOXYL-LABELED GLUTAMATE BY I.P. INJECTION

A. Glutamate-1-^{14}C

	Time interval after dose (min)	0–15	15–22·5	22·5–30	30–45	45–60
Glutamate-1-^{14}C; control	Counts in period	7,533	6,689	5,729	7,214	3,515
Dose = 37,000 counts;	Dose minus counts	29,467	22,778	17,049	9,835	6,320
n = 6 mice	Dose retained (%)	80	62	46	27	17
Glutamate-1-^{14}C 5	Counts in period	4,093	5,503	5,519	8,988	5,574
min after MS;	Dose minus counts	32,907	27,404	21,885	12,897	7,323
n = 5 mice	Dose retained (%)	89	74	59	35	20
Glutamate-1-^{14}C 3·5 hr. after MS;	Counts in period	9,634	7,232	5,276	6,577	3,076
n = 5 mice	Dose minus counts	27,366	20,134	14,858	8,281	5,202
	Dose retained (%)	74	54	40	22	14

B. Glutamate-5-^{14}C

	Time interval after dose (min)	0–15	15–30	30–45	30–45	45–60
Glutamate-5-^{14}C; control	Counts in period	4,720	9,844	6,142	6,142	3,304
Dose = 74,000 counts;	Dose minus counts	69,280	59,436	53,294	49,990	
n = 6 mice	Dose retained (%)	94	80	72	68	
Glutamate-5-^{14}C 5 min after MS;	Counts in period	2,263	7,362	7,501	5,068	
n = 4 mice	Dose minus counts	71,737	64,375	56,874	51,806	
	Dose retained (%)	97	87	77	70	

TABLE 2. HALFTIME REQUIRED TO EXCRETE HALF THE DOSE OF CARBOXYL ^{14}C-LABELED GLUTAMIC ACID; EFFECTS OF PRETREATMENT WITH MORPHINE

Substrate and pretreatment	No. of mice in group	Mean $t_{1/2} \pm$ S.D.	Deviation from control	Signif. of difference
Glutamate-1-^{14}C				
Control	8	27·19 ± 1·648		
5 min after morphine	6	36·00 ± 1·991	+ 8·81	< 0·001
3·5 hr after morphine	6	25·04 ± 2·065	− 2·15	N.S.*
Glutamate-5-^{14}C†				
Control	9	44·83 ± 9·245		
5 min after morphine	6	62·50 ± 13·70	17·67	0·025

* N.S. = not significant.

† Time to excrete one fourth of dose ($t_{1/4}$) is used with glutamate-5-^{14}C.

Glutamate-5-^{14}C

Glutamate labeled on the 5-carbon does not lose its label until additional enzymatic steps have taken place. Less than half the radioactivity of the injected labeled material is recovered during the experiments, in contrast to glutamate-1-^{14}C, of which 70%–75% is recovered in 1 hr; therefore $t_{1/4}$ is used as the descriptive parameter. The size of the standard deviations indicates that the variation from animal to animal is much greater than with the 1-C label. Five minutes after a dose of MS, the $t_{1/4}$ is prolonged considerably, and despite the large amount of variation present in the data, this prolongation is statistically significant ($P = 0{\cdot}025$).

DISCUSSION

It is evident from the data presented in Table 2 that shortly after pretreatment with MS there is a considerable slowing of the rate of glutamate-1-^{14}C metabolism. The metabolic rate 3·5 hr later is now slightly above control levels, but this difference is not statistically significant. This is in marked contrast to our unpublished observation that ethanol produces an initial inhibition of glutamate metabolism, followed 2 hr later by significantly accelerated activity.

Compared to glutamate-1-^{14}C, glutamate-5-^{14}C decarboxylation proceeds at a much slower rate, and less than half the dose administered is recovered as $^{14}CO_2$. The standard deviations of this group of measurements are many times larger than is the case with glutamate-1-^{14}C. None of this is unexpected, in view of the many additional metabolic steps which must take place before the 5=carbon is removed from the glutamate molecule. Our date (Table 2) show that glutamate-5-^{14}C metabolism is slowed by MS, and proportionally to about the same degree as is glutamate-1-^{14}C breakdown. One may postulate from this equivalence that the slowing of glutamate-5-^{14}C decarboxylation is the result of an inhibition early in the enzymatic sequence, which is also responsible for the inhibition of glutamate-1-^{14}C breakdown, and that no additional inhibition takes place later in the metabolic chain.

The effect of MS on glutamate metabolism demonstrated in these experiments may reflect the generalized slowing of oxidative metabolism produced by morphine, or it may demonstrate a more specific action of this drug on glutamate metabolism. The *in-vitro* studies of the effects of MS on glutamate and GABA metabolism in brain now in progress, should help clarify this point.

REFERENCES

1. P. O. Cohen and H. J. Sallach, in *Metabolic Pathways*, D. M. Greenberg, Ed., pp. 44–48. Academic Press, New York (1961).
2. D. M. Greenberg, *ibid.*, pp. 174–75.
3. K. A. C. Elliott and H. Y. Jasper, *Physiol Rev.* **39**, 383 (1959).
4. R. A. Ferrari and A. Arnold, *Biochim. biophys. Acta* **52**, 361 (1961).
5. E. Roberts, in *Inhibition in the Nervous System and Gamma-Aminobutyric Acid*, E. Roberts, Ed., p. 144. Pergamon Press, Oxford (1960).
6. E. Roberts, C. F. Baxter and E. Eiddberg, in *Structure and Function of the Cerebral Cortex*, D. B. Tower and J. P. Schade, Eds., p. 392. Elsevier, Amsterdam (1960).
7. C. F. Baxter and E. Roberts, *J. biol. Chem.* **236**, 3287 (1961).
8. R. A. Lovell and K. A. C. Elliott, *J. Neurochem.* **10**, 479 (1963).
9. L. S. Maynard and V. J. Schenker, *Int. J. Neuropharmacol.* **2**, 303 (1964).
10. S. Berl, A. Lajtha and H. Waelsch, in *Chemical Pathology of the Nervous System*, J. Folch-Pi Ed., pp. 361–68. Pergamon Press, Oxford (1958).