Biochemical Pharmacology, Vol. 21, pp. 2013–2019. Pergamon Press, 1972. Printed in Great Britain.

[2-^{14}C]ETHANOL AS A PRECURSOR OF GLUTAMINE, GLUTAMATE, γ-AMINOBUTYRIC ACID AND ASPARTATE IN HAMSTER BRAIN *IN VIVO*

MARY K. ROACH and W. N. REESE, JR.
Texas Research Institute, Texas Medical Center, Houston, Tex. 77025, U.S.A.

(*Received* 6 *October* 1971; *accepted* 3 *March* 1972)

Abstract—When [2-^{14}C]ethanol is injected intraperitoneally into hamsters, radioactive carbon is incorporated in the brain into glutamate and glutamine at a rate equivalent to 0·02 μmole ethanol/g/min and into aspartate and γ-aminobutyric acid (GABA) at a rate of 0·003 μmole/g/min. This incorporation is linear for at least 90 min after ethanol administration. Acetate formed from the hepatic oxidation of [2-^{14}C]-ethanol seems to be the principal source of radioactive carbon entering the amino acids. Significantly more ethanol-derived acetate is incorporated into glutamine and GABA after a high ethanol dose (2·50 g/kg) than after a low dose (0·62 g/kg).

RECENT studies indicate that ethanol administration alters glucose metabolism in the brain, resulting in increased glucose content and in decreased incorporation of labeled carbon from [U-^{14}C]glucose into the amino acids derived via the tricarboxylic acid cycle.[1,2] The incorporation of glucose carbon by glutamine decreases more markedly after ethanol than does the incorporation by glutamate, aspartate and γ-aminobutyric acid (GABA).[1] We have suggested that the effect of ethanol on glutamine metabolism in brain is due in part to the concomitant cerebral metabolism of ethanol-derived acetate[1] which, according to the concept of glutamate compartmentation,[3] should be incorporated into glutamine more than into the other amino acids.

To examine the role of ethanol-derived acetate in brain amino acid metabolism, we have measured the rate of incorporation and the distribution of ^{14}C from [2-^{14}C]-ethanol into the free amino acids of hamster brain.

MATERIALS AND METHODS

[2-^{14}C]Ethanol (New England Nuclear, Boston, Mass.) was combined with non-radioactive carrier ethanol to a specific activity of 2·0 μc/m-mole. Saline was added to give a final ethanol concentration of 25% (v/v).

Male golden Syrian hamsters (90–110 g) were injected intraperitoneally with the radioactive ethanol solution in doses of 0·62 or 2·50 g/kg. At appropriate times after ethanol administration, the animals were killed by immersion in liquid nitrogen. The brains were removed and weighed without thawing. The frozen brains were homogenized in 10 ml of ice-cold 5% perchloric acid and the homogenates centrifuged for 20 min at 10,000 *g* and 4°.

The perchloric acid extracts of brain were examined for radioactive amino acid content by the ion-exchange chromatographic method decribed previously.[1]

Blood acetate and ethanol levels were determined in another group of hamsters. Blood was collected from the retro-orbital sinus after i.p. administration of 0·62 and 2·50 g/kg of ethanol (25%, v/v). Using a disposable glass microsampling pipet (Corning Glass Works, Corning, N.Y.), 0·05 ml of blood was withdrawn from the sinus and transferred to a 1-ml serum bottle containing 0·05 ml propionic acid solution (0·5 mg/ml) as an internal standard. Five per cent $ZnSO_4$ and 0·3 N $Ba(OH)_2$, 0·05 ml of each, were added to deproteinize the blood. The serum bottles were stoppered and were centrifuged at 6000 rpm for 10 min. The colorless supernatant fluid was analyzed for acetate and ethanol content within 5 hr of preparation by the gas chromatographic method described below.

The specific activity of blood acetate was determined in a third group of hamsters. After the administration of radioactive ethanol, the animals were decapitated and the blood was collected in heparinized beakers and deproteinized with equal volumes of $ZnSO_4$ and $Ba(OH)_2$. Blood from two animals was pooled for each sample. After centrifugation, the deproteinized sample was applied to a column of Dowex 1 × 8 ion-exchange resin (50–100 mesh, Cl^- form) and the resin was washed with water. Acetate was eluted from the column with 1·0 N HCl. The acetate-containing eluate was assayed for radioactivity by liquid scintillation spectrometry and for acetate content by gas chromatography.

The gas chromatographic method of Mahadevan and Stenroos[4] for volatile fatty acids was modified for the simultaneous analysis of acetate and ethanol. A 6 ft × $\frac{1}{8}$ in. (i.d.) glass column was packed with Porapak Q (Waters Associates, Framingham, Mass.), 80–100 mesh, coated with 10% of H_3PO_4. The Porapak Q was coated by stirring it for 5 hr with the requisite amount of 85% H_3PO_4 dissolved in 70% methanol,

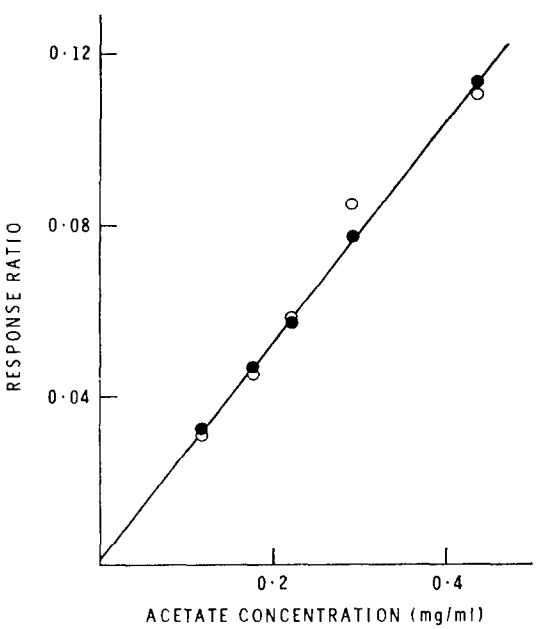

FIG. 1. Gas chromatographic determination of acetate. Closed circles represent standard curve from water; open circles, standard curve from blood. Each point represents four determinations.

followed by washing with methanol. After drying overnight at 110°, the resulting material was ready for packing. The packed column was conditioned for 48 hr at 170° with carrier gas flow.

For a Hewlett–Packard 402 gas chromatograph with flame ionization detector, the operating conditions were: carrier gas, helium; carrier gas flow rate, 100 ml/min; column oven, 170°; flash heater, 190°; detector, 220°. Under these conditions, retention times were 1·2 min for ethanol, 2·6 min for acetate and 6·0 min for propionate. A plot of response ratio (acetate peak area/propionate peak area) versus acetate concentration (Fig. 1) shows that the response was linear over the concentration range examined and that essentially 100 per cent recovery of acetate from blood was possible. Ethanol gave similar results.

RESULTS

Blood acetate content and specific radioactivity. Blood acetate levels in animals receiving a large ethanol dose (2·50 g/kg) were slightly higher than after a low dose (0·62 g/kg), and they remained maximally elevated throughout a 90-min time period (Table 1). At the lower dose, the acetate levels rose for 40 min and then declined as the blood ethanol level fell below that required to maintain saturation of liver alcohol dehydrogenase. Without alcohol administration, blood acetate concentration was less than 10 μg/ml.

TABLE 1. BLOOD ACETATE AND ETHANOL LEVELS IN HAMSTERS AFTER ACUTE ADMINISTRATION OF ETHANOL*

	Ethanol dose (g/kg)			
	0·62		2·50	
Time (min)	Acetate (μg/ml)	Ethanol (mg/ml)	Acetate (μg/ml)	Ethanol (mg/ml)
5	63 ± 11	0·90	87 ± 10	3·78
10	80 ± 11	0·83	92; 98	4·02
20	95 ± 5	0·74	108 ± 6	3·81
40	106 ± 5	0·48	120 ± 7	3·70
60	88 ± 8	0·21	132 ± 6	3·67
90			146 ± 18	3·28

* Acetate results are means ± S.E. of four animals unless indicated otherwise. Ethanol results are average of two animals. Animals received i.p. injection of ethanol as a 25% (v/v) solution in saline.

40 min after the administration of [2-^{14}C]ethanol, the specific activity of blood acetate was not significantly different in animals receiving 2·50 g/kg compared to those getting 0·62 g/kg, [2465 ± 359 (n = 6) and 2220 ± 197 (n = 8) dis./min/μmole respectively]. In another experiment, the specific activity of blood acetate was 3295 ± 700 dis./min/μmole (n = 4) at 20 min after 2·50 g/kg of ethanol and 3555 ± 434 dis./min/μmole (n = 4) at 40 min after the same dose, indicating that the specific activity was constant over time. In both experiments the specific radioactivity of blood acetate averaged 85 per cent of the specific activity of the administered ethanol.

Incorporation of ^{14}C from ethanol by brain amino acids. The micromolar equivalent of ^{14}C-ethanol incorporated into each of the four amino acids derived via the tricarboxylic acid cycle in brain was determined by dividing the total counts per minute of each amino acid by the specific activity of the administered ethanol. The incorporation was examined at various times after the administration of 2·50 g/kg of ethanol and was found to be linear for a period of at least 90 min (Fig. 2). Glutamate and glutamine incorporated the label from ethanol equally during this time at a rate equivalent to 0·02 μmole ethanol/min/g of brain. The incorporation into aspartate and GABA was also equimolar, at a rate of approximately 0·003 μmole/min/g.

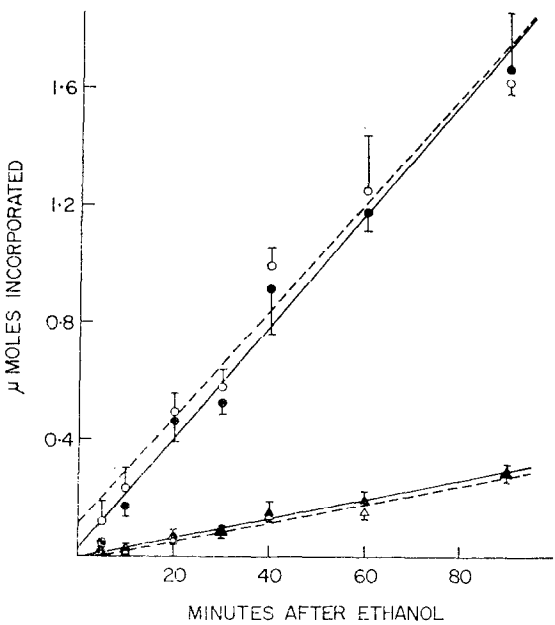

FIG. 2. Incorporation of radioactivity from [2-^{14}C]ethanol into amino acids in hamster brain afte i.p. injection of 2·50 g/kg of ethanol. Ordinate represents the micromole equivalent of [2-^{14}C]ethanol incorporated per gram. Vertical lines represent the plus or minus portion of standard deviation. Linear correlation analysis: glutamate (○), $\tilde{y} = 0{\cdot}729 + 0{\cdot}0193(x - 36{\cdot}62)$, $r = 0{\cdot}97$, $P \leq 0{\cdot}001$; glutamine (●), $\tilde{y} = 0{\cdot}778 + 0{\cdot}0184(x - 36{\cdot}62)$, $r = 0{\cdot}95$, $P \leq 0{\cdot}001$; aspartate (△), $\tilde{y} = 0{\cdot}120 + 0{\cdot}0033(x - 36{\cdot}62)$, $r = 0{\cdot}96$, $P \leq 0{\cdot}001$; GABA (▲), $\tilde{y} = 0{\cdot}105 + 0{\cdot}003(x - 36{\cdot}62)$, $r = 0{\cdot}96$, $P \leq {\cdot}001$.

Presumably, the ^{14}C from ethanol was incorporated into brain metabolites through the intermediate acetate. To determine the total incorporation of acetate into brain amino acids, rather than merely the incorporation of ethanol-derived acetate, obviously the calculations should be based on the specific activity of blood acetate. It was not feasible, however, to measure this activity for each animal. However, the specific activity of the blood acetate pool was relatively independent of ethanol dose and constant with time and, furthermore, was only 15 per cent lower than the activity of the administered ethanol. For these reasons, calculations of incorporation based on the specific activity of ethanol provide a good approximation of the uptake of acetate by the brain.

The incorporation of ethanol-derived ^{14}C was also examined at 40 min after doses of 0·62 and 2·50 g/kg of ethanol. The results presented in Table 2 indicate that glutamine and GABA incorporated significantly more ^{14}C at the high dose than at the low dose. Glutamate uptake of ^{14}C rose slightly but the increase was not statistically significant, aspartate incorporation did not change.

TABLE 2. EFFECT OF ETHANOL DOSE ON THE INCORPORATION OF ^{14}C DERIVED FROM [2-^{14}C]ETHANOL INTO GLUTAMATE, GLUTAMINE, ASPARTATE AND GABA IN HAMSTER BRAIN*

Ethanol dose (g/kg)	Glutamate	Glutamine	Aspartate	GABA
0·62 (5)	0·816 ± 0·097	0·799 ± 0·078	0·159 ± 0·030	0·116 ± 0·017
2·50 (8)	0·918 ± 0·159	0·993 ± 0·065	0·152 ± 0·034	0·136 ± 0·015
P	NS	≤0·005	NS	≤0·05

* Results are means ± S.D. as micromoles [2-^{14}C]ethanol incorporated per gram wet weight of brain. Numbers of animals are shown in parentheses. Hamsters were injected i.p. with 25% (v/v) ethanol in saline containing [2-^{14}C]ethanol (specific activity, 3·5 μc/m-mole) and were killed 40 min after injection.
NS = not significant.

The brain amino acid concentration did not differ significantly between the two dose levels. Previous studies have shown that aspartate is lower and glutamine higher in hamsters receiving ethanol compared to control animals without alcohol.[1]

The specific activities of glutamine, aspartate and GABA relative to the specific activity of glutamate were, respectively: 1·43 ± 0·16, 0·70 ± 0·05 and 0·66 ± 0·07, after the low ethanol dose and 1·63 ± 0·24, 0·63 ± 0·12 and 0·66 ± 0·08, after the high dose. Although the relative specific activity of glutamine was 20 per cent greater after the high ethanol dose, this difference was not statistically significant.

It should be noted here that when the source of ^{14}C is ethanol, the specific activity of glutamine relative to glutamate is greater than unity, whereas previous studies have shown that with ^{14}C-glucose this specific activity ratio is less than one.[1] According to the concept of glutamate compartmentation,[3] a glutamine-to-glutamate specific activity ratio of more than one would result if [2-^{14}C]acetate were the labeled precursor of acetyl-CoA in the brain. The possibility cannot be ignored, however, that ^{14}C derived from ethanol might also enter the brain as ^{14}C-glucose after gluconeogenesis in the liver. A small amount of radioactivity appears, in fact, in the chromatographic fractions containing glucose. The observed glutamine-to-glutamate specific activity ratio of more than one indicates, however, that acetate is the principal source of the ^{14}C derived from ethanol in these amino acids in brain.

DISCUSSION

Much research effort has been directed toward understanding the pharmacologic and pathologic actions of ethanol and of the first product of its metabolism, acetaldehyde. Studies of acetate, the product of the second step in alcohol metabolism, are few, however. As the results presented here confirm, alcohol ingestion leads to significant acetate levels in the blood. This is not surprising since 50–100 per cent of

hepatically metabolized alcohol has been shown to leave the liver as acetate.[5] The blood acetate levels reported here for hamsters were about 1·5–2 μmoles/ml and were somewhat higher than those of 0·8 μmole/ml reported for man.[5]

In the brain, metabolism of acetate via the tricarboxylic acid cycle can be distinguished from the metabolism of glucose carbon via the same pathway because acetate is one of a group of substrates that seems to be metabolized specifically by a slow-turning tricarboxylic acid cycle in equilibrium with a small pool of glutamate closely associated with glutamine synthesis.[6] Metabolism of labeled acetate results in the glutamine pool being more highly labeled than the total glutamate pool, the major portion of which seems to derive from a more active tricarboxylic acid cycle. As the results reported here show, brain glutamine is more highly labeled than glutamate when [2-^{14}C]ethanol is administered to hamsters, confirming that ethanol-derived acetate is the principal labeled precursor of these amino acids.

It is interesting that after an anesthetizing dose of ethanol (2·50 g/kg) the incorporation of ethanol-derived carbon into both glutamine and GABA is about 20 per cent greater than after a smaller ethanol dose (0·62 g/kg). This greater incorporation is unlikely to be due wholly to increased specific activity of the blood acetate pool, since acetate specific activity did not rise significantly with the higher ethanol dose. The possibility that increased ethanol oxidation in the brain could account for the greater incorporation is also remote because of the very low level of alcohol dehydrogenase activity shown by Raskin and Sokoloff[7] to occur in the soluble fraction of rat brain homogenate.

That ethanol-derived ^{14}C incorporation via the tricarboxylic acid cycle did not decrease after an anesthetizing dose of ethanol is perhaps more significant than that it increased. The incorporation of glucose carbon by this pathway is inhibited significantly during ethanol anesthesia,[1] and narcotizing doses of barbiturates and some other drugs also depress glucose carbon incorporation.[8,9] This decreased incorporation of glucose carbon generally has been assumed to reflect a decrease in the oxidative metabolism of glucose through the tricarboxylic acid cycle, the result of reduced energy requirements.

Assuming that there are two tricarboxylic acid cycles in brain, as is postulated from the evidence of glutamate compartmentation,[6] one explanation for the undiminished incorporation of label from ^{14}C-ethanol might be that the activity of the acetate-metabolizing tricarboxylic acid cycle is not depressed during narcosis, while the activity of the so-called "energy" cycle[3] is inhibited, leading to the observed decrease in glucose–carbon incorporation.

It is well known that glucose is the principal substrate for brain energy metabolism and amino acid synthesis. To compare the rate of incorporation of acetate with that of glucose, it is necessary to determine values for the rate of incorporation of glucose–carbon into the individual amino acids. Using [U-^{14}C]glucose, Flock *et al.*[2] present data for rats that provide a basis for an approximate calculation of these rates. This calculation provides an "acetate equivalent" of glucose incorporation for glutamate of 0·34, glutamine, 0·10, aspartate, 0·08 and GABA, 0·05 μmole/g/min.

The calculated rates of incorporation of acetyl units derived from [U-^{14}C]glucose cannot be equated directly with those measured for [2-^{14}C]ethanol because of the difference in molecular location of the labeled carbons and the problems associated with recycling through the tricarboxylic acid cycle.[6] This calculation, however,

provides at least some basis for comparing the rates of utilization of the two substrates. This comparison indicates that for glutamate, aspartate and GABA, acetate incorporation after alcohol is equivalent only to about 5 per cent of normal glucose metabolism. In contrast, acetate carbon can contribute about 20 per cent as much ^{14}C to the glutamine pool as does glucose. The considerable incorporation of acetate into glutamine, but not into the other amino acids, coincides with the earlier observation[1] that when ^{14}C-glucose is the labeled precursor, ethanol administration decreases the radioactive labeling of the glutamine pool more than that of the other amino acid pools.

Although the incorporation of ethanol-derived acetate into the amino acids other than glutamine is relatively minor compared to normal glucose utilization, an adaptive increase in acetate incorporation brought about by chronic ethanol consumption is an interesting possibility. During prolonged fasting, the brain has been shown to adapt its metabolic capability to use available substrates other than glucose.[10,11] It seems reasonable that the brain might also adapt its metabolism to utilize acetate during chronic alcohol intoxication while glucose utilization is partially inhibited. If an enhanced incorporation of acetate into the amino acids of brain occurred during prolonged alcohol consumption, this metabolic adaptation might have significance in the development of alcohol tolerance and physical dependence.

Acknowledgement—This investigation was supported in part by Grant MH 14434 and by General Research Support funds, United States Public Health Service. We thank Miss J. Corbin, Mrs. W. Pennington and Mrs. B. Stephens for their expert technical assistance.

REFERENCES

1. M. K. Roach and W. N. Reese, *Biochem. Pharmac.* **20,** 2805 (1971).
2. E. V. Flock, G. M. Tyce and C. A. Owen, *Proc. Soc. exp. Biol. Med.* **135,** 325 (1970).
3. S. Berl and D. D. Clarke, in *Handbook of Neurochemistry* (Ed. A. Lajtha), Vol. 2, p. 447. Plenum Press, New York (1969).
4. V. Mahadevan and L. Stenroos, *Analyt. Chem.* **39,** 1652 (1967).
5. F. Lundquist, N. Tygstrup, K. Winkler, K. Mellemgaard and S. Munck-Petersen, *J. clin. Invest.* **41,** 955 (1962).
6. C. J. Van Den Berg, L. J. Kržalić, P. Mela and H. Waelsch, *Biochem. J.* **113,** 281 (1969).
7. N. H. Raskin and L. Sokoloff, *J. Neurochem.* **17,** 1677 (1970).
8. H. S. Bachelard and J. R. Lindsay, *Biochem. Pharmac.* **15,** 1053 (1966).
9. Y. Yoshino and K. A. C. Elliott, *Can. J. Biochem. Physiol.* **48,** 228 (1970).
10. O. E. Owen, A. P. Morgan, H. G. Kemp, J. M. Sullivan, M. G. Herrera and G. F. Cahill, *J. clin. Invest.* **46,** 1589 (1967).
11. A. L. Smith, H. S. Satterthwaite and L. Sokoloff, *Science, N.Y.* **163,** 79 (1969).