the method of Metz *et al.* [9] in which varying concentrations of 2-deoxyuridine were added to monolayer cultures stimulated 22 hr previously with EGF in the presence of methotrexate or TP. After 1 hr incubation at 37°, 1 μCi [^{3}H]thymidine was added and after a further 2 hr incubation the cells were processed and harvested as described previously. The results are shown in Fig. 2.

Methotrexate is a pseudo-irreversible inhibitor of dihydrofolate reductase with high affinity for the enzyme so that the synthesis of thymidylate from deoxyuridine is suppressed thereby reducing the size of the intracellular thymidine pool. Consequently the incorporation of the exogenous [^{3}H]thymidine is increased. TMP on the other hand, does not significantly change the incorporation of [^{3}H]thymidine in this system. In two studies on normal human bone marrow a small inhibitory effect on dihydrofolate reductase has been noted at 1×10^{-4} M TP [10, 11] although another study only detected inhibition at 1×10^{-1} M [12].

This preliminary data would indicate that TP does not act to inhibit thymidine incorporation into EGF-stimulated human fibroblasts by virtue of an inhibition of dihydrofolate reductase. TP is a lipophilic compound and could well act directly at the cell membrane: if this is the case however it apparently does not affect the active uptake of amino acids which occurs during cell stimulation by EGF.

Acknowledgements—We are indebted to Dr. Morley Hollenberg for the gift of EGF and for much helpful advice and discussion. H. J. R. was a Wellcome travelling research fellow of the M.R.C.

* Address reprint requests to Dr. H. J. Rogers, Department of Pharmacology, Guy's Hospital Medical School, St. Thomas' Street, London, SE1 9RT.

Division of Clinical Pharmacology, Department of Medicine and Department of Pharmacology and Experimental Therapeutics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.

HOWARD J. ROGERS*
PAUL S. LIETMAN

REFERENCES

1. J. J. Burchall, *Ann. N.Y. Acad. Sci.* **186,** 143 (1971).
2. H. J. Rogers and P. S. Lietman, *Clin. Pharmac. Ther.* **17,** 242 (1975).
3. M. W. Ghilchik, A. S. Morris and D. S. Reeves, *Nature, Lond.* **227,** 393 (1970).
4. M. D. Hollenberg and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **70,** 2964 (1974).
5. R. H. Starkey, S. Cohen and D. N. Orth, *Clin. Res.* **22,** 350A (1974).
6. C. R. Savage and S. Cohen, *J. biol. Chem.* **247,** 7609 (1972).
7. D. E. Schwartz and W. H. Ziegler, *Postgrad. med. J.* **45,** Suppl. 32, (1969).
8. U. Mittwoch, D. J. Kirk and D. Wilkie, *J. med. Genet.* **11,** 260 (1974).
9. J. Metz, A. Kelly, V. C. Swett, S. Waxman and V. Herbert, *Br. J. Haematol.* **14,** 575 (1968).
10. J. Sive, R. Green and J. Metz, *J. clin. Path.* **25,** 194 (1972).
11. J. Koutts, M. B. Van der Weyden and M. Cooper, *Aust. N.Z. J. Med.* **3,** 245 (1973).
12. S. Waxman, *Clin. Res.* **19,** 434 (1971).

Biochemical Pharmacology, Vol. 25, pp. 2757–2759. Pergamon Press, 1976. Printed in Great Britain.

Subcellular site of acetaldehyde oxidation in monkey liver

(*Received* 17 *March* 1976; *accepted* 11 *June* 1976)

Until recently acetaldehyde was believed to be oxidized in the cytosol of the liver [1,2]. Evidence has now accumulated indicating that its oxidation occurs in mitochondria. Mitochondrial activities have been demonstrated in quantity and with kinetic characteristics adequate to account for acetaldehyde oxidation under most conditions [3–6]. Parrilla *et al.* [7], from changes in the state of reduction of cytosolic and mitochondrial nucleotides when acetaldehyde is utilized by perfused liver and isolated liver cells, concluded that acetaldehyde is oxidized to acetate predominantly in mitochondria. Rognstad and Clark [8], using isolated liver cells, and our laboratory [9], using liver slices and tracing with ^{3}H the fate of the *R* and *S* hydrogens of ethanol, have also arrived at this conclusion. Similarly Grunnet [10], from incorporation by hepatocytes of ^{3}H from [1,1-^{3}H-]ethanol into lactate and β-hydroxybutyrate, have concluded that acetaldehyde oxidation occurs in the mitochondria as well as in cytosolic compartments of the liver cell. In all these studies, the rat was the source for liver. Cytosolic and mitochondrial aldehyde dehydrogenases have been demonstrated to be in horse liver [11] and aldehyde dehydrogenases have been purified from livers of other species, including man and mouse [3], but their physiological role is uncertain.

We have, using slices of monkey liver, now obtained evidence that in this primate acetaldehyde oxidation is also predominantly in the mitochondrial compartment. In our approach [9], the *R* hydrogen of ethanol is accepted to be transferred to NAD with the formation of acetaldehyde, catalyzed by cytosolic alcohol dehydrogenase, and the *S* hydrogen to be transferred to NAD by acetaldehyde dehydrogenase either in the cytosolic and/or mitochondrial compartments. We have compared the incorporation of ^{3}H of [*R*1-^{3}H]ethanol and [*S*1-^{3}H]ethanol into lactate, lactate dehydrogenase being a cytosolic enzyme, and water, since NADH is oxidized via the electron transport system of the mitochondrion.

Livers were removed from three *Macaca fasicularis* (monkeys 1–3 of Table 1) and one *Macaca mulatta* (monkey 4 of Table 1). The first two monkeys were killed under ketamine anesthesia with a lethal dose of nembutal, the third was killed under ketamine anesthesia by exsanguination, and the fourth was under nembutal anesthesia at the time the liver was removed. Each was fasted overnight and

Table 1. Metabolism by monkey liver slices of [*R*1-^{3}H]ethanol and [*S*1-^{3}H]ethanol

Monkey	Hydrogen labeled	% Ethanol uptake	% Added ^{3}H incorporated into: Lactate	% Added ^{3}H incorporated into: H_2O	$\frac{Lactate_R/H_2O_R}{Lactate_S/H_2O_S}$
1	*R*	84.6	12.4	50.6	7.1
	S	80.3	1.1	31.8	
2	*R*	49.4	4.7	40.2	9.5
	S	48.6	0.6	48.8	
3	*R*	36.4	4.8	19.7	8.6
	S	29.4	0.6	21.2	
4	*R*	39.6	4.0	25.8	2.8
	S	36.5	1.4	25.4	

was being used for other purposes. The livers were transported in cold saline to the laboratory and slicing began within 20–40 min after removal. The procedures were essentially those previously detailed using slices of rat liver [9].

Briefly, 5 g of slices from the liver of each monkey was distributed into each of two flasks. The contents of the flasks were identical, containing ethanol and glucose, each at a concentration of 100 mg/dl in 30 ml of a high potassium–bicarbonate buffer, except that in one flask the ethanol was labeled with [*R*1-^{3}H]ethanol and in the other [*S*1-^{3}H]ethanol [12]. Two other flasks with identical contents, except without slices, were incubated and served as controls. After 2 hr of incubation with shaking at 37°, perchloric acid was added. The slices and incubation media were homogenized and determinations made on the supernatants obtained after neutralization and centrifugation. Ethanol uptake was determined enzymatically using alcohol dehydrogenase [13]. The supernatants were distilled and the radioactivity was determined in the ethanol in the initial fraction of the distillate. The ethanol was isolated as its *p*-nitrobenzoate derivative [14]. The ^{3}H activity was also determined in a final fraction of the distillate, and with correction for the small quantity of ^{3}H in ethanol in this fraction, this was taken as the measure of incorporation into water. In later experiments, a known volume of water was added to the supernatants so that there was a greater volume distilled between collection of the ethanol-containing initial fraction and the final fraction. The final fraction was then essentially devoid of ethanol. Acidified aliquots of the supernatants were extracted with ether and the lactate in the extracts was isolated as its phenacyl derivative [15]; this derivative was assayed for ^{3}H activity. Radioactivity in the ethyl *p*-nitrobenzoates, phenylacyl lactates, and water fractions was determined by combusting them in an oxidizer (model 306, Packard Instrument Co., Inc., Downers Grove, Ill.). The water was collected and analyzed in a liquid scintillation counter (Series 720, Searle Analytic, Inc., Des Plaines, Ill.), using internal standard to correct to dis./min.

The results for each of the four incubations are recorded in Table 1. In support of the adequate pairing, ethanol uptake was similar for both flasks of each pair. Of the ^{3}H in the ethanol taken up, a large percentage was recovered in water. Relative to incorporation into water, there was 2.8 to 9.5 times as much incorporation in lactate from the *R* as from the *S* hydrogen, when as much of the *S* as of the *R* hydrogen was incorporated into water. Thus, the major portion of the *S* hydrogen is not oxidized in the environment of lactate formation as compared to the *R* hydrogen. This result is similar to that observed using slices of livers from rats, and indicates that the *R* hydrogen had much greater access to the cytosol than the *S* hydrogen in the course of their oxidation. The significance, if any, of the fact that the lowest ratio, 2.8, was obtained with the liver from the *M. mulatta* is unknown, but differences in aldehyde dehydrogenase activities have been observed among strains of rats [5].

It seems reasonable to conclude from these data and the reports of acetaldehyde dehydrogenase activities in mitochondria, although from other species, that in the monkey oxidation occurs under our conditions predominantly via mitochondrial rather than cytosolic NAD-dependent acetaldehyde dehydrogenase(s). The alternatives remain that: (1) the oxidation of acetaldehyde occurs by other than NAD-dependent acetaldehyde dehydrogenase, although at least for rat liver the studies of Parrilla *et al.* [7] and Lindros *et al.* [16] would make this unlikely, or (2) the stereospecificity of the dehydrogenases are of the B rather than the A type, and the NADH has insufficient turnover so that unlabeled hydrogen is transferred via lactic dehydrogenase, an A-type enzyme. This second possibility also seems unlikely in view of the relatively small quantity of the nucleotide present in liver [17] relative to the quantity of ethanol utilized, and the fact that bovine liver aldehyde dehydrogenase is an A-type enzyme and the stereospecificity of a particular reaction is almost always independent of the source of the enzyme which catalyzes it [18].

Acknowledgements—This study was supported by grants from the National Science Foundation and the Distilled Spirits Council of the United States, Inc.

Department of Medicine,
Case Western Reserve University,
School of Medicine,
Cleveland, OH 44106, *U.S.A.*

PAMELA HAVRE
JOSEPH M. MARGOLIS
MARC. A. ABRAMS

REFERENCES

1. R. D. Hawkins and H. Kalant, *Pharmac. Rev.* **24,** 67 (1972).
2. H. I. D. Thieden, *Acta pharmac. tox.* **36** (1), 1 (1975).
3. S. O. C. Tottmar, H. Pettersson and K.-H. Kiessling, *Biochem. J.* **135,** 577 (1973).
4. A. A. Horton and M. C. Barrett, *Archs Biochem. Biophys.* **167,** 426 (1975).
5. R. A. Dietrich and C. Siew, in *Alcohol and Aldehyde Metabolizing Systems* (Eds. R. G. Thurman, T. Yonetani, J. R. Williamson and B. Chance), p. 125. Academic Press, New York (1974).
6. C. J. P. Eriksson, M. Marselos and T. Koivula, *Biochem. J.* **152,** 709 (1975).
7. R. Parrilla, K. Ohkawa, K. O. Lindros, U.-J. Zimmerman, K. Kobayashi and J. R. Williamson, *J. biol. Chem.* **249,** 4926 (1974).
8. R. Rognstad and D. G. Clark, *Eur. J. Biochem.* **42,** 51 (1974).
9. R. J. M. Corrall, P. Havre, J. M. Margolis, M. Kong and B. R. Landau, *Biochem. Pharmac.* **25,** 17 (1976).

10. N. Grunnet, B. Quistorff and H. I. D. Thieden, in *Alcohol and Aldehyde Metabolizing Systems* (Eds. R. G. Thurman, T. Yonetani, J. R. Williamson and B. Chance), p. 137. Academic Press, New York (1974).
11. H. Weiner, P. King, J. H. J. Hu and W. R. Bensch, in *Alcohol and Aldehyde Metabolizing Systems* (Eds. R. G. Thurman, T. Yonetani, J. R. Williamson and B. Chance), p. 101. Academic Press, New York (1974).
12. R. J. M. Corrall, H. M. Rodman, J. Margolis and B. R. Landau, *J. biol. Chem.* **249**, 3181 (1974).
13. R. K. Bonnischsen and H. Theorell, *Scan. J. clin. Lab. Invest.* **3**, 58 (1951).
14. H. Henstock, *J. chem. Soc.* **2**, 216 (1933).
15. J. B. Rather and E. E. Reid, *J. Am. chem. Soc.* **41**, 75 (1919).
16. K. O. Lindros, N. Oshino, R. Parrilla and J. R. Williamson, *J. biol. Chem.* **249**, 7956 (1974).
17. G. E. Glock and P. McLean, *Biochem. J.* **61**, 388 (1955).
18. G. Popják, in *The Enzymes* (Ed. P. D. Boyers), p. 116. Academic Press, New York (1970).

Biochemical Pharmacology, Vol. 25, pp. 2759–2760. Pergamon Press, 1976. Printed in Great Britain.

A simple micro-determination of type B monoamine oxidase

(*Received* 7 *April* 1976; *accepted* 2 *July* 1976)

The existence of type A and B monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4] (MAO) is demonstrable both *in vivo* and *in vitro* by their substrate specificity and their selective inhibition [1–3]. Type A MAO preferentially deaminates 5-hydroxytryptamine and norepinephrine, while type B MAO deaminates β-phenylethylamine (PEA) and benzylamine. For the determination of type A MAO, simple and sensitive methods are available [4, 5], and for that of type B MAO, spectrophotometric method using benzylamine as substrate [6] and fluorometric method using PEA as substrate [7] are available. However, the method for the determination of type B MAO using benzylamine is not highly sensitive and that using PEA involves an extraction procedure with organic solvent. Therefore, we have intended to obtain a more simple and sensitive method. The present paper deals with the details of a new, simple and sensitive method for the determination of MAO activity towards PEA by measuring phenylacetaldehyde, the reaction product from PEA catalyzed by MAO.

It has been shown by Samejima *et al.* [8, 9] that phenylacetaldehyde reacts with ninhydrin and a primary amine to yield a highly fluorescent compound, and this can be adopted for a sensitive measurement of phenylacetaldehyde. However, the substrate, PEA, can also react with ninhydrin to yield the same compound [10]. Accordingly, the reaction of PEA with ninhydrin should be suppressed. For this purpose, our previous finding that PEA does not react with ninhydrin below pH 6 [11] can be successfully adopted.

In the case of crude enzyme preparation such as tissue homogenate, the enzymes which destroy phenylacetaldehyde should be inhibited. The responsible enzymes are aldehyde dehydrogenase [12], aldehyde oxidase [13], aldehyde reductase and alcohol dehydrogenase [14]. We tested the inhibitors of each enzyme, *viz.* chloral hydrate, sodium azide, sodium phenobarbital and pyrazole, by adding them to rat brain and liver homogenates. However, only the addition of chloral hydrate to liver homogenate was effective, and the other inhibitors were not effective. It was confirmed that chloral hydrate has no effect on MAO activity.

When tissue homogenate was used as an enzyme source, the recovery of phenylacetaldehyde was found to be rather low. Taking into account the fact that aldehyde derivatives are adsorbed onto brain macromolecules [15], the low recovery was considered to be ascribed to similar adsorption phenomenon in the tissue homogenate, and we tried to avoid such adsorption by adding organic solvents. Among the organic solvents tested, *viz.* acetone, methanol and ethanol, methanol was found to be adoptable, since acetone inhibits ninhydrin reaction and ethanol causes turbidity, even though they seem to be effective.

The fluorescence excitation and emission spectra of the fluorescent compound obtained from the product formed from PEA by liver homogenate were found to be identical with those of the compound obtained from authentic phenylacetaldehyde, having the excitation and emission maxima at 390 nm and 495 nm, respectively.

On the basis of the above data, we propose the following procedure as a standard method for the determination of type B MAO in animal tissues.

Tissue was homogenized with 0.9% NaCl solution in a glass homogenizer fitted with a Teflon pestle. The incubation was carried out in a small centrifuge tube. To 0.5 ml of the homogenate containing 1–10 mg of the tissue, were added 0.15 ml of 0.5 M sodium phosphate buffer (pH 7.4), 0.1 ml of 0.05 M chloral hydrate solution, and 0.5 ml of PEA solution containing 50 μg (final concentration, 0.25 mM) of PEA–HCl. After incubation at 37° for 30 min, the enzyme reaction was stopped by adding 0.1 ml of 30% trichloroacetic acid, then mixed with 2 ml of methanol, shaken vigorously for 2 min and centrifuged (3,000 rpm, 5 min). A 0.5-ml aliquot of the supernatant was incubated with 1.5 ml of 0.5 M sodium phosphate buffer (pH 5.7), 0.5 ml of 0.05 M ninhydrin solution and 0.5 ml of 3 mM L-leucyl-L-alanine solution at 60° for 60 min in an oil bath, and left at room temperature for 15 min. The fluorescence intensity of the reaction product was measured with excitation at 390 nm and emission at 495 nm. As a blank test, the reaction mixture without substrate was incubated, and mixed with trichloroacetic acid and the same amount of PEA solution. Internal standard was taken by adding an appropriate amount of phenylacetaldehyde (Aldrich Chemical Co., Milwaukee, Wis.) to the assay mixture before incubation to permit direct calculation of results. The recovery of phenylacetaldehyde was over 80 per cent.

Figure 1 shows the relationship between MAO activity and the enzyme concentration with liver and brain homogenates as enzyme sources. The linear relationship was observed between MAO activity and the amount of enzyme in the range from 2.5 mg to 10 mg of the tissues in the assay mixture.

Figure 2 shows that there is a linear relationship