sin content, the following entire isolation procedure has to be carried out in a dim red light to prevent formation of opsin. This method permits the preparation of rod outer segments from 100–120 frozen bovine retinas. The retinas, suspended with 43% sucrose in 100 mM Tris-HCl buffer, pH 7.4, (1 ml/retina) were homogenized manually with a Kontes glass homogenizer. The suspension (180 ml) was divided between the tubes of a Beckman SW 27 rotor and overlaid with Tris buffer, pH 7.4. The discontinuous gradients were centrifuged at 113,000 × g for 60 min. The interfacial material was diluted with an equal volume of Tris buffer, pH 7.4, and centrifuged in a Beckman 35 rotor at 46,600 × g for 20 min to sediment rod outer segments. The sedimented pellet, suspended in 20 ml of 43% sucrose in 100 mM Tris-HCl buffer, pH 7.4, was layered between 15 ml of 43% sucrose buffered solution (bottom) and 15 ml of 100 mM Tris-HCl buffer, pH 7.4 (top). The Beckman SW 27 rotor was operated at 113,000 × g for 60 min. The interfacial red membranes were removed, diluted with Tris buffer and sedimented at 46,600 × g for 20 min. The sedimented red pellet, suspended in 12 ml of 1.10 g/ml density sucrose solution, was pipetted in 3 ml portions onto the tops of 4 gradients containing 1 ml of 1.2 g/ml buffered sucrose and 8 ml of a continuous density sucrose gradient 1.15 to 1.10 g/ml. Gradients were centrifuged in a Beckman SW 36 rotor at 182,000 × g for 60 min. A sharp red band at the top of the gradient was collected, care being taken to discard the superior phase containing synaptosomal particles. Rod outer segments were diluted and sedimented and the last step was repeated.

Membranes were stored at -20 °C and used within 3 weeks of preparation. The figure shows a schematic representation of the above procedure. Spectrophotometric analysis of rhodopsin content has been used as the purity criterium for rod outer segment preparations. As long as the rod disc membranes contain the bulk of the eye's rhodopsin, in pure preparations of rod outer segments, the ratio between absorbance at 280 nm and absorbance at 498 nm should correspond to a rhodopsin content of 65–70% of the total proteins[9]. Considering that cattle retinas are from animals not previously dark-adapted, the observed ratio of 2.4–2.6 is indicative of good disc purification.

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Methoxyphenylethylamines as substrates for type A and type B monoamine oxidase

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Summary. 4-Methoxyphenylethylamine was found to be a specific substrate for type B monoamine oxidase (MAO) in rat brain mitochondria, whereas 3,4-dimethoxyphenylethylamine was common for both types of MAO. These results suggest that O-methylation in the para-position increases the preference of the substrate for type B MAO, while a methoxy-group in the meta-position contributes to the substance being a type A substrate.

Mitochondrial monoamine oxidase [amine: oxygen oxidoreductase (deaminating, flavin-containing); EC 1.4.3.4] (MAO) is believed to exist in many animal tissues in 2 functional forms called type A and type B[1–3]. The relationship between the structure of a monoamine and its substrate specificity for type A and type B MAO has been investigated; a hydroxyl-group in the para- or meta-position may contribute to a substance being a type A substrate[4–6]. The β-hydroxylation of a monoamine seems to enhance its preference for type A MAO only slightly[7]; N-methylation causes almost no change in the substrate specificity[8]. In the present paper, we have studied the effect of a methoxy-group on substrate specificity for type A and type B MAO using 4-methoxyphenylethylamine (MPEA) and 3,4-dimethoxyphenylethylamine (DMPEA).

Materials and methods. A crude mitochondrial fraction was isolated from whole brains of male Sprague-Dawley rats weighing 150–250 g as described previously[7]. MPEA and DMPEA were purchased from Sigma Chemical Company, St. Louis, Mo. Clorgyline, a selective inhibitor of type A MAO[1], was generously supplied by May & Baker Ltd, Dagenham, England. Deprenyl, a selective inhibitor of type B MAO[9], was kindly donated by Prof. J. Knoll, Semmelweis University of Medicine, Budapest, Hungary.

MAO activities were determined fluorometrically by the method of Guilbault et al.[10] and Snyder and Hendley[11]. For each assay (final volume, 3.0 ml), 0.35 mg of protein was used. The assays were carried out at pH 7.4 for 30 or 60 min. Under the conditions used, the assays were linear during incubation for 60 min. For kinetic analyses, the concentration ranges of MPEA and DMPEA were 15–1000 µM and 0.3–10 mM, respectively.

For inhibition experiments with clorgyline and deprenyl, the assay mixtures were preincubated with each inhibitor at 37 °C for 10 min to ensure maximal enzyme inhibition. It was confirmed that each inhibitor neither interfered with the formation of the fluorescent compound nor quenched its fluorescence when hydrogen peroxide was added directly. Protein was measured by a modification[12] of the conventional biuret method.

Results. Both MPEA and DMPEA were active substrates for MAO in rat brain mitochondria. Their Michaelis-Menten kinetic constants were determined from Lineweaver-Burk plots as shown in figure 1. The K_m value for MPEA was much lower than that for DMPEA; the V_{max} value for MPEA was higher than that for DMPEA. There was slight substrate inhibition at the highest substrate concentrations for both substrates.

In order to determine the specific MAO type responsible for the oxidation of the amines, the sensitivity to clorgyline and deprenyl was studied as illustrated in figure 2. All the curves with MPEA as substrate were single sigmoidal; clorgyline, at a concentration of 10^{-8} M, hardly inhibited MPEA deamination, but deprenyl at 10^{-7} M completely inhibited the activity. There was almost no difference in the inhibition pattern between the 2 concentrations of MPEA. These results clearly indicate that MPEA is highly specific for type B MAO over a wide concentration range of the substrate.

The curves with DMPEA as substrate are also presented in figure 2. In the curves with clorgyline, clear plateaus appeared at 10^{-8}–10^{-7} M for both concentrations of the substrate. The sensitivity to clorgyline with 10 mM DMPEA was higher than with 500 µM DMPEA. In the curves with deprenyl, plateaus were not clear, and the deamination of 10 mM DMPEA was more resistant to deprenyl than that at 500 µM.

Discussion. In the present paper, we have characterized MPEA and DMPEA, the simplest methoxy-derivatives, as substrates for type A and type B MAO in order to study the effect of O-methylation on substrate specificity for both types. Since in our previous papers[7,13,14] it was reported that substrate concentration affects the inhibition patterns with clorgyline and deprenyl especially when phenylethylamine and phenylethanolamine were used as substrates, we tested, in the present study, 2 different substrate concentrations for each substrate.

We have demonstrated that MPEA is specific for type B MAO (figure 2). para-Tyramine, the precursor of MPEA, is a common substrate for both types of MAO in rat brain mitochondria[5], with type A activity being much higher than type B activity. These findings suggest that O-methylation in the para-position causes the inactivation of type A activity or increases affinity for type B MAO since the K_m value (figure 1) of MPEA for type B MAO is much lower than that of para-tyramine for type B MAO[15].

Using DMPEA as substrate, clear plateaus appeared in the curves with clorgyline at different substrate concentrations, but not in the curves with deprenyl (figure 2). The lack of plateaus in the curves with deprenyl was previously observed, despite the presence of both types, when enzyme preparations such as rabbit[16] and chick tissues[17,18], and substrates such as meta-octopamine[6] were used. It can be concluded, therefore, that DMPEA is common for both types. Houslay and Tipton[4] classified DMPEA into a type B substrate without any presentation of experimental data using a photometric assay method. Since the present fluorometric assay is much more sensitive than the photometric one, the discrepancy may be due to the low sensitivity of their method. Our result with DMPEA suggests that the methoxy-group in the meta-position contributes to its being a substrate for type A MAO. Roth and Feor[19] reported that MAO activity towards 3-methoxytyramine, the meta-O-methylated compound from dopamine, was much more sensitive to clorgyline than that towards dopamine, which is consistent with our idea.

MPEA, to our knowledge, has not been identified in biological samples. However, it seems possible that this

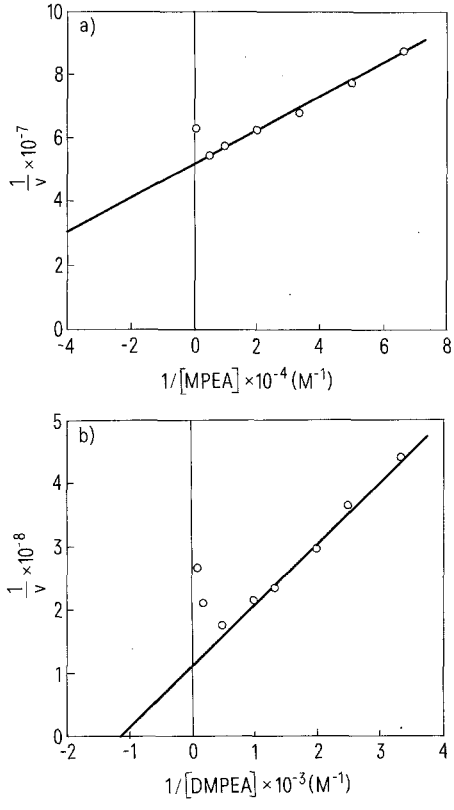

Fig. 1. Lineweaver-Burk plots for rat brain mitochondrial MAO with MPEA (a) and DMPEA (b) as substrates. The K_m values for MPEA and DMPEA were 10.2 and 869 µM, respectively; the V_{max} values were 19.2 and 9.00 nmoles/mg protein/30 min. The symbol v indicates moles H_2O_2 formed/mg protein/30 min. Each point represents the mean obtained from duplicate determinations on a single enzyme source prepared from the pooled brains of 6 rats.

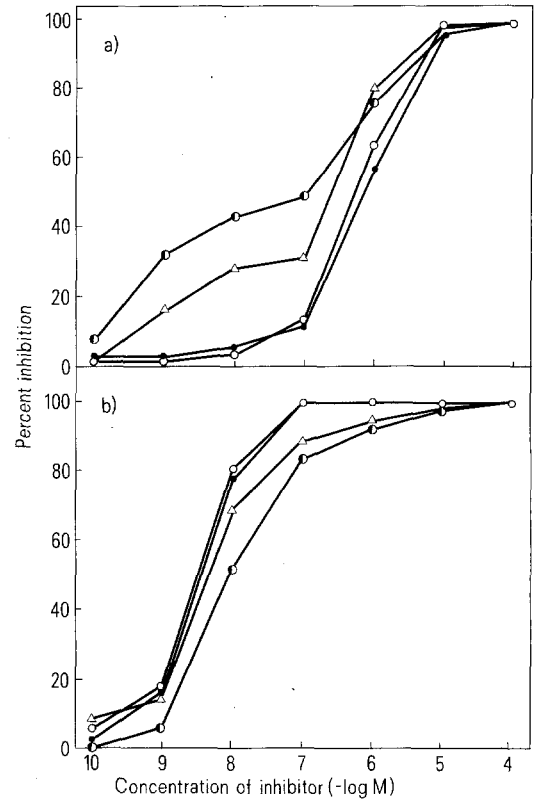

Fig. 2. Inhibition of MAO in rat brain mitochondria by clorgyline (a) and deprenyl (b) using different concentrations of MPEA and DMPEA as substrates. Key: 10 µM MPEA (○——○); 1000 µM MPEA (●——●); 500 µM DMPEA (△——△); 10 mM DMPEA (◐——◐). Each point represents the mean obtained from duplicate determinations on a single enzyme source prepared from the pooled brains of 6 rats.

amine is formed from para-tyramine by non-specific transmethylase[20]. DMPEA has been identified in the urine of both schizophrenic and normal subjects[21]. Therefore, these amines might be actively metabolized by MAO in vivo in mammalian tissues.

Many workers employ phenylethylamine as a type B-specific substrate. However, since phenylethylamine loses its specificity for the type B enzyme under various conditions[13,14], MPEA as well as benzylamine[13] is to be recommended as a more specific substrate for type B MAO.

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Levels of erythrocyte 2,3 DPG and ATP in heavy hashish smokers

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Summary. The variations in concentration of ATP and 2,3 DPG, and in lactic acid production as a function of time, were measured in the erythrocytes of heavy hashish smokers. Results indicate that the most remarkable reduction in concentration of the compounds examined occurred at 30 and 45 min after hashish smoking. The findings are discussed in connection with the possible influence of hashish components, especially Δ^9-THC, on the erythrocyte glycolytic pathway.

Hashish compounds are known to affect the metabolism of proteins, nucleic acids and lipids[1-3], and therefore probably influence carbohydrate metabolism. The connection between hunger – especially for sweet foods – and hashish smoking[4], led investigators to search for a hypoglycemic effect of the drug; none was observed[5,6]. On the contrary, Podolsky et al.[7] have detected hyperglycemic changes which were attributed to hashish constituents. Another interesting observation is that hashish compounds can inhibit glucose transport from the plasma into the erythrocytes[8]. Functional and structural changes induced by cannabinoids have also been described[3,9]. These observations prompted us to examine the possible variation of erythrocyte ATP and 2,3 DPG, and lactic acid production in chronic hashish users, under experimental conditions.

Materials and methods. 25 volunteers, heavy hashish smokers (aged 45–56), were examined in this study. These subjects had been using hashish for 20–30 years. Some of them had obvious signs of chronic cannabism. According to the information given by them they had every kind of hashish product (pure resin, marihuana etc.) that could possibly be found, always using a narghile pipe. Some of them reported smoking up to 100 g of crude hashish at a time. The hashish product used in this experiment was pure hashish resin extracted from the flowering tops of female cannabis plants. The hashish resin used was first analyzed by chromatographic methods and thus the concentration of the active constituents, especially Δ^9-THC, was accurately known (3,6%). Each of the hashish users was allowed to smoke 20 g of pure resin using a narghile pipe for a period not exceeding 10 min. In all cases blood samples were drawn from an antecubital vein prior to smoking and at 15, 30, 45 and 60 min following smoking, and transferred immediately into siliconized capillary tubes. After the whole blood leukocytes and platelets were separated, the erythrocyte residues were mixed with a 9:1 v/v 1.5% EDTA solution in 0.9% NaCl and centrifuged at 13,000 rpm for 30 min at 4 °C (Sorvall RC-2B). In the erythrocyte pellet ATP was estimated as described by Lamprecht and Trantschold[10]; 2,3 DPG was measured spectrophotometrically following the enzymatic method of Krimsky[11]. Finally lactic acid production was measured according to the method of Hohorst et al.[12]. The statistical analysis employed was Student's t-test.

Results. The values of erythrocyte 2,3 DPG, ATP and lactic acid production are presented in the table. It can be seen that the greatest alteration in 2,3 DPG and ATP as well as in lactic acid production are observed 30 and 45 min after smoking hashish. After 60 min the values tend to return to the level prior to smoking.

Discussion. The results obtained reflect the biochemical action of hashish components, especially Δ^9-THC, on erythrocyte glycolysis in heavy hashish smokers. The concentrations of ATP, 2,3 DPG and lactic acid production decreased in the erythrocytes of these subjects, with the greatest reduction being observed at 30 and 45 min after hashish smoking, when the highest concentration of the drug in the blood stream is noted[13]. This correlation indicates that the observed changes could be attributed to the direct and rapid effect of hashish constituents on erythro-