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METABOLISM OF PHYSOSTIGMINE IN MOUSE LIVER MICROSOMAL INCUBATIONS STUDIED BY LIQUID CHROMATOGRAPHY WITH DUAL-ELECTRODE AMPEROMETRIC DETECTION

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SUMMARY

The metabolism of physostigmine was studied by its incubation with the microsomal fraction of mouse liver. The metabolites formed were separated by reversed-phase ion-pair liquid chromatography and detected amperometrically by dual electrodes. Two major and six minor metabolites were found. Retention times and electrochemical characteristics were studied for these and compared with the hydrolysed products of physostigmine: eseroline and rubreserine. It can be concluded that none of the major metabolites is identical with these standards, and this is in contradiction to what has previously been assumed. Conjugation reactions of the metabolites with glucuronic acid and glutathione were also attempted, but no indication was found that these reactions take place during microsomal incubation.

INTRODUCTION

Physostigmine (eserine; Phy) is an alkaloid extracted from the plant *Physostigma venenosum* (Calabar bean). This drug is well absorbed in the gastrointestinal tract and readily enters the central nervous system, where it acts as an acetylcholinesterase inhibitor [1]. It is used as an antidote to anticholinergic drugs, such as atropine, scopolamine and tricyclic antidepressants [2], and to reverse other drug-induced anticholinergic effects, e.g. in connection with anaesthesia [3]. Recently, investigations have been undertaken on its possible prophylactic use in organofluorophosphate intoxication [4] and for the treatment of senile dementia in Alzheimer's disease [5]. It is also used as a miotic in ophthalmology [6].

Phy was isolated from Calabar beans as early as in 1863 [7]. At the same time

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the spontaneous formation of coloured decomposition products was evident. Depending on the reaction conditions, several different compounds can form and these have been characterized and their preparations described [8,9]. The hydrolysed products eseroline (Ese) and rubreserine (Rub) can also be generated at an electrode surface. The electrochemical pathways between these and the parent compound have been investigated with cyclic voltammetry, coulometry and chronoamperometry [10]. Pharmacological studies have shown that the action of Phy is terminated by metabolism rather than by urinary or biliary elimination [11] and that the metabolism takes place predominantly in the liver [12]. Furthermore, the pharmacokinetics of Phy have been studied after intramuscular administration of ^{3}H-labelled Phy to rats [13]. Fractions were collected after liquid chromatographic (LC) separation, and three metabolites were found of which one was believed to be Ese.

In this study we examined the metabolites of Phy formed by the incubation of Phy with the microsomal fraction of mouse liver. The microsomal fraction contains the enzyme cytochrome P-450, which is a mixed function oxidase with a central role in the body's defence against many chemical agents [14]. The metabolites formed after incubation were separated by reversed-phase ion-pair LC and detected amperometrically. By using on-line detection the high chromatographic resolution can be maintained, which is not the case when fraction collection is involved. The electrochemical properties of the metabolites were studied with dual electrodes and compared with those of standard solutions of Ese and Rub. The combined information from retention times and electrochemistry was then used in the discussion of the possible identity of the metabolites. The possibility of enzyme-catalysed conjugation reactions of the metabolites with glutathione or glucuronic acid were investigated by adding the necessary substrates to the microsomal fraction before incubation. A further attempt to mimic animal studies more closely was made by incubating Phy with mouse liver slices.

EXPERIMENTAL

LC instrumentation

The liquid chromatograph was a Bioanalytical Systems LC-304 with an LC-23A column heater and an LC-22A temperature controller. Two LC-4B amperometric controllers were used for the dual-electrode detection together with glassy carbon working electrodes and an Ag/AgCl reference electrode. A Rheodyne Model 7125 injector valve equipped with a 20-μl injector loop was used for injecting samples, and a Biophase Octyl column (250 mm$\times$4.6 mm I.D., particle size 5 μm) was used for the separations. Additional cyclic voltammetry (CV) was performed on a BAS 100 electrochemical analyzer with an Auto Cell stand, a glassy carbon working electrode, a platinum wire auxiliary electrode and an Ag/AgCl reference electrode. All equipment was from Bioanalytical Systems (West Lafayette, IN, U.S.A.).

Chromatographic conditions

The mobile phase was acetonitrile (Mallinckrodt, St. Louis, MO, U.S.A.; redistilled)–0.1 *M* phosphate buffer [pH 3.0; prepared with sodium dihydrogenphos-

phate (Mallinckrodt) and 85% phosphoric acid (Fisher, Fair Lawn, N.J., U.S.A.)] (40:60) containing 17 m*M* (0.5%, w/v) sodium dodecylsulphate (SDS) (Fluka, Buchs, Switzerland). All water was deionized and distilled. The flow-rates used were 1 and 2 ml/min, and the column temperature was set at 35°C. The dual electrodes were used in series or parallel adjacent mode, with potentials applied as specified in the figure legends.

Hydrodynamic voltammograms (HDVs)

If not otherwise specified, the HDVs were obtained with series dual electrodes by keeping E_1 at +1.0 V and varying the potential of E_2. Sample was injected at each potential setting and the peak-current ratios were calculated and plotted versus the potential of E_2.

Standards

Phy (free base) was obtained from Sigma (St. Louis, MO, U.S.A.), Ese was prepared by alkaline hydrolysis of Phy in the absence of oxygen in the following manner: 2 ml of 10^{-4} *M* Phy were deoxygenated by purging it with nitrogen for a few minutes and then made alkaline by adding two drops of 10% sodium hydroxide. The nitrogen purging was continued for a further 10 min before the reaction was stopped by the addition of 6 ml of 0.1 *M* phosphoric acid. This solution was kept sealed in a nitrogen atmosphere. Rub was also prepared by alkaline hydrolysis of Phy, but in the presence of oxygen in the following and otherwise similar manner. Two drops of 10% sodium hydroxide were added to 2 ml of $5 \cdot 10^{-5}$ *M* Phy. After 5 min of stirring the reaction was stopped by the addition of 6 ml of 0.1 *M* phosphoric acid.

Microsomal incubations

ICR male mice (Harlan Sprague–Dawley, Indianapolis, IN, U.S.A.) were decapitated and their livers were removed and rinsed with cold saline solution. The livers were homogenized in cold 20 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) (Sigma) buffer (pH 7.4) containing 1.15% potassium chloride and the homogenates were centrifuged at 10 000 *g* for 20 min. The supernatants were transferred to clean tubes and centrifuged for 60 min at 100 000 *g*. The resulting pellets were resuspended in fresh buffer and centrifuged again at 100 000 *g* for 60 min, while the supernatants containing the cytosol were saved and used in some of the experiments. The final pellets containing the microsomal fraction could be kept frozen in liquid nitrogen for a few days or used at once. Prior to use, each pellet was homogenized in 6 ml of 26 m*M* phosphate buffer (pH 7.4), 98 m*M* potassium chloride solution. The protein concentration was determined by the method of Lowry et al. [15] in one experiment, using bovine serum albumin as standard, and was found to be 0.6 mg/ml.

For the incubations, 500 µl of the microsomal proteins were added to tubes containing 25 µl of Phy and 25 µl of water. The metabolism was initiated by the addition of 100 µl of NADPH (Sigma) in magnesium chloride solution. The final concentrations were $2 \cdot 10^{-4}$ *M* Phy, $4 \cdot 10^{-4}$ *M* NADPH, 15 m*M* magnesium chloride, 75 m*M* potassium chloride and 20 m*M* phosphate. The tubes were incubated

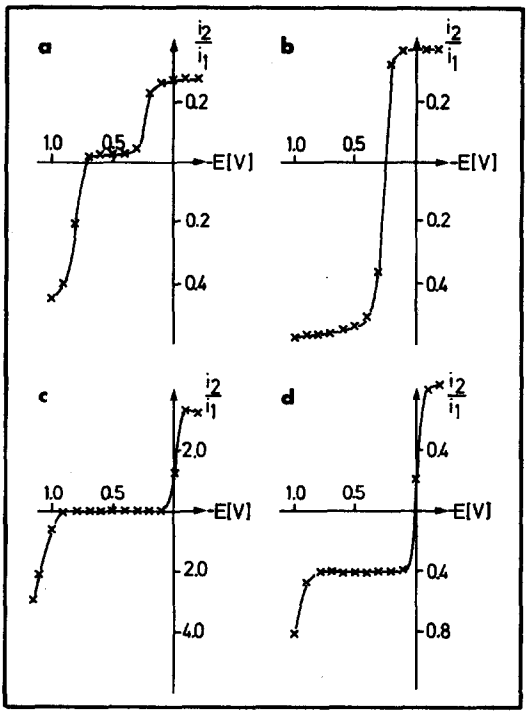


Fig. 1. Hydrodynamic voltammograms at pH 3.0 of standard solutions of (a) Phy, (b) Ese, (c) Rub and (d) Rub. Obtained with series dual-electrode detection with $E_1 = +1.0$ V and E_2 varied, except in d, where $E_1 = -0.2$ V.

during shaking for 30 min, after which 350 μl of ice-cold quenching solution (made up from 5.8 ml of methanol and 0.2 ml of 1 *M* perchloric acid) were added. They were then centrifuged for 10 min, and the supernatant was injected directly on the chromatograph. Control incubations with Phy, NADPH, magnesium chloride or microsomes omitted were also carried out. For the conjugation studies 25 μl of water were replaced by 25 μl of glutathione (reduced, from Sigma) or uridine diphosphoglucuronic acid (UDPGA) (Sigma). The final concentrations were 2.4 and 0.7 m*M*, respectively. In one set of incubations the cytosol (contained in the supernatant saved during the differential centrifugation) replaced half the volume of microsomal proteins. In another set of incubations the cytosol was used completely instead of microsomal protein.

Mouse liver slice incubations

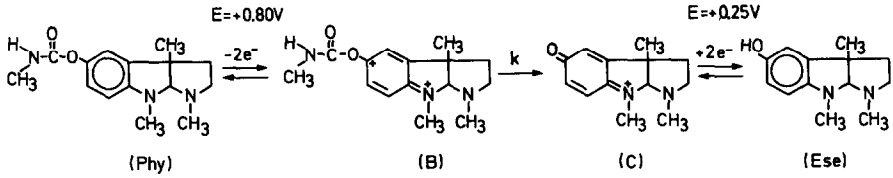
A thin liver slice was incubated in 1 ml of potassium chloride–phosphate (pH 7.4) and 25 μl of $1 \cdot 10^{-2}$ *M* Phy for 30 min at 37°C. Blank incubations were also carried out as well as incubations on previously frozen liver slices.

RESULTS AND DISCUSSION

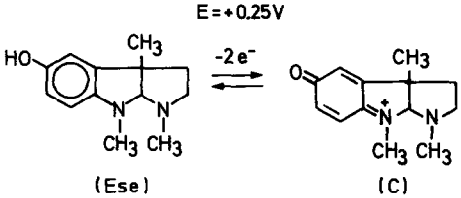
Electrochemistry and LC of standards

HDVs were recorded at pH 3 for Phy and its hydrolysed products Ese and Rub. Phy shows an oxidation wave at +0.8 V, which appears to be irreversible (Fig.

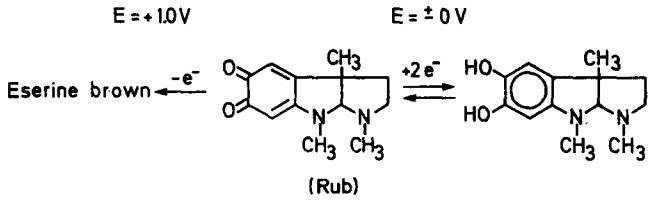
1a). A reduction wave appears at +0.25 V, and this is from a product formed at the first series electrode, since no reduction wave appears if there is no previous oxidation. This behaviour is in agreement with an ECE reaction mechanism. A previous study on the electrochemistry of Phy, Ese and Rub has been performed employing cyclic voltammetry, coulometry and chronoamperometry [10]. The results presented from that study can be used to explain the features of the HDVs. In the scheme below it is implied that Phy is oxidized to B at the first of the series electrodes. B undergoes a fast irreversible chemical reaction to yield C, and C can in turn be reduced to Ese at a more negative potential at the second of the series electrodes.



The HDV of Ese (Fig. 1b) shows a reversible oxidation wave at +0.25 V. This is explained by the same reversible reaction that was observed at +0.25 V for Phy in accordance with the following scheme:



Rub shows a reversible reduction wave at ±0 V (Fig. 1c and d). Furthermore, an irreversible oxidation wave appears at +1.0 V, and this feature had not been recognized in the previous CV study. Rub is known to undergo further oxidation to yield eserine brown, possibly a polymerization product [7]. Hence, this is a probable explanation of what is observed at +1.0 V, as outlined in the following reaction scheme:



A chromatogram of a mixture of Phy, Ese and Rub is shown in Fig. 2. In this case a parallel electrode configuration was used. Only Rub shows a reduction peak at the electrode monitored at −0.2 V and only Phy and Ese show oxidation peaks at the electrode monitored at +1.0 V. The three components are chromatographically completely resolved and the ion-pairing system gives good peak shape for

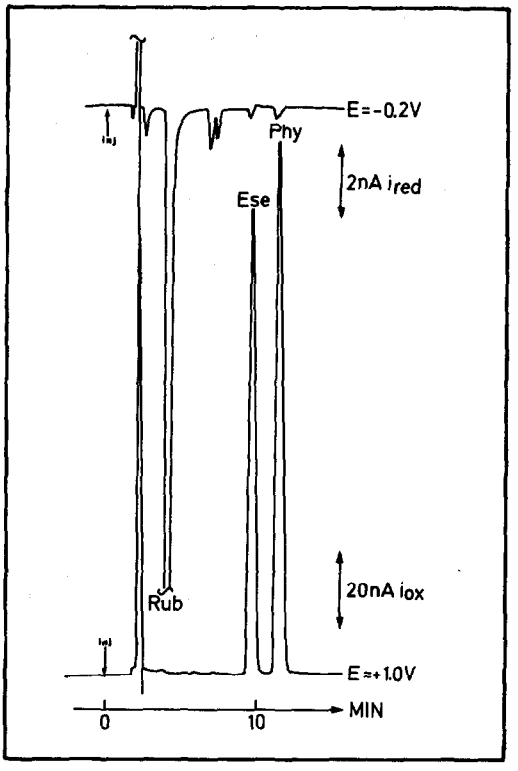


Fig. 2. Chromatogram of a mixture of Phy, Ese and Rub. Parallel dual-electrode detection; flow-rate, 2 ml/min; other LC conditions as in Experimental.

these compounds, of which Phy is protonated and positively charged throughout the pH range normally used with bonded-phase silica columns. It was observed that the retention of Phy increases with increasing concentration of SDS in the mobile phase. The dominating retention mechanism therefore is by ion-pairing rather than micellar chromatography. The high concentration of organic solvent prevents the formation of micelles, although the concentration of 17 m*M* SDS in the mobile phase is well above the reported critical micellar concentration of 8 m*M* for this detergent in water. The acidic pH used was chosen to lie within the range where Phy has its optimum stability to avoid the decomposition to Rub, which is known to take place rapidly in alkaline solution.

Mouse liver microsomal incubations

Mouse liver microsomal incubations were performed as described in Experimental. This included a series of blanks that proved that no peaks in the chromatograms originated from any of the reagents used. It was found that no decomposition of Phy took place unless both microsomes and NADPH were present. This suggests that cytochrome P-450 is responsible for the metabolism, since

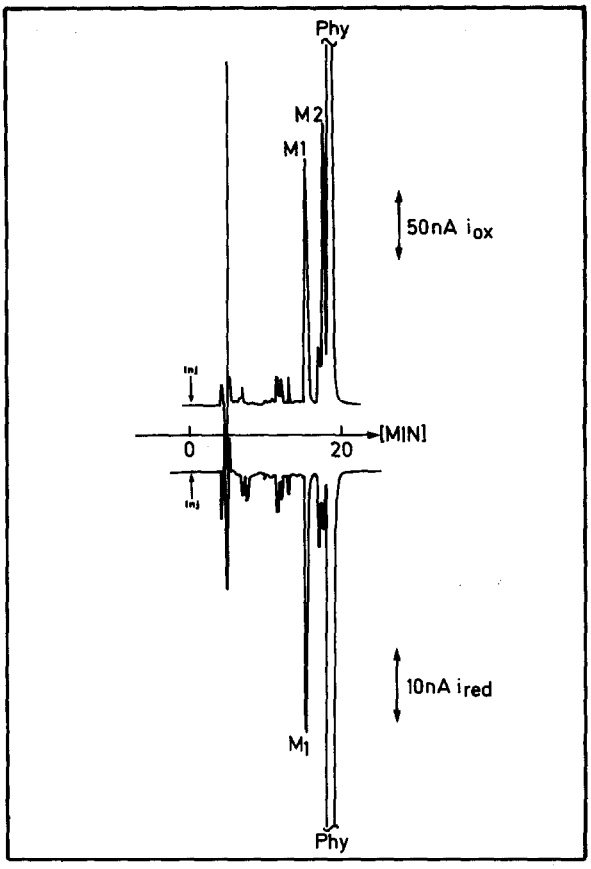


Fig. 3. Chromatogram of mouse liver microsomal incubation. Series dual-electrode detection with $E_1 = +1.0$ V and $E_2 = -0.2$ V; flow-rate, 1 ml/min, other LC conditions as in Experimental.

this enzyme is dependent on the presence of oxygen and NADPH for its functioning [16]. A chromatogram of a microsomal incubation of Phy is shown in Fig. 3. Examination of the oxidative, upper trace of this figure shows two major metabolites, which are denoted M_1 and M_2. In addition there are six minor metabolites present. In the previous study on Phy metabolism, ^{3}H-labelled Phy was intramuscularly administered to rats and the pharmacokinetics was studied [13]. The similarity between our microsomal incubations and the animal study is the formation of the metabolite M_1, which in the latter case was tentatively identified by retention time as Ese. The published work involved fraction collection before detection, yielding a broad peak for the parent drug, which might have hidden M_2 and one of the minor metabolites observed in Fig. 3. The striking difference between these studies is that our chromatogram lacks what is their main metabolite(s) eluting with the void volume. A possible explanation for this discrepancy is that the non-retained peak could consist of tritiated water or other small molecules containing the tritium that has been replaced during the metabolism. Another possibility is a product of some conjugation reaction not available in a microsomal incubation.

The possibility of further metabolism through conjugation reactions with glucuronic acid and glutathione was investigated as described in Experimental. Conjugation with glucuronic acid is the most commonly found reaction converting a compound into a more hydrophilic form that is more easily excreted from the body [20]. The enzyme glucuronide transferase is present in the microsomal fraction, but the substrate, glucuronic acid, is dissolved in the cytosol. Therefore, either cytosol contained in the supernatant saved during the differential centrifugation or UDPGA was added before incubation. However, no new peak could be detected in the chromatograms, indicating that no conjugation product has formed. The amount of the previously observed metabolites was found to be the same whether cytosol and UDPGA were added or not. Glutathione alone or glutathione plus cytosol was added to other incubations. This nucleophile is known to trap intermediates to form conjugates. This has been studied for many other compounds e.g. *p*-benzoquinone [21]. In the case of Phy no adducts formed, as is indicated by the fact that no new peaks appeared in the chromatograms. Instead the amounts of the metabolites formed significantly decreased and more of the Phy remained unchanged. Here glutathione may be acting as a reducing agent preventing the formation of oxidized metabolites. This function of glutathione has previously been noted in microsomal incubations of aniline [22].

Tissue slices have been used to study metabolism in cases where the liver microsomal system does not completely mirror what occurs in the whole organism [23]. A liver slice contains more enzyme systems than the microsomal fraction and should mimic an animal experiment more closely. Mouse liver slice incubations were carried out as described above, also on previously frozen liver. Cytochrome P-450 lies deeply buried in the microsomes, and pretreatment by freezing and thawing, alternatively the addition of a surfactant, has been noted to increase the availability of the enzyme [20]. However, very little metabolic action was obtained in comparison with the microsomal incubations, and it was not possible from our experiments to conclude if any new metabolite had formed.

A brief kinetic study of the formation of metabolites during microsomal incubation was performed by drawing small aliquots of sample solution at close time intervals. These were drawn from one large batch of microsomal incubation into small tubes containing quenching solution and centrifuged before injection of the clear supernatants. The results showed that all the metabolites appeared simultaneously (Fig. 4). As other workers have noted, the metabolism of Phy is fast. Somani and Khalique [13] have reported half-life values of 17 and 16 min in the plasma and brain, respectively, after intramuscular distribution to rats. In our experiment a steady state was reached after ca. 15 min of incubation time, after which the concentration of the metabolites remained constant during the rest of the experimental time, 2 h.

Nature of the metabolites

To obtain further information on the possible nature of the metabolites formed, HDVs were recorded. In these HDVs the current ratios are plotted versus E_2 when E_1 in a series mode is held at a constant potential of +1.0 V. The calculation of these current ratios compensates for different sample concentrations to yield simply qualitative information. The HDVs for M_1 and M_2 are shown in Fig. 5,

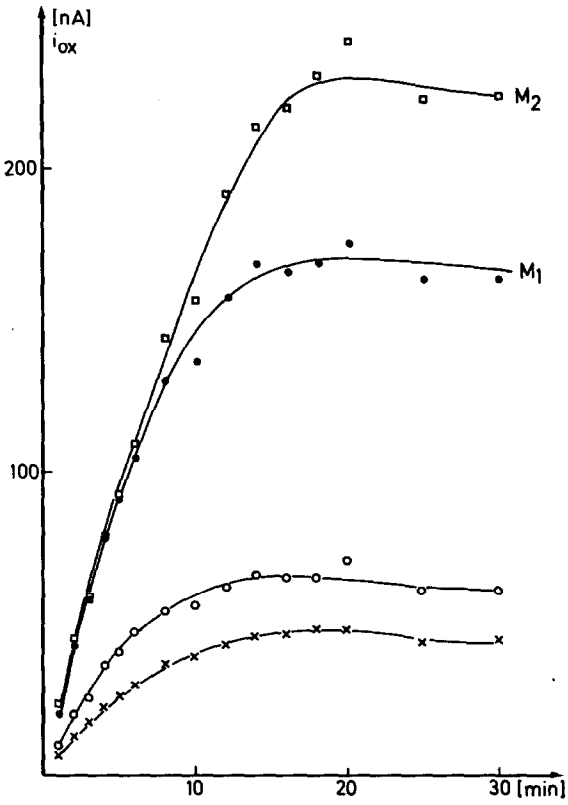


Fig. 4. Time dependence of the formation of metabolites of Phy in a mouse liver microsomal incubation. The two major metabolites, M_1 and M_2, and two of the minor metabolites are shown.

and these can be compared with the HDVs in Fig. 1 recorded for the standard compounds. The striking difference between the HDVs of M_1 and Ese standard strongly suggests that these two compounds are not the same. Further evidence for this conclusion was obtained when the incubation products were spiked with Ese prior to injection. M_1 and Ese were here found to separate with a resolution of ca. 0.8, with M_1 slightly less retained than Ese.

It has also been suggested that the active form of Phy is the open-ring indolium cation. This is formed by a reversible process in 6 *M* hydrochloric acid and could be formed in enzyme-catalysed reactions [19]. To study the effect of this ring opening, the open ring forms of Phy and Ese were generated in 6 *M* hydrochloric acid. However, when the solutions were diluted and injected onto the liquid chromatograph at pH 3.0, only the closed ring forms were found. Therefore, it seems that the structure of M_1 is not as simple as being the open ring form of Ese.

Different possibilities of dealkylation and hydroxylation commonly attributed to cytochrome P-450 are possible for the Phy molecule. Since the metabolites show about the same oxidation characteristics as the parent drug, a suggestion is that they still contain the methyl urethane moiety and have undergone different N-dealkylation and (or) hydroxylation of the aromatic ring. Another study has

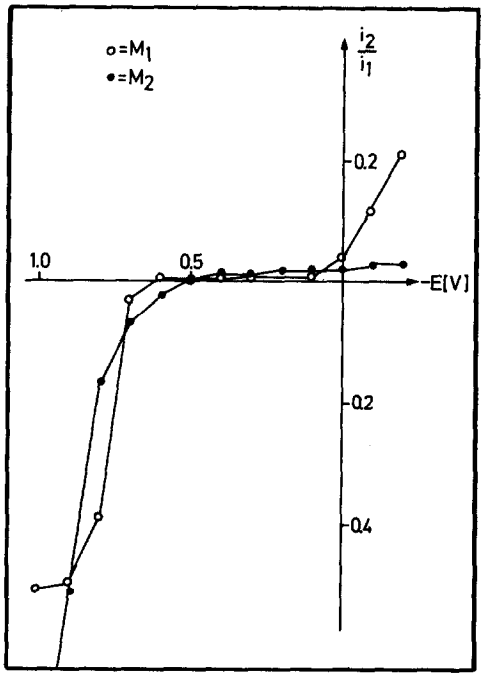


Fig. 5. Hydrodynamic voltammograms at pH 3.0 of the two major metabolites of Phy formed during mouse liver microsomal incubation.

shown that the anticholinesterase activity of Phy is coupled to its urethane group with some indication that an amino group also needs to be present [17]. This means that the observed metabolites, or some of them, could still be biologically active.

CONCLUSIONS

In an LC analysis, two main and six minor metabolites were obtained after mouse liver microsomal incubation of Phy. With on-line electrochemical detection the high chromatographic resolution is maintained. In addition, the electrochemical properties of the metabolites can be studied and compared with those of the known standards Ese and Rub. The results from HDVs and retention times strongly indicate that none of the major metabolites is identical with these two standards. This contradicts the results obtained by other workers, who have used identification by retention time only.

No evidence could be found for conjugation reactions between the metabolites and added glucuronic acid. Neither could any conjugation with glutathione be discovered. Instead glutathione acted as a reducing agent, decreasing the amount of metabolites formed. These results further limit the discussion of the probable nature of the metabolites. Incubations with cytosol did not result in any metabolism, indicating that no other enzyme than those present in the microsomal fraction was responsible for any metabolism of Phy.

The similar oxidation potentials exhibited by Phy and all of the investigated metabolites, which differed from the oxidation potentials of the hydrolysed products, lead us to suggest that the urethane moiety is still attached to the aromatic ring on these structures. If this is correct then these metabolites may also be biologically active. There are several possibilities of N-dealkylation, N-hydroxylation and aromatic hydroxylation that can be attributed to the action of the enzyme believed to be responsible for this metabolism, cytochrome P-450. The positive structural identification of the metabolites remains a significant challenge.

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