*Biochemical Pharmacology*, Vol. 37, No. 20. pp. 3867–3876, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00


# HYDROLYSIS OF $^3$H-BAMBUTEROL, A CARBAMATE PRODRUG OF TERBUTALINE, IN BLOOD FROM HUMANS AND LABORATORY ANIMALS *IN VITRO*

ANDERS TUNEK, EVA LEVIN and LEIF-Å. SVENSSON
Research and Development Department, AB Draco,* P.O. Box 34, S-221 00 Lund, Sweden

(*Received* 17 *November* 1987; *accepted* 28 *April* 1988)

**Abstract**—Tritiated bambuterol, a bis-dimethylcarbamate prodrug of terbutaline, was incubated *in vitro* with blood from both sexes of the following species: man, guinea pig, rat, mouse, dog and rabbit. The rates of hydrolysis of bambuterol to its monocarbamate derivative and further to terbutaline were measured. Large species variations were observed, e.g. blood from two of the human subjects was 15-fold more active than blood from the male rats. The rate of terbutaline formation as a function of initial bambuterol concentration was investigated in human plasma, and was found to describe a bell-shaped curve.

Several pieces of evidence indicated that butyrylcholinesterase (EC 3.1.1.8) is the blood enzyme predominantly responsible for hydrolysis of bambuterol, although minor contributions from other esterases cannot be excluded. An exception may be blood from the rabbit, where the kinetics of the hydrolysis was different than in blood from the other species.

The kinetics of bambuterol hydrolysis is discussed on basis of the established mechanism of carbamate interactions with cholinesterases, and the high affinity of bambuterol for butyrylcholinesterase.

Bambuterol (1-3,5-bis-(*N*,*N*-dimethylcarbamoyloxy)phenyl)-2-*t*-butylaminoethanol hydrochloride) is a carbamate prodrug of the adrenoreceptor agonist terbutaline (Fig. 1) [1]. The dimethylcarbamate group was used to obtain built-in cholinesterase inhibitory properties in the prodrug in order to improve its presystemic hydrolytic stability. Clinical trials have demonstrated prolonged duration of action and reduced side effects, and pharmacokinetic studies have shown lower peak-trough ratios for plasma terbutaline concentrations, after intake of bambuterol as compared to plain terbutaline [2].

The bioconversion of bambuterol to terbutaline involves, in the simplest case, a two-step hydrolysis with the monocarbamate derivative as intermediate (Fig. 1). Since bambuterol is a carbamate derivative, inhibition of the cholinesterase will occur simultaneously with hydrolysis. It is believed that inhibition is caused by rapid carbamylation of a serine residue at the esteratic active site to generate an inactive carbamylated esterase intermediate which is only slowly hydrolyzed back to active esterase, i.e. $k_3$ in equation 1 is rate determining. Thus, the "inhibition" is a result of a slow turnover of the catalytic cycle.

$$E + CM \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - CM \underset{M}{\overset{k_2}{\rightarrow}} EC \overset{k_3}{\rightarrow} E + C \qquad (1)$$

where E = esterase, CM = carbamate substrate, M = phenolic product of hydrolysis, EC = carbamylated esterase and C = carbamic acid derivative.

We recently demonstrated that bambuterol has an extremely high affinity for BuChE† (EC 3.1.1.8) in human blood, reflected by an $IC_{50}$ of 17 nM [3]. The present *in vitro* study was designed to investigate bambuterol as a substrate for hydrolytic enzymes in blood from various species. Knowledge of the type obtained in this study is important for the interpretation and understanding of pharmacological effects of bambuterol obtained *in vivo*. In addition, we have investigated in more detail the kinetics of bambuterol hydrolysis in human plasma, with particular emphasis on the effect of the initial bambuterol concentration on the rate of terbutaline formation.

## MATERIALS AND METHODS

### *Animals*

Sprague–Dawley rats and NMRI mice, weighing around 250 and 25 g, respectively, were obtained from Möllegaard (Copenhagen, Denmark), Dunkin Hartley guinea pigs weighing 400–500 g from Sahlin (Malmö, Sweden), New Zealand White rabbits weighing around 2 kg from S. Hansson (Dörröd, Sweden), and Beagle dogs, 15–16 months old, from (Turbo-hundar, Sweden). The human subjects were healthy volunteers, 27–34 years of age.

### *Blood samples*

Blood was collected into heparinized Vacutainer tubes. The mouse blood was collected from eight animals of each sex, rat and guinea pig blood from two animals of each sex, and the blood from each sex pooled. Of the other species blood from one single individual of each sex was used for each incubation. The incubations and enzyme activity determinations were started at the most 2 hr after

* Subsidiary of AB Astra.

† Abbreviations used: LC, liquid chromatography; BuChE, butyrylcholinesterase (plasma cholinesterase, acylcholine acylhydrolase, EC 3.1.1.8); $IC_{50}$, concentration resulting in 50% inhibition of enzyme activity.

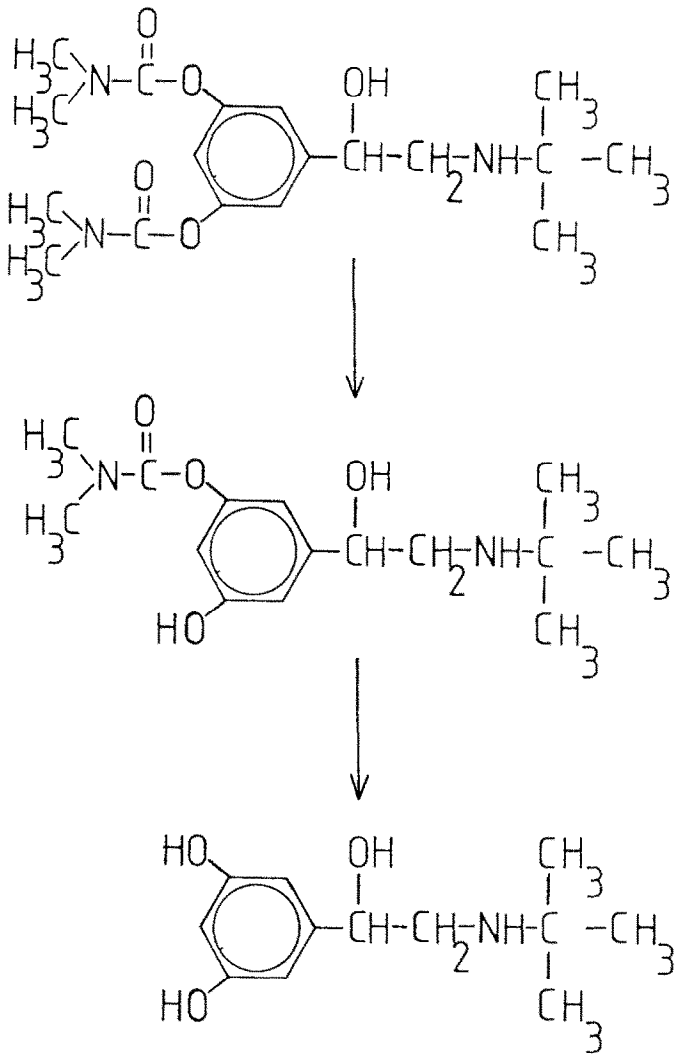

Fig. 1. Two-step hydrolysis of bambuterol to terbutaline.

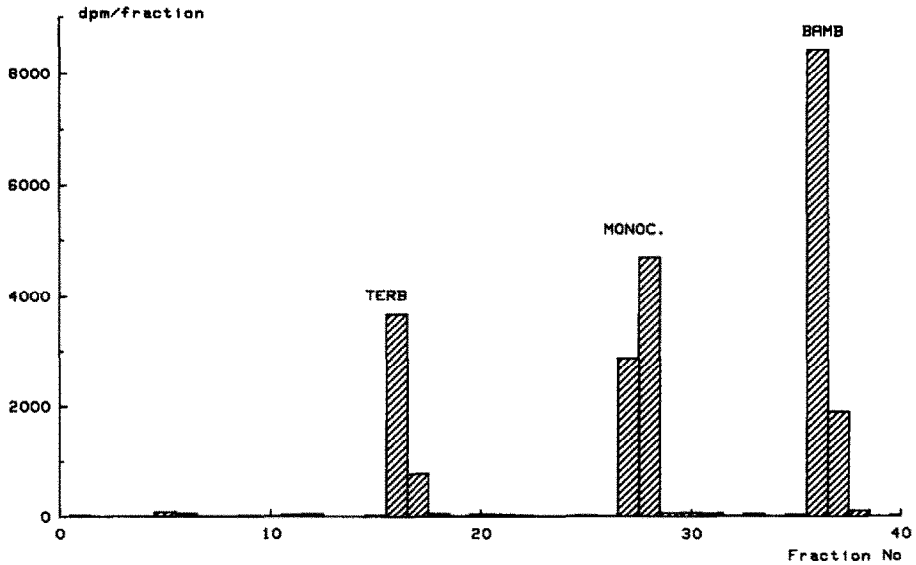


Fig. 2. A typical radiochromatogram. This particular tracing was obtained after incubation with blood from rabbit I for 120 min.

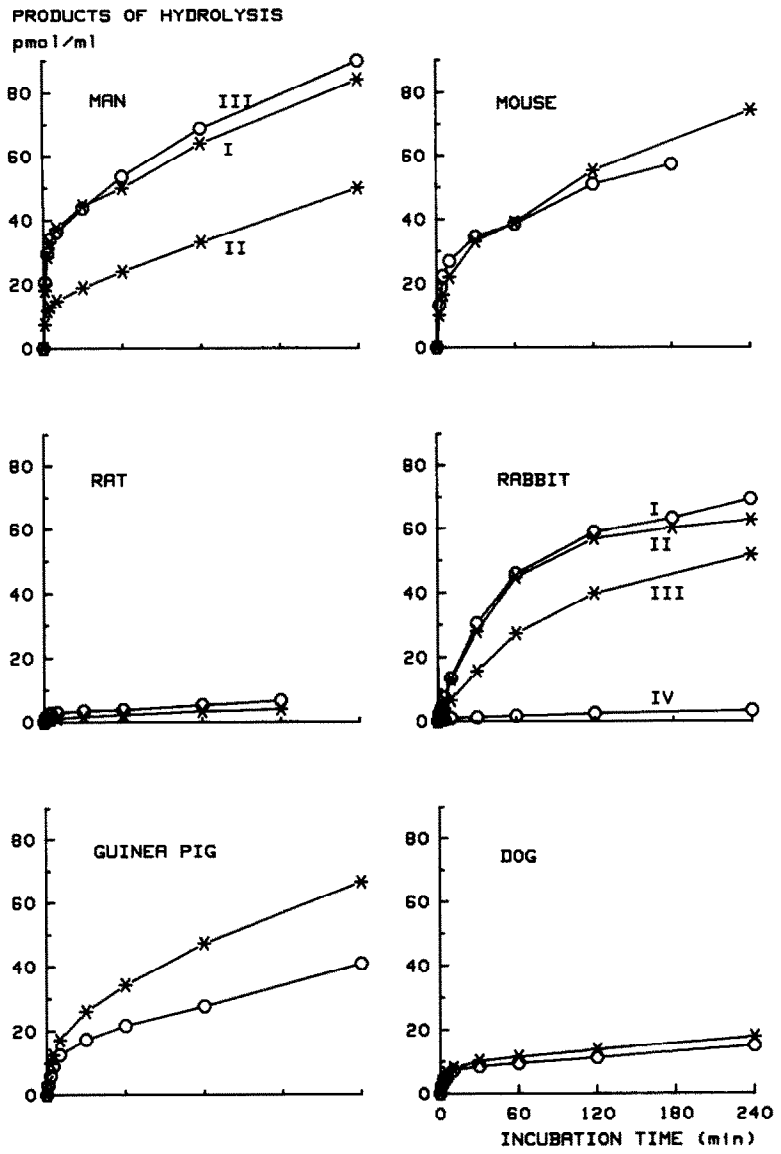


Fig. 3. Hydrolysis of bambuterol in blood from six species. Crosses are males and circles females. Products of hydrolysis are the monocarbamate + terbutaline.

collection of blood. Blood from three humans was tested, and these are referred to as I, II (males) and III (female).

*Human blood plasma*

Blood from one individual (man I) was collected into vacutainer tubes. Plasma was prepared by centrifugation of the blood for 10 min at 3700 rpm.

*Compounds*

Tritiated bambuterol hydrochloride ($^3$H-bambuterol, batch TRQ 2750) with a specific activity of 1.02 Ci/mmol was obtained from the Radiochemical Centre (Amersham, U.K.). The label was at the benzylic carbon in the ethanolamine side chain. The radiochemical purity was found to be 96% when the substance was tested on the LC-gradient system described below. One peak of impurity, containing about 1% of the radioactivity, comigrated with the monocarbamate. The stock solution of $^3$H-bambuterol was 8.4 mM in ethanol. Prior to incubations this stock solution was appropriately diluted with physiological saline. During the incubations performed no tritiated water was detected, thus little or no tritium exchange occurred.

Other chemicals were of analytical grade and purchased from commercial sources.

*Preparation of $^3$H-monocarbamate*

The tritiated monocarbamate was prepared by incubating 1 $\mu$M $^3$H-bambuterol with 10 ml freshly prepared human blood plasma for 7 hr at 37° and then for 13 hr at room temperature. Bambuterol and its metabolites were then extracted on a SepPak $C_{18}$-cartridge (Waters Associates). The metabolite eluate was evaporated to dryness, dissolved in 300 $\mu$l of

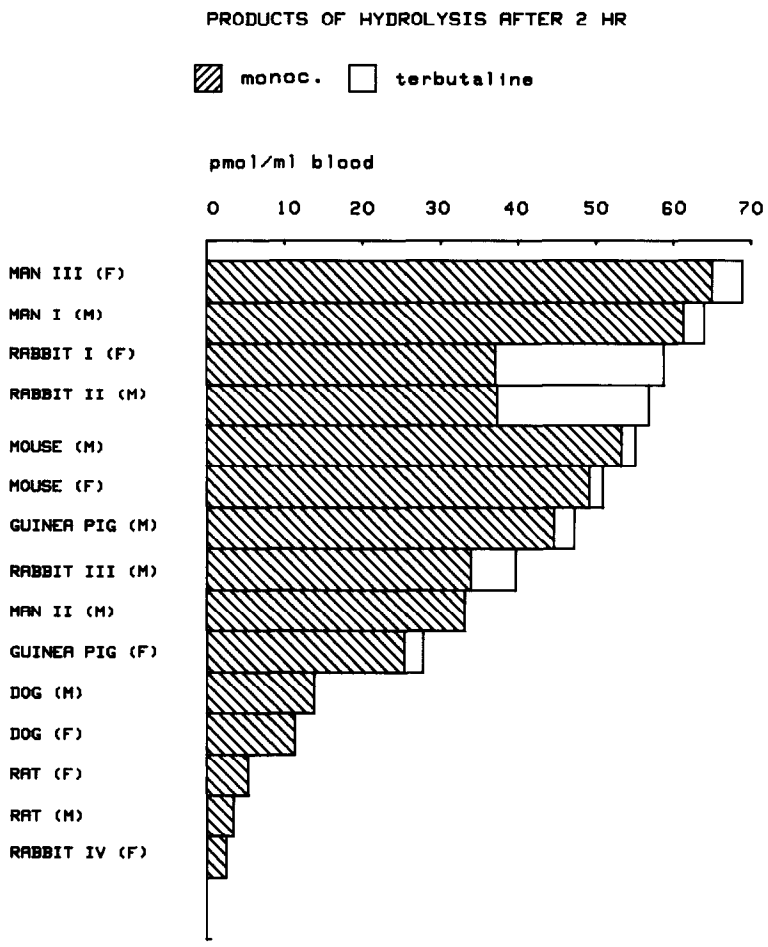


Fig. 4. Products of hydrolysis of bambuterol in blood after 2 hr at 37°. Open parts of the bars are terbutaline and hatched parts of the bars are the monocarbamate.

water and metabolites separated on the LC-system. The fraction with a retention time resembling that of synthetic unlabelled monocarbamate was collected, evaporated to dryness, and dissolved in water.

*Incubations*

*Whole blood.* Six millilitres of the blood was temperated at 37° for about 10 min on a shaking water bath. Then the reaction was initiated by adding 300 μl of a 2 μM $^3$H-bambuterol solution, thus yielding a bambuterol concentration of around 95 nM. After incubation times indicated in the figures aliquots of 500 μl were added to 500 μl 5% perchloric acid. After mixing on a vortex mixer the samples were centrifuged. An aliqout of 200 μl of the supernatant was then injected onto the liquid chromatography system described below.

When the inhibition of bambuterol hydrolysis by physostigmine was tested, the blood from man III was preincubated at 37° with $10^{-6}$ M physostigmine for 15 min before addition of the bambuterol. The physostigmine was added dissolved in 100 μl saline. In a control incubation a blood specimen was preincubated with 100 μl physiological saline.

Except for the mouse, man III and rabbits I and II, where only single incubations were performed, the incubations were run in duplicates. The results given are averages between the duplicate experiments.

*Human plasma.* These incubations were performed with 5 ml of plasma, to which 250 μl of $^3$H-bambuterol or $^3$H-monocarbamate was added. The samples were processed as described above.

*Liquid chromatography (LC) system*

The LC system consisted of two Waters M-45 pumps, a Waters Intelligent Sample Processor 710 B, and a Waters Automated Gradient controller Model 680. The column was Nucleosil $C_{18}$, 5 μm (150 × 4·6 m i.d.). Mobile phase A consisted of 3.86 g of ammonium acetate and 3.5 ml of acetic acid diluted with 1000 ml water. Mobile phase B was 3.86 g of ammonium acetate and 3.5 ml of acetic acid diluted with 1000 ml 90% methanol in water (v/v). The flow rate was 1.0 ml/min. The gradient program was as follows: Initial conditions 80% A/20% B, linear change to 50% A/50% B during the first 10 min; linear change to 10% A/90% B during 10–20 min; linear change to 80% A/20% B during 23–26 min. At least 7 min of equilibration time at initial condition was used between the runs.

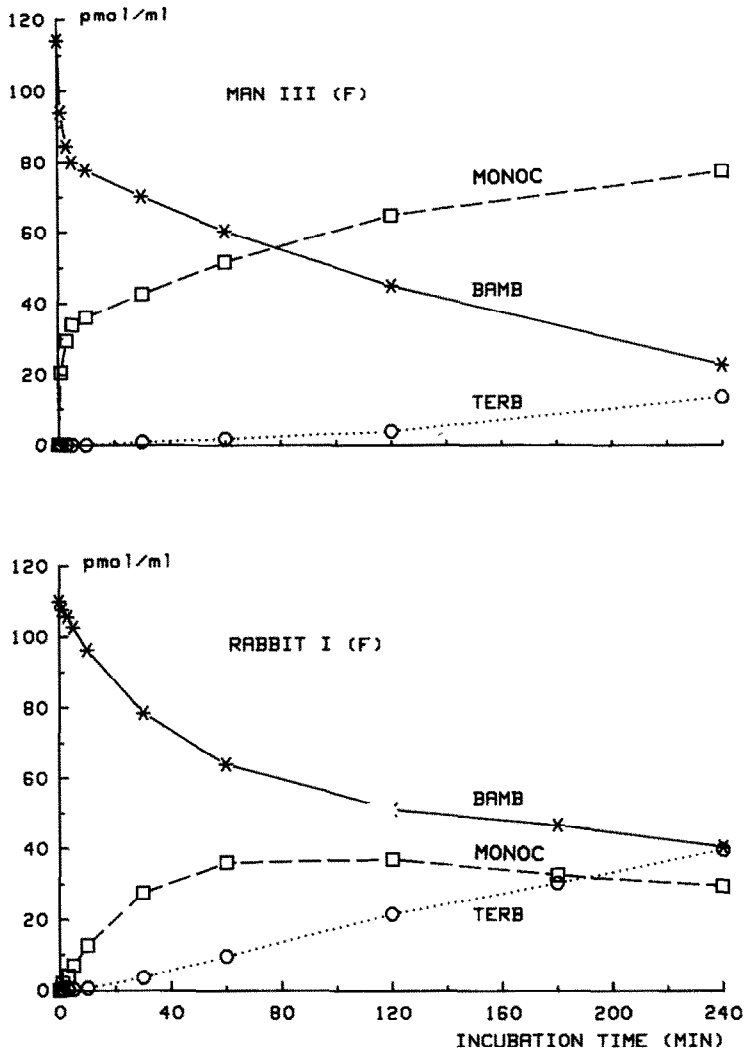


Fig. 5. Formation of the monocarbamate and terbutaline from bambuterol in blood from man III and rabbit I *in vitro*.

*Fractionation and quantitation of bambuterol and i. metabolites*

Bambuterol and its metabolites were quantitated by collecting 0.5-min fraction of the LC-eluate with a LKB Redirac 2112 fraction collector. To each fraction 10 ml Optifluor (Packard) was added, and the radioactivity of the samples counted in a Packard Tri Carb Scintillation Spectrometer. Radiochromatograms such as the one shown in Fig. 2 were obtained. The amount of each compound was then estimated by the sum of the radioactivity of three fractions.

*Recovery study*

$^3$H-bambuterol (1 $\mu$M) was incubated with human whole blood (1 ml) for 6 hr at 37°. The incubations (N = 3) were terminated as described above, and the total amount of radioactivity, and the metabolite composition, of the supernatant was determined. The pellets were then suspended in 2.5% perchloric acid by shaking and sonication. After centrifugation the supernatant was analysed as described above. This procedure was repeated three times.

*Determination of BuChE activity*

BuChE activity was determined as described in detail elsewhere [3]. The substrate used was butyrylthiocholine. The optimal substrate concentration was found to be 1 mM except for the rat where it was 4 mM. The blood specimens were hemolyzed by 4–20-fold dilution with deionized water, and 100 $\mu$l of the hemolyzed blood added to the cuvettes (total volume 3.0 ml).

## RESULTS

*Recovery studies*

The recovery of radioactivity as $^3$H-bambuterol + $^3$H-monocarbamate + $^3$H-terbutaline in the supernatant after precipitation with perchloric acid was in blood 83.6 ± 4·2% (N = 222) and in plasma 93.5 ± 4·4% (N = 34). The recovery study

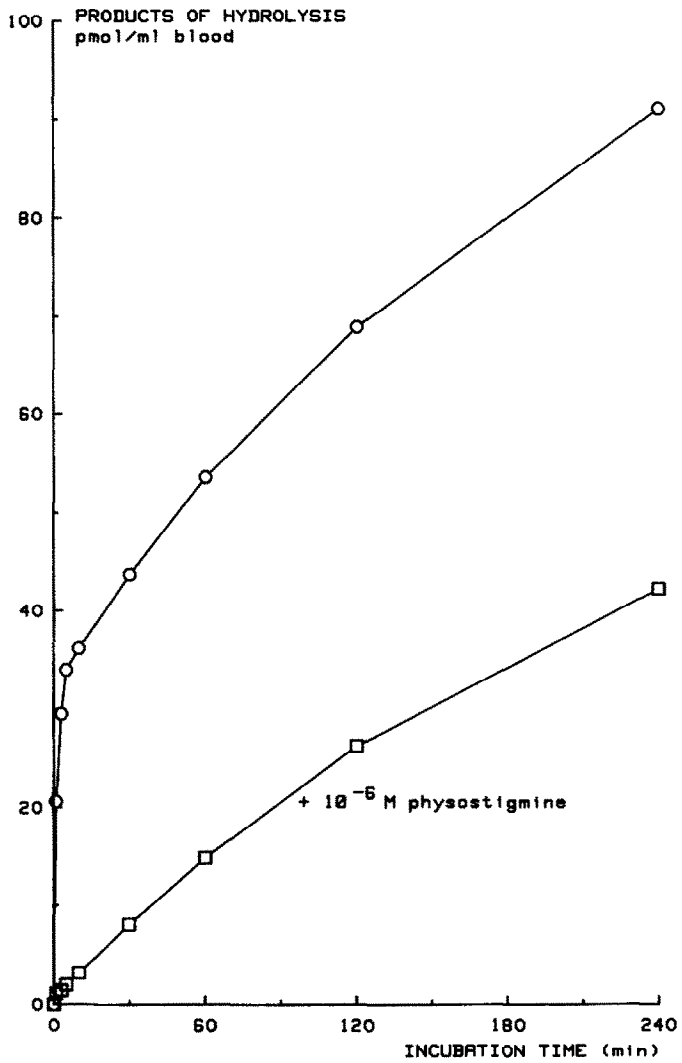


Fig. 6. Inhibition by physostigmine of bambuterol hydrolysis in human blood (man III).

demonstrated that no particular metabolite accumulated in the pellet. Thus, the overall recovery in each LC-run was used for each of these compounds, and all values given have been compensated accordingly.

*Hydrolysis of bambuterol in blood from various species*

The hydrolysis of approximately 95 nM $^{3}$H-bambuterol in blood from males and females of six species is shown in Figs 3 and 4. The blood from the various species had dramatically different capabilities to hydrolyze bambuterol, e.g. two of the human blood specimens were around 30-fold more active in this respect than was blood from one of the female rabbits and 15-fold more active than blood from the male rat.

The hydrolysis of bambuterol was non-linear with time as is seen in Fig. 3. The probable biochemical mechanisms are discussed below.

The first product of hydrolysis of bambuterol is the monocarbamate derivative (cf. Fig. 1) and, in five of the species, under the conditions chosen, only a small fraction of this compound was further transformed to terbutaline as illustrated in Fig. 4. The exception was blood from the rabbit. The rates of hydrolysis of bambuterol and the fate of the monocarbamate and terbutaline in blood from man III and rabbit I are compared in Fig. 5. The initial rate of bambuterol hydrolysis and monocarbamate formation was much faster in human than in rabbit blood. However, as is also seen in Fig. 5, the hydrolysis of the monocarbamate to yield terbutaline took place to a much larger extent in rabbit blood.

*Correlation between BuChE activity and bambuterol hydrolysis*

BuChE activity, with butyrylthiocholine as substrate, was measured in aliquots of the blood specimens from all species used in the bambuterol hydrolysis experiments. There was a good correlation ($e = 0.82$) between the rapid initial phase (0–5 min) of bambuterol hydrolysis and BuChE activity. BuChE activities in blood from man I, II and III

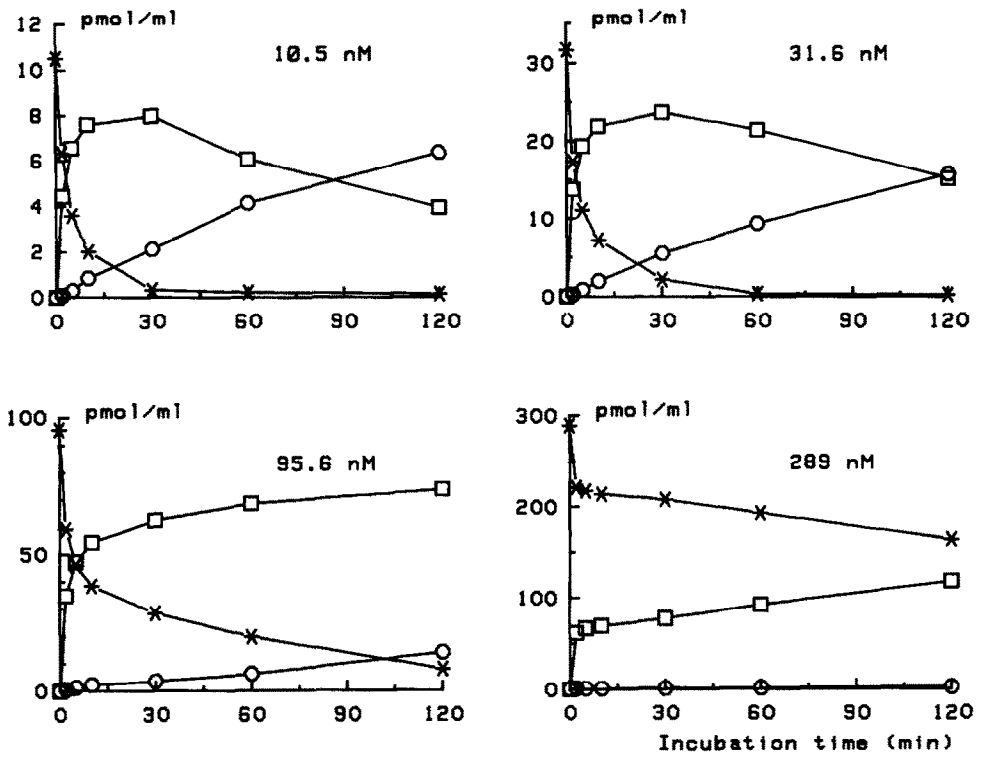


Fig. 7. Hydrolysis of $^3$H-bambuterol in human plasma at four different bambuterol concentrations. *, bambuterol; □, the monocarbamate, ○, terbutaline.

(Figs 3 and 4) were 14.3, 6.8 and 14.0 nmol thiocholine/min/5 μl blood, respectively.

*The effect of physostigmine on the hydrolysis of bambuterol in human blood*

The effect of preincubation of blood from man III with $10^{-6}$ M of the potent cholinesterase inhibitor physostigmine is shown in Fig. 6. The rapid initial phase of bambuterol hydrolysis was completely abolished by physostigmine, while the slow phase was only mildly affected.

*The effect of bambuterol concentration on monocarbamate and terbutaline formation in human plasma*

The hydrolysis of $^3$H-bambuterol in human plasma, at four different bambuterol concentrations, is illustrated in Fig. 7. It is very clear, particularly at higher concentrations, that the hydrolysis of bambuterol was a non-linear reaction. The possible biochemical mechanisms underlying this behaviour are discussed below. The capacity of the rapid initial phase was around 60 pmol/ml plasma, a value which is in good agreement with reported concentrations of BuChE in human plasma [4]. At the highest bambuterol concentration this phase was completed within 2 min. The slow phase of hydrolysis proceeded at a rate of around 25 pmol/ml plasma/hr.

The rate of terbutaline formation at the four bambuterol concentrations are compared in Fig. 8. Terbutaline was produced at the highest rate from 31.6 nM bambuterol (9 pmoles terbutaline/ml plasma/hr), while formation was very slow at the highest bambuterol concentration (289 nM).

The rates of hydrolysis of $^3$H-bambuterol and $^3$H-monocarbamate were compared as illustrated in Fig. 9. The initial burst so typical for bambuterol hydrolysis was much less pronounced for monocarbamate hydrolysis.

## DISCUSSION

Esterases are a heterogeneous group of enzymes, known to differ greatly qualitatively and quantitatively between species [5, 6]. This is true also for BuChE [7]. In humans several genotypes of the enzyme exist, and rare but healthy individuals apparently completely lack this enzyme activity [8, 9]. As indicated in this study, BuChE is probably the enzyme in blood responsible for hydrolysis of bambuterol. Therefore, it was not too surprising to observe the large difference in the rate of bambuterol hydrolysis between blood from the various species (cf. Figs 3 and 4).

The product of hydrolysis of bambuterol is the monocarbamate derivative which may be further hydrolyzed to terbutaline (cf. Fig. 1). It was recently shown that bambuterol has a higher affinity for human BuChE than the monocarbamate [3], since the monocarbamate was found to be a 10-fold less efficient inhibitor of this enzyme. This difference in affinity to BuChE is also reflected by the results presented in Fig. 9. The hydrolysis of the monocarbamate almost completely lacked the initial burst

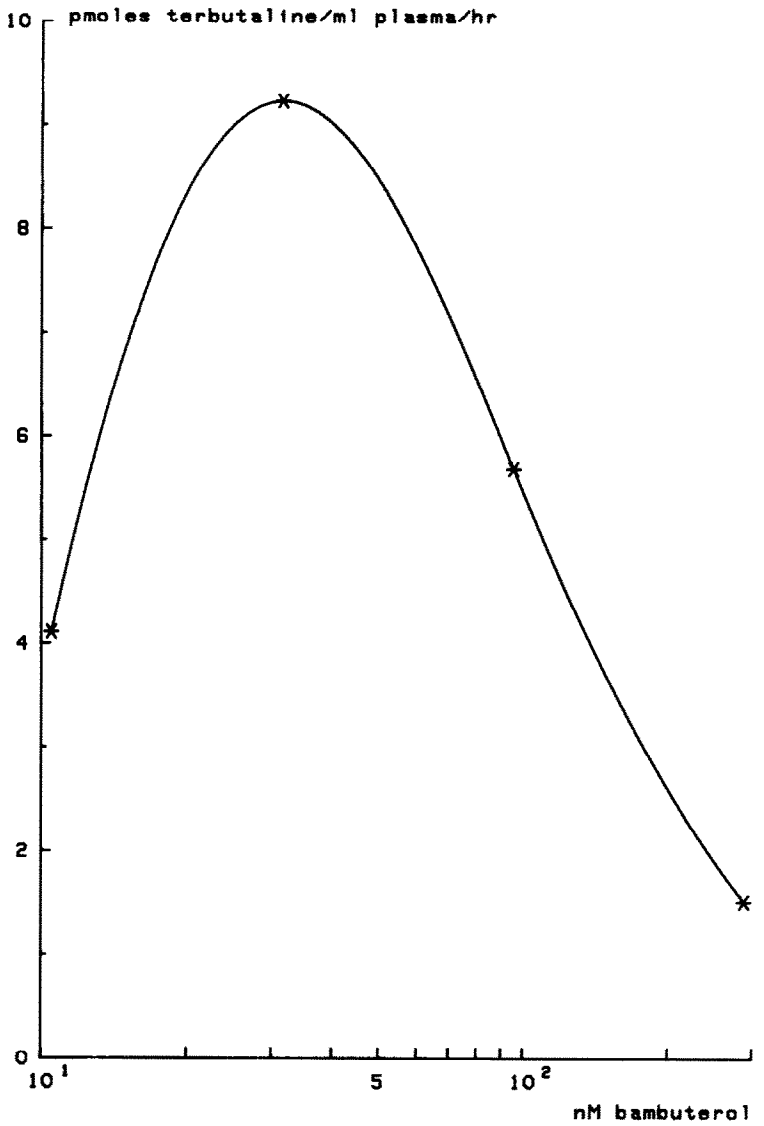


Fig. 8. The formation in human plasma of terbutaline from $^3$H-bambuterol. These results are calculated from Fig. 7.

so typical for hydrolysis of bambuterol. This should further imply that as long as bambuterol is present in appreciable amounts, the first step of hydrolysis will dominate, and little terbutaline will be formed. The results presented in this study are in accordance with these expectations as illustrated in Figs 5 and 7. In blood from rabbit the situation was different as seen in Fig. 5. Here, the monocarbamate derivative appeared to be a better substrate than bambuterol for the esterases.

In this and a recent report [3] several pieces of evidence (listed below) strongly indicate that BuChE is the most important blood enzyme involved in the hydrolysis of bambuterol, the possible exception is the rabbit. The evidence for a crucial role of BuChE in the hydrolysis of bambuterol in human blood (and plasma) is numerous. Firstly, the capacity of the inital burst of hydrolysis was in the order of 60 pmol/ml (Fig. 7), which corresponds very well with reported values for the concentration of BuChE active sites in human blood plasma [4]. The rate of hydrolysis in the slow phase (Fig. 7) also allows us to calculate the half-time for regeneration of the BuChE activity at hydrolysis of bambuterol. Thus, if the total concentration of enzyme is assumed to be 60 pmol/ml and the rate of hydrolysis in the slow phase was 25 pmol/ml plasma/hr, $t_{1/2}$ will be 72 min for the reactivation of BuChE. This is in excellent agreement with results calculated from inhibition kinetics [3]. This kinetic behaviour of hydrolysis and inhibition is in accordance with the extensively investigated interaction between cholinesterases and carbamates [10, 11]. Thus, $k_1$ and $k_2$ in equation 1 control the rapid binding and hydrolysis steps, and $k_3$ the slow regeneration of the active site. Secondly, there was a good correlation ($r = 0{\cdot}82$) between the rate of bambuterol hydrolysis during the initial phase and the rate of hydrolysis of the BuChE substrate

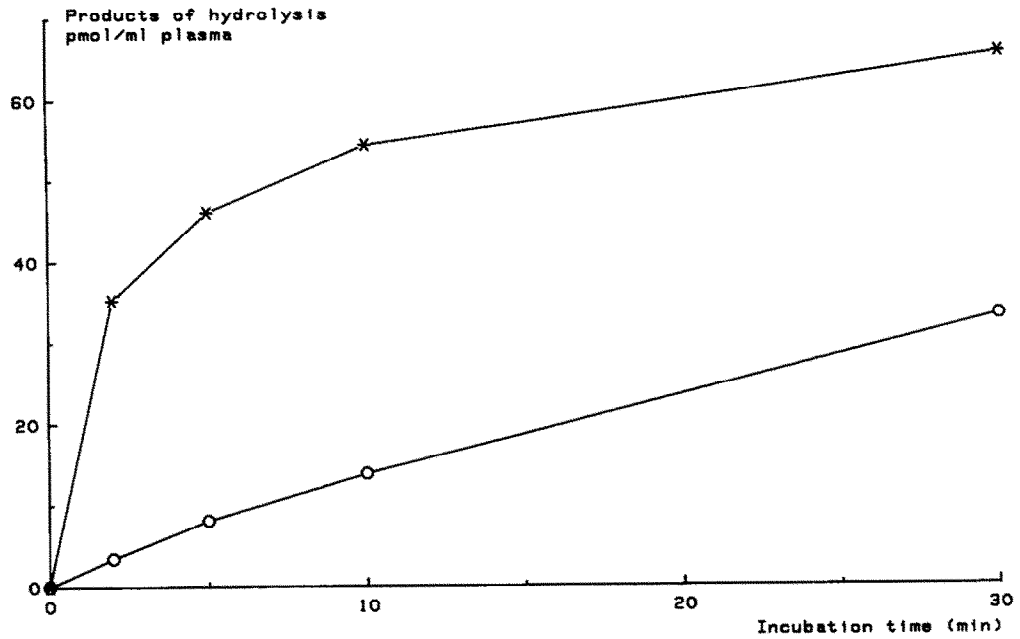


Fig. 9. Hydrolysis of bambuterol and of the monocarbamate in human blood plasma. The substrate concentrations were 100 nM. Results shown represent monocarbamate formed from bambuterol (*), and terbutaline formed from the monocarbamate (O).

butyrylthiocholine. Thirdly, carbamylation of the esterase by preincubation of human blood with physostigmine, a well known reversible cholinesterase inhibitor [10, 11], completely blocked the rapid initial phase of bambuterol hydrolysis (Fig. 6). Bambuterol, however, is a better inhibitor than physostigmine of BuChE and thus probably has a higher affinity to the enzyme's active site [3], so once the *N*-methylcarbamate residue from physostigmine has left the enzyme, bambuterol successfully competed for binding to the active site of the regenerated esterase. Consequently, the slow phase of bambuterol hydrolysis was only mildly affected by physostigmine. The crucial role of BuChE in both the rapid and the slow phase of bambuterol hydrolysis is further supported by experiments in progress in our laboratory using plasma from humans homozygous for the atypical form of BuChE ($E_aE_a$, kindly provided by Dr J Viby Mogensen, Copenhagen). Both phases of bambuterol hydrolysis are much slower in such atypical plasma.

The kinetics of terbutaline formation from bambuterol has the implication that the amount of terbutaline formed in plasma does not increase with the concentration of bambuterol (Fig. 8). Instead, a level must exist where the rates of inhibition and regeneration of the esterase balance each other to give an optimal rate of terbutaline formation. As can be seen from Fig. 8, of the four bambuterol concentrations used in this study, 31.6 mM resulted in the highest rate of terbutaline formation, while lower and higher concentrations resulted in slower terbutaline formation.

The present study gives insight into the kinetics of hydrolysis in blood and plasma of bambuterol to the monocarbamate derivative and further to the pharmacologically active terbutaline. The blood is, however, only one of many tissues active in biotransformation of bambuterol as will be demonstrated in future reports. It must therefore be remembered, that the dramatic differences in the rate of bambuterol hydrolysis in blood observed in this study will not reflect differences between various animals or individuals in their total capacity of forming terbutaline from bambuterol. Nevertheless, the features of the interaction between bambuterol and BuChE probably positively influence the biological stability of this prodrug. These factors most likely contribute to the sustained generation of the active bronchodilator, terbutaline, resulting in a long duration of action allowing bambuterol to be dosed only once daily in man [12].

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