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THE EFFECT OF BILE SALTS ON THE ESTERASE ACTIVITIES OF PLASMIN AND SOME OTHER ENZYMES

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SUMMARY

Various bile salts were tested for their effects on the plasmin-catalyzed hydrolysis of tosyl-L-lysine methyl ester. Only 3,7,12-trihydroxycholane-24-oate(cholate), its amino acid conjugates (glycocholate and taurocholate), and 3,12-dihydroxycholane-24-oate (deoxycholate) produced significant rate enhancement, although the latter became an inhibitor at higher concentrations. Of the others, 3-hydroxycholane-24-oate (lithocholate) inhibited plasmin much more strongly than 3,7-dihydroxycholane-24-oate (chenodeoxycholate). Lineweaver–Burk plots showed that a K_m(app) decrease alone was responsible for the observed rate enhancement by glycocholate. With a series of different substrates, enhancement by glycocholate was proportional to K_m values reported in the literature. Conductivity measurements showed no significant interaction between the substrate itself and cholate. The effects of temperature, ionic strength and pH indicated the suggested interaction of glycocholate with plasmin to be hydrophobic rather than ionic in nature. Plasmin samples obtained by different procedures, and plasminogen preparations at various stages of streptokinase activation, were all equally accelerated by glycocholate in their hydrolysis of tosyl-L-lysine methyl ester. By comparison, thrombin-catalysed hydrolysis was enhanced more markedly, and that of trypsin to a lesser extent. Deoxycholate had an inhibitory effect on plasmin's tosyl-L-arginine methyl esterase activity, but still strongly accelerated that of thrombin.

INTRODUCTION

The role of bile salts as emulsifiers and promoters of lipolytic action in the intestinal absorption of lipids is well known[1]. Their protective effects on various lipolytic enzymes have also been investigated[2–4]. However, there have been widely

Abbreviations: ALMe, $^{\alpha}N$-acetyl-L-lysine methyl ester; Cbz-LMe, $^{\alpha}N$-Cb_2-L-lysine methyl ester; TAMe, $^{\alpha}N$-tosyl-L-arginine methyl ester; TLMe, $^{\alpha}N$-tosyl-L-lysine methyl ester; BAMe, $^{\alpha}N$-benzoyl-L-arginine methyl ester.

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differing results reported concerning the effects of bile salts on the activities of non-lipolytic enzymes. Thus, in one study, chymotrypsinogen was activated by trypsin more rapidly in the presence of bile salts than in their absence[5] while in another, trypsin was found to digest hemoglobin (but not casein or fibrinogen) more rapidly with bile salts present[6]. There have also been reports of tryptic proteolysis being inhibited by bile salts. Some attempt has been made to reconcile the various observations through a complex pH dependence of cholate micellar charge and its supposed interaction with substrate[5].

On the other hand, the esterase activities of trypsin and chymotrypsin towards specific amino acid ester substrates were found to be unaffected by taurocholate or glycocholate[7]. Although an enhancement of esterase activity by conjugated bile salts has been reported for rat pancreatic juice and serum acting on *p*-nitrophenyl and *β*-naphthyl acetates[8,9], the only kinetic analysis of enhancement by cholate seems to be one involving thrombin esterase activity[10]. A balance between the activities of the fibrin-forming enzyme, thrombin, and the fibrinolytic enzyme, plasmin, appears to be important for maintaining normal hemostasis. In view of the presence of elevated blood cholates levels in disorders such as obstructive jaundice, and because of the apparently significant effect of bile salts on thrombin, the effects of a variety of these compounds on plasmin were investigated.

EXPERIMENTAL PROCEDURE

Enzymes

Plasminogen was obtained from human Cohn Fraction III (gift of Dr A. R. Pappenhagen, Cutter Laboratories, Berkeley, Calif., U.S.A.), by the acid-extraction procedure of Robbins and Summaria[11]. Activation of the proenzyme to plasmin was usually carried out by incubation for 30 min at 37 °C with streptokinase (250 units/ml, Lederle Laboratories, Pearl River N.J. U.S.A.) in 50% glycerol (pH 7.5). Using the method of Johnson *et al.*[12] the fully activated enzyme was found to have an activity of 3.0 CTA units per optical absorbance unit.

Native plasminogen was prepared from Cohn Fraction III by using affinity chromatography according to Liu and Mertz[13]. This product had an activity of 4.0 CTA units per optical absorbance unit after complete activation by streptokinase. Some of this material was allowed to activate spontaneously by incubation for 10 days in 50% glycerol at 30 °C (ref. 14). However, it only developed an activity of 1.5 CTA units per absorbance unit in this time.

Thrombin was purified by the method of Lundblad[15] from "Topical Thrombin" of bovine origin (Parke-Davis and Co. Detroit Mich. U.S.A.) Urokinase was obtained from Calbiochem. (La Jolla Calif. U.S.A.). Trypsin was a 2 times crystallized product from Worthington Biochemical Corp. (Freehold N.J. U.S.A.).

Substrates

Substituted L-lysine and L-arginine ester substrates used throughout were obtained from Cyclo Chemical Corp. (Los Angeles Calif. U.S.A.).

Bile salts

All bile salts used were commercial products obtained, in some cases, from more than one source. Their purity, as determined by thin-layer chromatography

and certified by the suppliers, ranged from a minimum of 94% to "highest purity" (approx. 99%). Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.) supplied sodium cholate (94%) and sodium taurocholate (containing small amounts of glycocholate). Mann Research Labs (New York, N.Y., U.S.A.) supplied sodium cholate (M.A. grade, highest purity), sodium deoxycholate (M.A. grade, highest purity), sodium glycocholate (95%) and sodium taurocholate (95%). Sigma Chemical Co. (St. Louis, Mo., U.S.A.) supplied lithocholic (95%) and chenodeoxycholic (95%) acids. 1% (w/v) stock solutions of the latter were neutralized by NaOH prior to use.

Measurement of esterase activity

The activity of the enzyme was assayed by titrimetic procedure[10]. The titration equipment consisted of a Model M26 pH meter, a Model 11 titrator and a Model SBR-2 titrigraph recorder (Radiometer, Copenhagen, Denmark). Experiments were usually performed at pH 7.5, 37 °C, in the presence of 0.15 M NaCl and in a total volume of 1.0 ml.

In a typical experiment, the enzyme to be studied was preincubated with the desired bile salt for 1 min and the titration initiated by the addition of substrate. The rate of addition of 0.01 M NaOH, required to maintain pH constant, was recorded. Initial rates were obtained from the initial slopes recorded during the first minute of the reaction. Observations made with glycocholate only, showed little difference in enzyme activity rates with changes in the order of addition of reagents and, subsequently, enzyme was usually added last, to pre-incubates of substrates and bile salts.

Conductivity measurements

Conductivity experiments were carried out using a portable conductivity bridge, (Model PM-70-CB, Barnstead Still and Sterilizer Co., Boston, Mass., U.S.A.) fitted with a 1 cm conductance cell (Model 3403, Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.).

RESULTS

Activation of plasminogen

Incubation of acid-extracted plasminogen with a relatively low streptokinase concentration (50 units/ml) permitted the measurement of esterase activity developing during the process of activation. At specific intervals during activation, 0.05-ml aliquots of the activation mixture were removed and tested for esterase activity in the absence and presence of glycocholate. Following determination of the initial rate of hydrolysis (v_1) of 10^{-3} M tosyl-L-lysine methyl ester (TLMe) in the titrimetric assay system, glycocholate was added to a final concentration of 10^{-2} M. The rate observed under these conditions was also recorded (v_2), and the enhancement in the rate due to glycocholate (v_2/v_1) was calculated. Values v_1 and v_2/v_1 were plotted against time as shown in Fig. 1.

The enhancement of plasmin activity due to glycocholate was observed at all stages of the formation of the enzyme from its precursor, and the ratio v_2/v_1 seems almost constant (2.8–3.0) throughout the progress of activation. A slight diminution in the maximum attainable enhancement of activity was noted when the activation

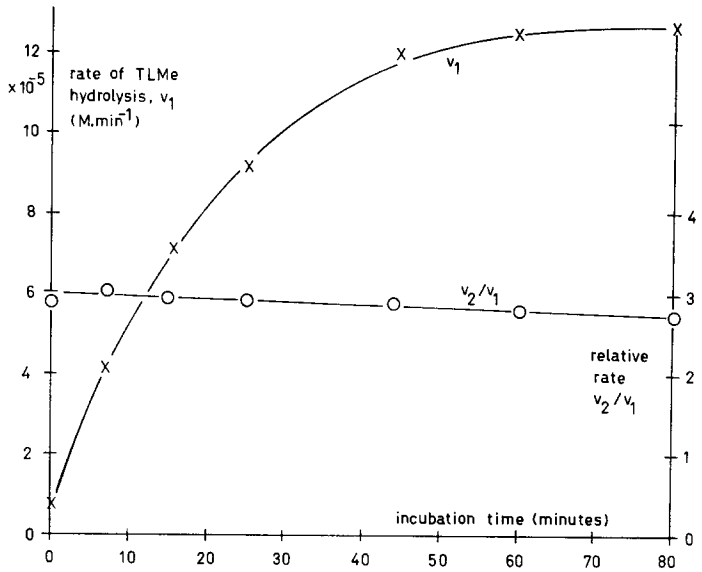

Fig. 1. Incubation time of plasminogen with 50 units/ml of streptokinase plotted against: activity on 10^{-3} M TLMe (rate of hydrolysis, v_1) (×—×); and relative rate of hydrolysis (v_2/v_1) after further addition of 10^{-2} M sodium glycocholate to each assay mixture (○—○).

of plasminogen was prolonged over 90 min. However, it is apparent that any variation in the degree of plasminogen activation should not cause any significant error in experiments concerned with the glycocholate enhancement of plasmin's esterase activity.

Effects of various bile salts on plasmin's TLMe esterase activity

Similar experiments on the enhancement of plasmin's TLMe esterase activity were carried out at various concentrations of the conjugated bile salts taurocholate and glycocholate, as well as sodium cholate itself and the related bile salts, deoxycholate, chenodeoxycholate and lithocholate. Rates of TLMe (10^{-3} M) hydrolysis by plasmin, relative to those obtained in the absence of bile salts (v_2/v_1), were plotted against the concentrations of these agents as shown in Figs 2 and 3.

It is apparent that only sodium cholate itself, and the conjugated bile salts, taurocholate and glycocholate, enhanced the plasmin-catalysed hydrolysis of TLMe. Deoxycholate at low concentrations gave a significant rate enhancement, but at higher levels proved to be a potent inhibitor.

Effects of variations in substrate structure and concentration on plasmin's esterase activity in the presence of various cholates

The rate of TLMe hydrolysis by plasmin was measured at various substrate concentrations ranging from $0.4 \cdot 10^{-3}$ to $10 \cdot 10^{-3}$ M, with and without sodium glycocholate present. The measurements with glycocholate were made using three different concentrations, *viz.* $4 \cdot 10^{-3}$, $10 \cdot 10^{-3}$ and $20 \cdot 10^{-3}$ M of this bile salt. The effects of $4 \cdot 10^{-3}$ M sodium cholate, deoxycholate and glycocholate on the rate of plasmin-

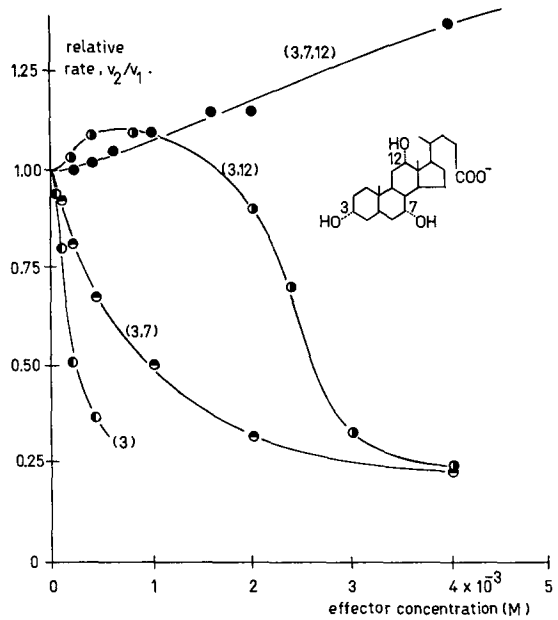

Fig. 2. Rates of hydrolysis (v_2), relative to those in the absence of bile salts (v_1), of 10^{-3} M TLMe by plasmin, plotted against the molar concentration of the effectors: sodium cholate (3,7,12-trihydroxy-cholan-24-oate) (●); deoxycholate (3,12-dihydroxy-cholan-24-oate) (◑); chenodeoxycholate (3,7-dihydroxy-cholan-24-oate) (◓); and lithocholate (3-hydroxy-cholan-24-oate) (◐).

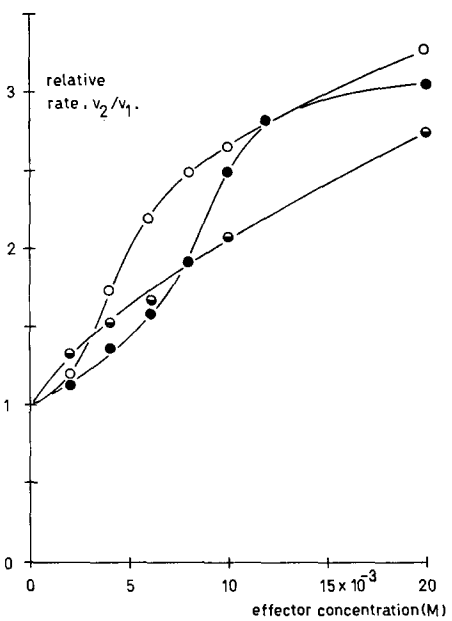

Fig. 3. Rates of hydrolysis (v_2), relative to those in the absence of bile salts (v_1), of 10^{-3} M TLMe by plasmin, plotted against the molar concentration of the effectors; sodium cholate (●); sodium taurocholate (◒); and sodium glycocholate (○).

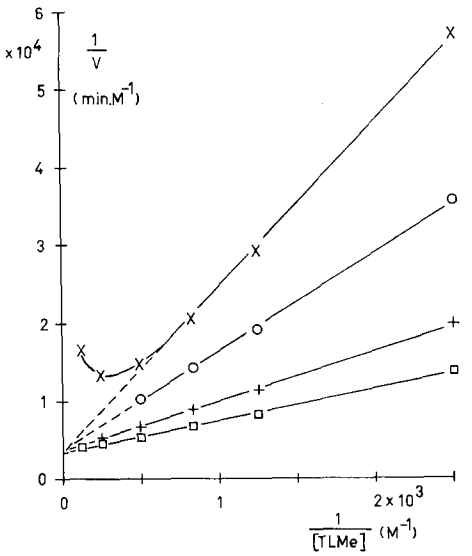

Fig. 4. Lineweaver–Burk plots ($1/v$ plotted against $1/[S]$) for plasmin-catalysed TLMe hydrolysis in systems containing the following concentrations of sodium glycocholate: 0 M (×): $4 \cdot 10^{-3}$ M (○); 10^{-4} M (+); and $2 \cdot 10^{-4}$ M (□).

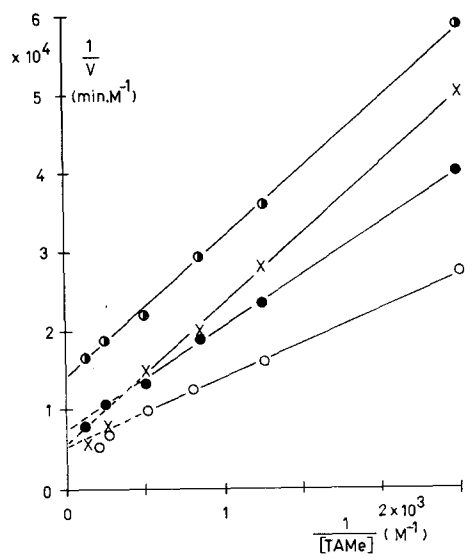

Fig. 5. Lineweaver–Burk plots for plasmin-catalysed hydrolysis of TAMe in systems containing $4 \cdot 10^{-3}$ M of each following cholate: no cholate (×); sodium cholate (●); sodium deoxycholate (◑); and sodium glycocholate (○).

catalyzed tosyl-L-arginine methyl ester (TAMe) hydrolysis were similarly determined at several substrate concentrations. Results were expressed as reciprocal plots ($1/v$ *vs* $1/S$) according to Lineweaver and Burk[16] as shown in Figs 4 and 5.

It is apparent from these data that only glycocholate decreased K_m(app)

without decreasing V. Sodium cholate decreased V and inhibited the rate of TAMe hydrolysis at high substrate concentrations, but had the opposite effect at TAMe concentrations below $2.5 \cdot 10^{-3}$ M. Sodium deoxycholate inhibited proportionally more at higher substrate concentrations, apparently in the manner of an uncompetitive inhibitor, acting only on the enzyme–substrate complex[17]. However, its effect is probably more complicated, as this type of plot may also indicate a mixture of non-competitive inhibition combined with a decrease in K_m(app).

The observation of plasmin esterase inhibition by TLMe conflicts with the recent findings by Weinstein and Doolittle[19] of substrate activation. Perhaps the difference in results is due to the use of plasmin derived from acid-extracted plasminogen in this study, while their plasmin was obtained from native precursor. The substrate activation due to TAMe is in agreement with previously reported results[19] and has also been reported for trypsin[20] and thrombin[10].

At this stage, the possibility of some unique property of the $^{\alpha}N$-tosyl substituent causing enzymatic enhancement, as suggested for product and substrate activation of trypsin by Kallen-Trummer *et al.*[21], led to a study of other ester substrates of plasmin. Several $^{\alpha}N$-substituted L-lysine and L-arginine methyl esters, all at a concentration of 10^{-3} M, were hydrolysed by plasmin in the presence of varying concentrations of sodium glycocholate. Rates of hydrolysis relative to those obtained in the absence of glycocholate (v_2/v_1) were plotted against glycocholate concentration as shown in Fig. 6.

It is apparent that the plasmin-catalysed hydrolyses of substrates other than TAMe and TLMe were also enhanced by glycholate, though to a lesser degree.

Some of the above data were rearranged into the form previously used by

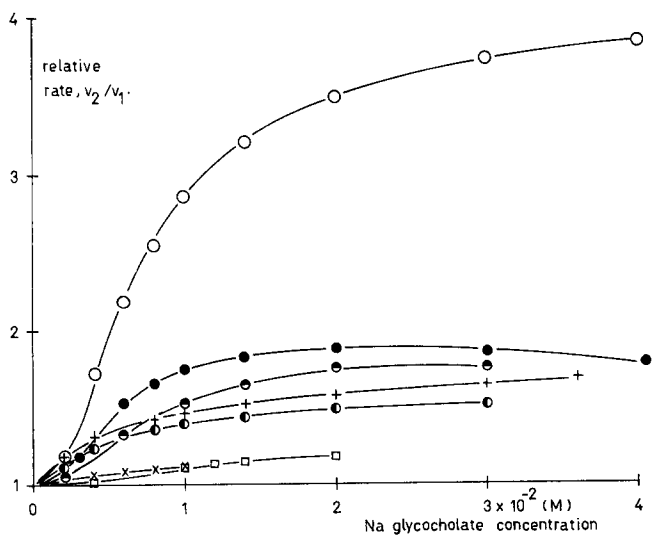

Fig. 6. Rates of hydrolysis relative to those in the absence of glycocholate (v_2/v_1) plotted against glycocholate concentration for various substrates at 10^{-3} M hydrolysed by plasmin. ○, TLMe; ●, TAMe; ◓, ALMe; +, acetyl-L-arginine methyl ester; ◐, Cbz-LMe; ×, Cbz-L-arginine methyl ester; □, BAMe.

Inagami and Murachi for studying the enhancement of trypsin esterase activity by amines[22]. Thus,

$$\frac{v_2}{v_1} = -K_a\left(\frac{v_2}{v_1} - 1\right)/[A] + v_{lim}/v_1,$$

where $[A]$ is the effector (in this case, glycocholate) concentration, K_a is the dissociation constant of the enzyme–effector or the enzyme–substrate complex–effector complex, and v_{lim}/v_1 is the extrapolated relative rate at infinite effector (glycocholate) concentration. The results obtained in this study are shown in Fig. 7. Points obtained at equal glycocholate concentrations could be conveniently joined by straight lines passing through the origin, in a manner similar to the modification suggested by Coleman[23] for Eadie plots.

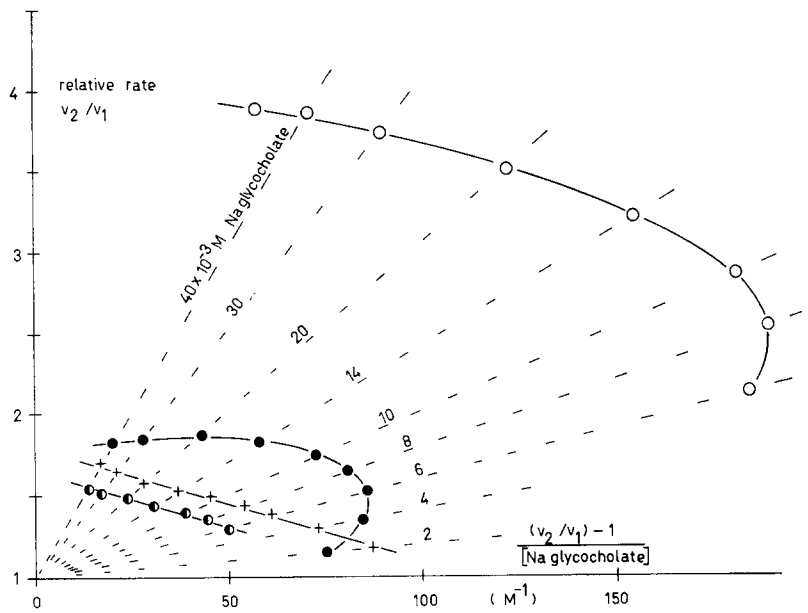

Fig. 7. Relative rates of hydrolysis (v_2/v_1) plotted against $[(v_2/v_1)-1]$/[sodium glycocholate] for several substrates hydrolysed by plasmin at varying sodium glycocholate concentration. ○, TLMe; ● TAMe; +, acetyl-L-arginine methyl ester; ◐, Cbz-LMe.

Only two substrates gave a linear relationship, *viz.* acetyl-L-lysine methyl ester (ALMe) and its Cbz-substituted analogue (Cbz-LMe). The plots were parallel and evaluation of their slope yielded a value for K_a of $7.2 \cdot 10^{-3}$ M. However, the significance of this value is uncertain since there is some doubt whether the Inagami–Murachi model is valid when K_m(app) is not constant (Brubacher, L. J., unpublished results). The non-linear relationships observed for most substrates appeared to correspond to the sigmoid portions of the plots shown in Fig. 6.

Investigation of cholate–substrate interaction

One convenient explanation for the observed enhancement of plasmin and thrombin esterase activities by cholates invokes the formation of a substrate-cholate

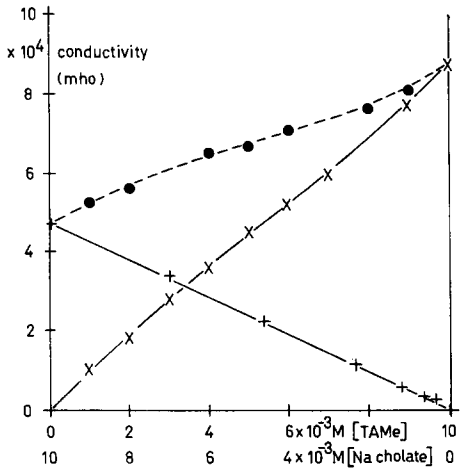

Fig. 8. Conductivity plotted against concentration (o–10^{-2} M) of individual components and of the mixture containing a constant total concentration of 10^{-2} M at 22 °C and pH 7.5 of sodium cholate (+) and TAMe (×). – – – –, sum of above contributions indicated by dashed line; ●, mixture.

complex which may be bound by the enzyme more readily than the free substrate (Cole, E. R., personal communication). This idea is made even more attractive by the direct crystallization of such a complex from relatively concentrated solutions of sodium cholate and TAMe (Cole, E. R., unpublished results).

Due to the many ambiguities possible in the interpretation of kinetic data, a separate, physical method had to be used to investigate potential complex formation under conditions known to produce enhanced rates of enzyme-catalysed hydrolysis. Measurements were carried out of the conductance in solution of the individual components and a comparison was made between their sum and the observed conductances of the actual mixtures. The combined concentrations of TAMe and sodium cholate were maintained constant at 10^{-2} M, thus including compositions known to produce enhanced rates of thrombin-catalysed hydrolysis[10]. Temperature was held constant at 22 °C, and pH was maintained at 7.5 in these experiments. The results obtained are shown in Fig. 8.

Any binding or affinity between TAMe and cholate should have resulted in a neutralization of charge and a decrease in conductance. In the absence of any significant decrease in the latter, it is unlikely that such complex formation is important in the enhancement of enzyme esterase activity by bile salts.

Effects of temperature, ionic strength and pH on the enhancement of plasmin's TLMe esterase activity by glycocholate

In each case, the rate of TLMe hydrolysis by plasmin was determined first in the absence of glycocholate (v_1), and then in the presence of 10^{-2} M sodium glycocholate. Since values of V appear to be unchanged in this type of rate enhancement, they can be cancelled from the ratio of the Michaelis–Menten expressions[24] for v_1 and v_2. Thus, the relative rate enhancement, v_2/v_1, gives an indirect indication of

how K_m(app), or more specifically, the substrate binding affinity, had been changed by the glycocholate.

$$\frac{v_2}{v_1} = \frac{K_m(\text{app})_1 + [S]}{K_m(\text{app})_2 + [S]}$$

Substrate concentration [S] was held constant at 10^{-3} M throughout. Relative rates (v_2/v_1) were then plotted against the variable considered as shown in Figs 9 and 10.

In the first experiment, temperature was varied between 10 and 55 °C, in the presence of a constant NaCl concentration (0.15 M) and at constant pH 7.5. The rate enhancement (v_2/v_1), and therefore the effectiveness of glycocholate in decreasing K_m(app), increased with temperature to a maximum near 45 °C. This is significantly higher than the optimum temperature of 37 °C for the overall rate observed in the absence of glycocholate (v_1).

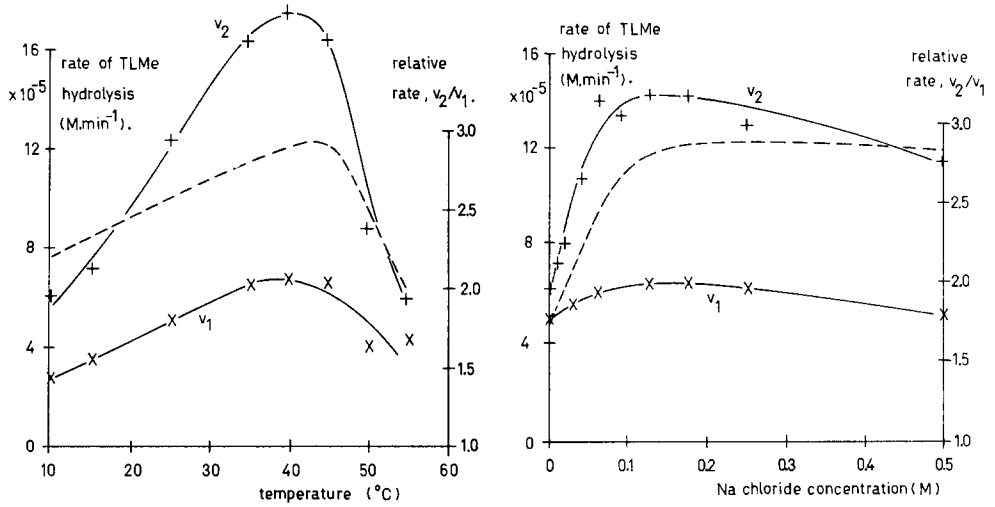

Fig. 9. Rates of hydrolysis with (v_2, +) and without (v_1, ×) 10^{-2} M sodium glycocholate present, and their ratio (v_2/v_1, indicated by dashed line) for the hydrolysis of 10^{-3} M TLMe by plasmin plotted against temperature.

Fig. 10. Rates of TLMe hydrolysis, (v_2 and v_1) and their ratio (v_2/v_1, indicated by dashed line) as for Fig. 9. plotted against NaCl concentration.

In the second experiment, the effect of NaCl concentration up to 2 M was studied similarly, but at constant pH 7.5 and constant temperature 37 °C. Clearly, rate enhancement increased rapidly to a plateau above a NaCl concentration of 0.15 M.

The effects of pH levels ranging from 6.5 to 9.0 were also investigated at constant 37 °C and in 0.15 M NaCl. However, in this case, although both v_1 and v_2 varied with pH and gave an optimum rate at pH 7.8, their pH dependence was parallel resulting in a constant value of 2.9 for v_2/v_1 throughout.

The effect of cholates on the esterase activities of other enzymes

Enzymes used were purified bovine thrombin, spontaneously activated native human plasmin, trypsin and urokinase. Sufficient enzyme was used in each case to

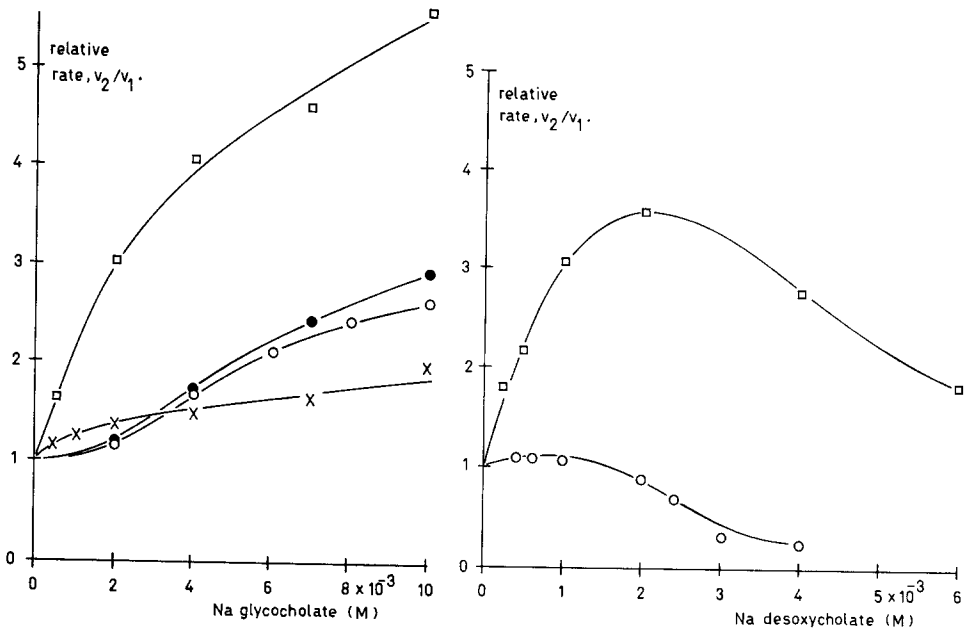

Fig. 11. Relative rates of hydrolysis of 10^{-3} M TLMe by various enzymes plotted against concentration of sodium glycocholate. □, thrombin; ●, spontaneously-activated native plasminogen (native plasmin); ○, streptokinase-activated, acid-extracted plasminogen (acid-extracted plasmin); ×, trypsin.

Fig. 12. Relative rates of hydrolysis of 10^{-3} M TAMe by thrombin and acid-extracted plasmin plotted against concentration of sodium desoxycholate. □, thrombin; ○, acid-extracted plasmin.

give a conveniently measurable rate of hydrolysis of 10^{-3} M TLMe by the usual titrimetric procedure. Sodium glycocholate concentration was progressively increased, and relative rates (v_2/v_1) were plotted against glycocholate concentration as shown in Fig. 11, together with the data obtained earlier for streptokinase-activated, acid-extracted plasminogen (detail from Fig. 1).

Glycocholate enhancement of TLMe esterase activity decreased in the order thrombin, native plasmin, acid-extracted plasmin, trypsin, urokinase, with the latter enzyme not being noticeably enhanced (and thus not recorded in Fig. 11).

The same procedure was repeated to investigate the effects of sodiumdeoxycholate on the TAMe esterase activities of thrombin and plasmin. Earlier results indicated plasmin to be inhibited by deoxycholate (Fig. 5), whereas thrombin's TAMe esterase activity was reported to be enhanced even more noticeably by deoxycholate than by sodium cholate (Cole, E. R., unpublished).

As shown in Fig. 12, both these findings were substantiated.

DISCUSSION

The activation of plasminogen by streptokinase is still incompletely understood. Early studies suggested that streptokinase combined with a "proactivator" in human plasma, forming a plasminogen activator with esterase activities and

inhibition profiles different from those of plasmin itself[25]. Other work indicates "proactivator" may be trace concentrations of plasmin[26], or plasminogen itself[27]. It is known that the activation process involves cleavage of an arginyl–valine bond in the plasminogen molecule (mol. wt 81 000), with liberation of a 7000 mol. wt peptide, and with perhaps further autolysis on incubation[28].

The data obtained here show the degree of glycocholate enhancement of TLMe esterase activity to be almost constant throughout the activation process. Even before streptokinase addition, the trace esterase activity showed the same glycocholate enhancement. Since enzymes capable of hydrolysing TLMe were found to vary appreciably in their glycocholate-enhancement characteristics (Fig. 11), with urokinase, in particular, showing no enhancement effect, the esterase activity present throughout the activation could be tentatively identified as plasmin. If this initial esterase activity could be regarded as a function of the streptokinase–activator complex[25], this would support the concept that plasmin itself may be involved in the activation process.

Only sodium cholate (the 3,7,12-trihydroxycholane-24-oate) and its conjugated derivatives produced significant enhancement of plasmin's TLMe esterase activity. Lithocholate (the 3-hydroxy derivative) was a more potent inhibitor than chenodeoxycholate (the 3,7-dihydroxy derivative) while deoxycholate (the 3,12-dihydroxy derivative) at low concentrations produced enhancement, but at higher levels was found to be an uncompetitive inhibitor. From these results the presence of hydroxyl groups in all three Positions 3, 7 and 12 appears necessary for a significant enhancement effect. Substitution by hydroxyl at the 3 positions is probably vital, while that at Position 12 appears to be more important for the enhancement than that at Position 7. In physiologically-occurring bile salts, the hydroxy-groups are usually in the α position, *i.e.* they all face one side of the molecule, with the hydrophobic C–H groups facing the other side[18]. In clustering to form more stable micelles in aqueous solution, only a few molecules of the trihydroxycholate derivatives can arrange their hydrophobic surfaces together, whereas the less hydroxylated dihydroxycholates, *e.g.* deoxycholate, are able to form much larger aggregates. Such variations in micelle-size of cholates have recently been confirmed experimentally by Small[29].

Thus, with the exception of lithocholate which does not form micelles at the temperatures studied, the inhibitions observed for the less hydroxylated cholates may be due to the interaction of large, negatively-charged micelles with the enzyme molecule, leading to inactivation. There is also the possibility that binding of cholates to the enzyme occurs at two or more sites; one enhancing substrate hydrolysis, the other(s) inhibiting it. Increased hydrophobic character may simply increase the binding of cholate at the second site preventing further activity.

The reciprocal plots of plasmin-catalysed TAMe and TLMe hydrolysis at several glycocholate concentrations show clearly that the intercept $1/V$ was unaffected by glycocholate.

Assuming the usual mechanism for enzyme catalysis, and nomenclature from reference[30],

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES \overset{k_3}{\rightleftharpoons} E + P.$$

Since

$$V = \frac{k_2 k_3}{k_2 + k_3} \cdot [E]_0,$$

and V and $[E]_0$ were constant throughout, k_2 or k_3 must also be constant, or else change in a compensatory way.

In a previous investigation into the effect of sodium cholate on thrombin's esterase activity, Curragh and Elmore[10] found that cholate inhibited the hydrolysis of αN-benzoyl-L-arginine methyl ester (BAMe). Since the latter reaction was known to have k_3 rate-limiting, the effect of cholate was probably to decrease k_3. On the other hand, they found V for thrombin-catalysed TAMe hydrolysis to be increased by sodium cholate, while K_m(app) was decreased. Here, k_3 was not rate-limiting, and since

$$K_m \text{ (app)} = \frac{k_3}{k_2 + k_3} \cdot K_m, \left(\text{where } K_m \doteqdot \frac{k_{-1}}{k_1}\right),$$

and

$$V \doteqdot k_2[E]_0, \text{ (since } k_3 > k_2),$$

the cholate was considered to be increasing k_2 as well as decreasing k_3 (ref. 10).

Our results show that for glycocholate acting on the plasmin-catalysed hydrolysis of many substrates, presumably having a range of k_2/k_3 values, there was not a single case of inhibition. This indicates either that none of the mechanisms involved has k_3 rate-limiting, or that glycocholate has no effect on k_3. If the latter is correct, and V is independent of glycocholate, then k_2 is probably also unaffected by glycocholate. In that case, glycocholate enhancement can only be due to a decrease in K_m. A similar suggestion was made by Curragh and Elmore for the cholate enhancement of thrombin esterase activities on substrates having relatively high K_m(app) values[10].

The actions of sodium cholate and deoxycholate on plasmin's TAMe esterase activity appear to be more complex than that of sodium glycocholate (Fig. 5). Values of V were significantly decreased, perhaps due to the relatively greater degree of hydrophobic character shown by these effectors. It is for this reason, that further experiments to investigate the mechanism of cholate-induced rate enhancement, were carried out with glycocholate, the most hydrophilic, and least likely to be denaturing, effector.

The greatest enhancement of plasmin esterase activity by glycocholate was observed with TLMe, and it was found that, generally, hydrolysis of the various lysine esters was affected more than that of their arginine analogues (Fig. 6). This suggested plotting susceptibility to glycocholate enhancement against K_m(app) values recorded for the various substrates by Sherry *et al.*[25]. As is apparent from Fig. 13, a nearly linear relationship was obtained which suggests once again that glycocholate enhancement may be related to enzyme–substrate binding affinity.

The distinction between activations by amines[22,31], ester substrates[20] and their products[21] of trypsin esterase activity on one hand, and that by glycocholate of the esterase activity of plasmin is quite clear. Whereas the former are due finally to increases in k_{cat}, the glycocholate effect seems to be due only to decreased K_m(app) values.

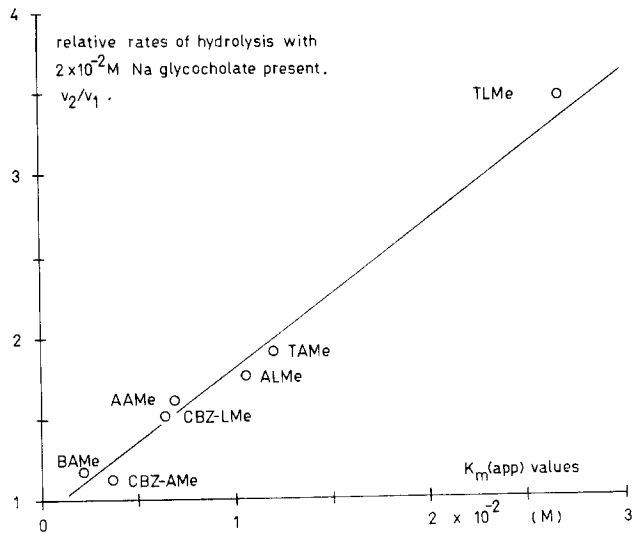

Fig. 13. Relative rates of hydrolysis of various substrates (abbreviations as shown in legend of Fig. 6) by acid-extracted plasmin with $2 \cdot 10^{-2}$ M sodium glycholate present plotted against K_m(app) values. AAMe, acetyl-L-arginine methyl ester.

Such an effect on K_m(app) values might be considered to be due either to increased affinity by enzyme for "altered" substrate, (*i.e.* the product of glycocholate–substrate interaction), or to increased affinity by a glycocholate-modified enzyme for substrate. However, a separate physical method, namely measurement of conductivity, showed that there was no appreciable interaction between TAMe, a typical substrate and sodium cholate.

Further work, carried out to investigate the glycocholate–plasmin interaction, indicated it to be hydrophobic in nature. Firstly, the effectiveness of glycocholate in decreasing K_m(app) for the hydrolysis of TLMe increased with temperature to a maximum near 45 °C, which is significantly higher than the optimum temperature of 37 °C for the overall rate observed in the absence of glycocholate. Generally, hydrophobic interactions increase with temperature, an example being the solubilities of phenols, nonionic detergents and other partially-hydroxylated compounds, which decrease with temperature. On the other hand, ionic interactions diminish with temperature, an example being the solubilities of ionic salts. Increased rate enhancement at higher temperatures could thus indicate hydrophobic interaction, possibly between the steroid nucleus and some hydrophobic amino acid residues in the enzyme molecule.

Secondly, increasing ionic strength was also found to promote the glycocholate effect. An increase in the dielectric constant of solvent-media is well-known to result in the weakening of ionic bonds. As well as functioning in this manner, an increase in the ionic salt concentration promotes the "salting-out" of hydrophobic compounds, thus providing further support for the proposed model.

The absence of a glycocholate effect on the pH profile indicates no involvement of pH-sensitive groups, such as histidine residues, in the enhancement mechanism. It also incidates that there is no interaction between the ε-amino group of the TLMe

substrate and the carboxyl group in glycocholate, since the former has a pK value within the range investigated, and its dissociation would have weakened any binding between the substrate and glycocholate.

Although proteins were formerly thought not to bind cholates and their derivatives[32], more recent observations do indicate the binding of cholanic acids by human plasma proteins[33]. In addition, detergent-binding capacities of proteins have been used as an indication of lipophilic character. In proteins derived from lipoprotein (membranes or otherwise), detergents such as deoxycholate are considered to bind to the sites formerly occupied by lipids[34]. Using the Inagami–Murachi model, the dissociation constant for glycocholate–plasmin interaction was estimated to be $7.2 \cdot 10^{-3}$ M, which corresponds to a relatively strong binding compared to that reported for various amine effectors of trypsin[21,30].

The enhancement of TLMe esterase activity by glycocholate was not restricted to plasmin, but also occurred to a much greater degree with bovine thrombin, and to a lesser extent with trypsin. Plasminogen prepared by "affinity" chromatography and spontaneously activated in 50 % glycerol, showed an effect very similar, but slightly more pronounced, than that found for the streptokinase-activated "acid-extracted" plasminogen used throughout this investigation.

The greater susceptibility of thrombin to cholates is interesting. In some instances, plasmin activity may in fact be inhibited, while that of thrombin is enhanced. For example, the differential effects of sodium deoxycholate in the hydrolysis of TAMe by these two enzymes are shown in Fig. 12. Whether or not such opposing effects are of regulatory significance in the vascular system is still not known.

The similarity in hydrophilic–lipophilic balance, (HLB value), if not the actual positions of hydroxylation, between cholate, deoxycholate, the digitoxigenins and related sapogenins suggests that these latter cardiac stimulants may also act as enzyme esterase accelerators.

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