3. The results also show that the primary structure of the lysine-rich histone is probably irregular with respect to the lysine residues.

I thank Professor J. A. V. Butler, F.R.S., for his advice and encouragement and Miss Pamela Simson for the amino acid analyses. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

- Crampton, C. F., Stein, W. H. & Moore, S. (1957). *J. biol. Chem.* **225**, 363.
- Cruft, H. J., Hindley, J., Mauritzen, C. M. & Stedman, E. (1957). *Nature, Lond.*, **180**, 1107.
- Davison, P. F. & Butler, J. A. V. (1956). *Biochim. biophys. Acta*, **21**, 568.
- Desnuelle, P. & Casal, A. (1948). *Biochim. biophys. Acta*, **2**, 64.
- Johns, E. W. (1962). Thesis: Royal Institute of Chemistry.
- Johns, E. W. (1963). *Proc. World Conf. Histone Biology and Chemistry*, p. 52. San Francisco: Holden-Day Inc.
- Johns, E. W. (1964). *Biochem. J.* **92**, 55.
- Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
- Phillips, D. M. P. (1963*a*). *Biochem. J.* **87**, 258.
- Phillips, D. M. P. (1963*b*). *Proc. World Conf. Histone Biology and Chemistry*, p. 46. San Francisco: Holden-Day Inc.
- Phillips, D. M. P. & Johns, E. W. (1959). *Biochem. J.* **72**, 538.
- Phillips, D. M. P. & Simson, P. (1962). *Biochem. J.* **82**, 236.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.

Biochem. J. (1964), **93**, 163

Kinetics and Mechanism of Catalysis by Proteolytic Enzymes

2. KINETIC STUDIES OF THROMBIN-CATALYSED REACTIONS AND THEIR MODIFICATION BY BILE SALTS AND OTHER DETERGENTS*

BY E. F. CURRAGH AND D. T. ELMORE

Department of Biochemistry, Queen's University, Belfast, N. Ireland

(Received 19 March 1964)

Thrombin, which converts fibrinogen into fibrin, is capable of hydrolysing suitable derivatives of amino acids. The present work was undertaken to compare the specificities and kinetics of action of bovine thrombin and bovine pancreatic trypsin (Baines, Baird & Elmore, 1964). Most of the potential substrates examined belong to one of the series of compounds (I) or (II). It has been shown that bile salts have an inhibitory effect on the blood-clotting process (Garagnani & Facchini, 1956). This might be of clinical significance in obstructive jaundice when the plasma concentration of bile salts is increased. We have studied the

effects of bile salts and other detergents on the kinetics of several thrombin-catalysed reactions. A preliminary account of some of this work has appeared (Curragh & Elmore, 1963).

EXPERIMENTAL

Materials

The thrombin used in the present investigation was 'topical thrombin' obtained from Parke, Davis and Co. Ltd.; its activity was assayed with α -N-toluene-*p*-sulphonyl-L-arginine methyl ester (II*b*; R = O·CH₃, *n* = 3) and expressed in the units defined by Ehrenpreis & Scheraga (1957). Syntheses of several of the potential substrates and inhibitors have been reported by Baines *et al.* (1964).

* Part 1: Baines, Baird & Elmore (1964).

α -*N*-Toluene-*p*-sulphonyl-L-arginine amide (IIb; R = NH₂, *n* = 3) was synthesized by the method of Bergmann, Fruton & Pollok (1939). The hydrochlorides of α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester (I; R = O·CH₃, *n* = 4), α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester (I; R = O·CH₃, *n* = 3) and methyl γ -amino-L- α -toluene-*p*-sulphonamidobutyrate (I; R = O·CH₃, *n* = 2) were synthesized by the method of Barrass & Elmore (1957). 5-(3-Guanidinopropyl)-2-thiohydantoin hydrochloride was synthesized by the method of Elmore, Ogle & Toseland (1956). β -Dipalmitoyl-L- α -kephalin and α -lecithin (micro-analytical grade) were purchased from Mann Research Laboratories (New York). The source of fibrinogen was bovine plasma (fraction I) (Armour Pharmaceutical Co. Ltd.). Syntheses of the remaining compounds are described below.

α -*N*-Toluene-*p*-sulphonyl-L-arginine *n*-propyl ester hydrochloride. α -*N*-Toluene-*p*-sulphonyl-L-arginine trihydrate (4.0 g.) was converted into the *n*-propyl ester hydrochloride (IIb; R = O·CH₂·CH₂·CH₃, *n* = 3) (4.0 g.) by treatment with a solution of anhydrous HCl in *n*-propanol. Recrystallized from propan-1-ol-ether, it had m.p. 127–128°, [α]_D²⁵ -10.9 ± 0.2° (c 4 in water) (Found: C, 47.2; H, 6.7; N, 13.7. C₁₆H₂₇ClN₄O₄S requires C, 47.2; H, 6.7; N, 13.8%).

α -*N*-Benzoyl-L-arginine cyclohexyl ester hydrochloride. α -*N*-Benzoyl-L-arginine (2.0 g.) was converted into the cyclohexyl ester hydrochloride (IIa; R = O·C₆H₁₁, *n* = 3) (2.0 g.) by treatment with a saturated solution of anhydrous HCl in cyclohexanol. Recrystallized from methanol-ether, it had m.p. 200–201° (Found: C, 57.3; H, 7.0; N, 14.1. C₁₈H₂₈ClN₄O₃ requires C, 57.6; H, 7.1; N, 14.2%).

ω -*N*-Nitro- α -*N*-toluene-*p*-sulphonyl-L-arginine. ω -*N*-Nitro-L-arginine (2.5 g.) in aqueous NaOH at pH 9.0 was treated with toluene-*p*-sulphonyl chloride (2.0 g.) in ether. Acidification to pH 4.0 gave ω -*N*-nitro- α -*N*-toluene-*p*-sulphonyl-L-arginine (4.0 g.). Recrystallized from water, it had m.p. 170–171° (Found: C, 40.6; H, 5.4; N, 18.6. C₁₃H₁₉N₅O₈· $\frac{1}{2}$ H₂O requires C, 40.9; H, 5.2; N, 18.4%).

ω -*N*-Nitro- α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride. ω -*N*-Nitro- α -*N*-toluene-*p*-sulphonyl-L-arginine (2.0 g.) was converted into the methyl ester hydrochloride (1.3 g.) by treatment with methanol saturated with anhydrous HCl. Recrystallized from methanol, it had m.p. 200–201° (Found: C, 43.4; H, 5.5; N, 18.3. C₁₄H₂₁N₅O₈S requires C, 43.5; H, 5.5; N, 18.1%).

N-Toluene-*p*-sulphonyl-L-glutamyl- γ -(*N*²-benzyloxycarbonyl)hydrazide. *N*-Toluene-*p*-sulphonyl-L-pyrrolid-5-one-2-carboxylic acid (10.0 g.) in water (16 ml.) was heated under reflux for 10 min. with hydrazine hydrate (20 ml.). The solution was evaporated under reduced pressure to a gum which was redissolved in water (50 ml.). The pH was adjusted to 8.0, and the solution was treated with benzyl chloroformate (40 ml.) in ether (50 ml.) with periodic addition of NaOH to maintain constant pH. The resulting benzyl *N*-(benzyloxycarboxyamido)carbamate (29.2 g.),

having m.p. 79–80°, was collected and the filtrate was twice extracted with ethyl acetate and acidified. The resulting oil was extracted with ethyl acetate and the extract was dried (over Na₂SO₄) and evaporated under reduced pressure. The residual *N*-toluene-*p*-sulphonyl-L-glutamyl- γ -(*N*²-benzyloxycarbonyl)hydrazide (13.6 g.) was crystallized from ethyl acetate–light petroleum (b.p. 40–60°), when it had m.p. 126.5–127.5° (Found: C, 53.1; H, 4.8; N, 9.0. C₂₀H₂₃N₃O₇S requires C, 53.4; H, 5.2; N, 9.4%).

N-Toluene-*p*-sulphonyl-L-glutamyl- γ -(*N*²-benzyloxycarbonyl)hydrazide α -methyl ester. The foregoing acid (11.1 g.) in ethanol (100 ml.) was treated with a slight excess of diazomethane in ether. Evaporation and the addition of light petroleum (b.p. 40–60°) yielded the ester (11.2 g.), which had m.p. 117.0–117.5° after recrystallization from aqueous methanol (Found: C, 53.9; H, 6.0; N, 8.9. C₂₁H₂₅N₃O₇S requires C, 54.4; H, 5.4; N, 9.1%).

N-Toluene-*p*-sulphonyl-L-glutamyl- γ -hydrazide α -methyl ester hydrochloride. The foregoing ester (2.32 g.) was hydrogenolysed in a mixture of methanol (70 ml.) and *n*-HCl (5 ml.) over palladous oxide. Evaporation and crystallization from methanol-ether afforded *N*-toluene-*p*-sulphonyl-L-glutamyl- γ -hydrazide α -methyl ester hydrochloride (1.40 g.), which had m.p. 146–147° (Found: C, 42.5; H, 5.6; N, 11.0. C₁₃H₂₀ClN₃O₆S requires C, 42.7; H, 5.5; N, 11.5%).

Benzyl glycocholate. A mixture of cholic acid (2.50 g.) and triethylamine (0.606 g.) in dimethylformamide–dioxan (1:1, v/v) (10 ml.) was cooled to 0° and treated with a cold solution of ethyl chloroformate (0.65 g.) in dimethylformamide–dioxan (1:1) (10 ml.). After 15 min., a mixture of triethylamine (0.61 g.) and glycine benzyl ester hydrobromide (1.48 g.) in cold dimethylformamide–dioxan (1:1) (10 ml.) was added. The mixture was left to warm up overnight, stirred for a day and then evaporated under reduced pressure. A solution of the residual solid in ethyl acetate was washed successively with aqueous NaHCO₃ and water and then dried (over MgSO₄). The addition of ether gave benzyl glycocholate (1.80 g.), which had m.p. 145–148° (Found: C, 70.9; H, 9.5; N, 3.0. C₃₃H₄₉NO₆ requires C, 71.3; H, 9.1; N, 2.5%).

Glycocholic acid. Hydrogenolysis of benzyl glycocholate (3.0 g.) in methanol over palladous oxide gave glycocholic acid (2.1 g.), which had m.p. 169° after recrystallization from ethyl acetate–ether (Found: C, 65.6; H, 9.3; N, 3.0. Calc. for C₂₈H₄₃NO₆· $\frac{1}{2}$ H₂O: C, 65.7; H, 9.3; N, 3.0%).

Kinetic and computational techniques

Hydrolysis of esters was followed by means of a pH-stat and recorder (Baines *et al.* 1964). Values of $K_m(\text{app.})$, $k_3(\text{app.})$ and $pK(\text{app.})$ were computed as before (Elmore, Kingston & Shields, 1963; Baines *et al.* 1964). The initial velocity of hydrolysis of the amide was determined by measuring the volume, α_1 , of alkali required to maintain a constant pH (8.0) for a given time interval, t . Excess of

Scheme 1

neutral formaldehyde solution was then added and the volume of alkali, a_2 , required to restore the pH to 8.0 was determined. The initial velocity was calculated from the expression $(a_1 + a_2)/t$. Clotting experiments were carried out by the technique of Johnson & Seegers (1955) with silicone-treated tubes.

THEORETICAL

The catalytic mechanism of thrombin has not been intensively studied. In view of the close resemblance between thrombin, trypsin and chymotrypsin, however, it is probable that a similar three-step mechanism is involved (Scheme 1). At the optimum pH, the apparent zero-order rate constant, $k_{3(\text{app.})}$, and apparent Michaelis constant, $K_{m(\text{app.})}$, are given (Gutfreund & Sturtevant, 1956*a, b*) by the equations:

$$1/k_{3(\text{app.})} = 1/k_2 + 1/k_3 \quad (1)$$

$$K_{m(\text{app.})} = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)} \quad (2)$$

In addition, it has been shown by Baines *et al.* (1964) that

$$K_{(\text{app.})} = \frac{K_1 K_2 (k_2 + k_3)}{k_2 K_1 + k_3 K_2} \quad (3)$$

The parameters $k_{3(\text{app.})}$, $K_{m(\text{app.})}$ and $K_{(\text{app.})}$ can be measured experimentally, and it is necessary to determine the effects on these parameters of alterations in the various rate constants in order to interpret the kinetic results of experiments conducted in the presence of kinetic modifiers such as bile salts. It is obvious from eqn. (1) that an increase in k_2 or k_3 or both will increase $k_{3(\text{app.})}$. If there is a large difference between k_2 and k_3 , however, $k_{3(\text{app.})}$ will be relatively insensitive to an increase in the larger rate constant. If either k_2 or k_3 is increased and the other is decreased, $k_{3(\text{app.})}$ may be increased or decreased depending on (a) the relative magnitudes of k_2 and k_3 and (b) the amount by which they are altered.

From eqn. (2) it can be seen that an increase in k_1 decreases $K_{m(\text{app.})}$, whereas an increase in k_{-1} increases $K_{m(\text{app.})}$. The effects on $K_{m(\text{app.})}$ of alterations to k_2 and k_3 can be determined most easily by partial differentiation:

$$\frac{\partial K_{m(\text{app.})}}{\partial k_2} = \frac{k_3(k_3 - k_{-1})}{k_1(k_2 + k_3)^2}$$

If $k_3 < k_{-1}$, which is very probable for an enzyme-catalysed reaction, $\partial K_{m(\text{app.})}/\partial k_2 < 0$, and an increase in k_2 will decrease $K_{m(\text{app.})}$. On the other hand, if $k_3 > k_{-1}$, or if $k_3 \gg k_2$ when

$$K_{m(\text{app.})} \sim (k_{-1} + k_2)/k_1$$

an increase in k_2 will increase $K_{m(\text{app.})}$.

Similarly:

$$\frac{\partial K_{m(\text{app.})}}{\partial k_3} = \frac{k_2(k_{-1} + k_2)}{k_1(k_2 + k_3)^2} > 0$$

Hence an increase in k_3 will result in an increase in $K_{m(\text{app.})}$ unless $k_3 \gg k_2$, when $K_{m(\text{app.})}$ will be insensitive to a change in k_3 .

Partial differentiation of eqn. (3) gives:

$$\frac{\partial K_{(\text{app.})}}{\partial k_2} = \frac{k_3 K_1 K_2 (K_2 - K_1)}{(k_2 K_1 + k_3 K_2)^2}$$

If $K_1 > K_2$, as seems likely for reactions catalysed by the related enzymes trypsin and chymotrypsin, $\partial K_{(\text{app.})}/\partial k_2 < 0$, and an increase in k_2 will decrease $K_{(\text{app.})}$ and increase $pK_{(\text{app.})}$.

Similarly:

$$\frac{\partial K_{(\text{app.})}}{\partial k_3} = \frac{k_2 K_1 K_2 (K_1 - K_2)}{(k_2 K_1 + k_3 K_2)^2}$$

If $K_1 > K_2$, $\partial K_{(\text{app.})}/\partial k_3 > 0$, and an increase in k_3 will increase $K_{(\text{app.})}$ and decrease $pK_{(\text{app.})}$. Changes in $pK_{(\text{app.})}$ are likely to be small and will be further modified if K_1 and K_2 are altered.

RESULTS AND DISCUSSION

Specificity. In the hydrolysis of the methyl, ethyl and cyclohexyl esters of α -N-benzoyl-L-arginine, $k_{3(\text{app.})}$ is sensibly constant (Table 1, II*a*) and it is probable that deacylation of (α -N-benzoyl-L-arginyl)-thrombin, i.e. (IV) $\rightarrow$ (V), is rate-determining. For these substrates, therefore, $k_2 \gg k_3$. Similar behaviour was found with trypsin (Schwert & Eisenberg, 1949). In contrast, $k_{3(\text{app.})}$ was not constant for a series of esters of α -N-toluene-*p*-sulphonyl-L-arginine (Table 1, II*b*). It can be concluded that deacylation of the common intermediate, (α -N-toluene-*p*-sulphonyl-L-arginyl)-thrombin, is not itself rate-determining, but that

Table 1. $k_{3(\text{app.})}$ values at pH 8.4 and 25° for the hydrolysis of various substrates in 0.1M-sodium chloride

Values are given as means $\pm$ S.D., with the numbers of determinations in parentheses.

Substrate	$10^7 k_{3(\text{app.})}$ (mole.min. ⁻¹ enzyme unit ⁻¹)
(I; R = O·CH ₃ , $n = 4$)	10.43 $\pm$ 0.60 (6)
(II <i>a</i> ; R = O·CH ₃ , $n = 3$)	3.233 $\pm$ 0.071 (5)
(II <i>a</i> ; R = O·C ₂ H ₅ , $n = 3$)	3.246 $\pm$ 0.013 (15)
(II <i>a</i> ; R = O·C ₆ H ₁₁ , $n = 3$)	3.305 $\pm$ 0.058 (5)
(II <i>b</i> ; R = O·CH ₃ , $n = 3$)	6.754 $\pm$ 0.431 (4)
(II <i>b</i> ; R = O·C ₂ H ₅ , $n = 3$)	2.988 $\pm$ 0.051 (10)
(II <i>b</i> ; R = O·CH ₂ ·CH ₂ ·CH ₃ , $n = 3$)	2.611 $\pm$ 0.056 (6)
(II <i>b</i> ; R = O·C ₆ H ₁₁ , $n = 3$)	6.538 $\pm$ 0.088 (6)
(II <i>b</i> ; R = NH ₂ , $n = 3$)	0.207 $\pm$ 0.026 (3)

$k_{3(\text{app.})}$ contains a contribution from the acylation step, i.e. (III) $\rightarrow$ (IV), which is governed by k_2 . This observation is in contrast with the results obtained with trypsin (Baines *et al.* 1964). The values of $k_{3(\text{app.})}$ do not fall in the order that would be expected for chemical hydrolysis: thus the cyclohexyl ester is hydrolysed considerably faster than might have been expected. A plausible explanation can be provided by the theory of induced fit (Koshland, 1958). The bulky cyclohexyl group may alter the conformation of the enzyme in such a way that acylation of the enzyme is facilitated.

Values of $K_{m(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and α -*N*-benzoyl-L-arginine ethyl ester by thrombin (Table 2) are very small and comparable with those for the trypsin-catalysed reactions. Sherry & Troll (1954) and Ronwin (1959), on the other hand, obtained values of $K_{m(\text{app.})}$ for the thrombin-catalysed hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester that are much higher than ours. A similar situation arose with the trypsin-catalysed reaction and this discrepancy was apparently due to a failure by earlier workers to detect the occurrence of activation by substrate (Trowbridge, Krehbiel & Laskowski, 1963; Baines *et al.* 1964).

Evidence has been obtained for the occurrence of activation by substrate in the thrombin-catalysed reaction. Alternatively, it is possible that the high values of $K_{m(\text{app.})}$ obtained by the earlier workers result from the use of buffers. The observation that the position of the optimum pH for the thrombin-catalysed hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester varied with the nature of the buffer (Sherry & Troll, 1954) supports this view. The use of the pH-stat avoids the use of buffers.

α -*N*-Toluene-*p*-sulphonyl-L-homoarginine methyl ester (IIb; R = O·CH₃, n = 4) was not perceptibly hydrolysed by thrombin, although it has been found to be a fairly sensitive substrate for trypsin (Baines *et al.* 1964). This observation seems to provide the simplest method to date for distinguishing between thrombin and trypsin by using synthetic substrates. ω -*N*-Nitro- α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester was not appreciably hydrolysed by either thrombin or trypsin, and it is likely

that the low basicity of the nitroguanidino group is responsible. The latter will be unprotonated at the pH values used for enzyme studies and the presence of a positive charge, within a fairly narrow range of distance from the bond to be cleaved, appears to be an essential requirement for a sensitive substrate of trypsin and thrombin.

α -*N*-Toluene-*p*-sulphonyl-L-lysine methyl ester (I; R = O·CH₃, n = 4) was found to be the most sensitive substrate for thrombin under the conditions used. The relative contributions of k_2 and k_3 to $k_{3(\text{app.})}$ are as yet unknown for this substrate. α -*N*-Toluene-*p*-sulphonyl-L-ornithine methyl ester (I; R = O·CH₃, n = 3) was also hydrolysed by thrombin. In view of its ready lactamization at slightly alkaline pH values (Curragh & Elmore, 1962), the hydrolysis of this substrate was studied at pH 7.0. Under these conditions, the kinetics of thrombin-catalysed hydrolysis were first-order with respect to substrate with substrate concentrations up to 6 mM (k = $7.46 \times 10^{-6} \pm 0.03 \times 10^{-6}$ min.⁻¹ thrombin unit⁻¹.l.); $K_{m(\text{app.})}$ must therefore be quite high. Catalysis of the hydrolysis of the lower homologue, methyl γ -amino- α -L-toluene-*p*-sulphonamidobutyrate (I; R = O·CH₃, n = 2) by thrombin could not be detected. *N*-Toluene-*p*-sulphonyl-L-glutamyl- γ -hydrazide α -methyl ester, which may be regarded as an isostere of α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester in which part of the side chain is held in a planar configuration, was not hydrolysed by thrombin. This contrasts with the observation that *N*-benzyloxy-carbonyl-L-glutamyl- γ -hydrazide α -methyl ester is slowly hydrolysed by trypsin (Ebata, 1961). The hydrazide group has pK_a 3–4 and would not therefore be protonated at the pH values used for enzyme studies. α -*N*-Toluene-*p*-sulphonyl-L-arginine amide (IIa; R = NH₂, n = 3), although hydrolysed by thrombin, is considerably less sensitive than the corresponding esters. 5-(3-Guanidinopropyl)-2-thiohydantoin, which may be regarded as a cyclic α -*N*-acylated amide of arginine, was not hydrolysed by thrombin. Locking of the α -*N*-acyl and amide groups in the same plane may explain the stability of this compound to enzymic hydrolysis.

Table 2. $K_{m(\text{app.})}$ values at pH 8.4 and 25° for the hydrolysis of some arginine derivatives by thrombin

Substrate				$K_{m(\text{app.})}$ (μM)		
				(IIa; R = O·CH ₃ , n = 3)	(IIb; R = O·CH ₃ , n = 3)	(IIb; R = NH ₂ , n = 3)
Bile salt						
None				16.1 $\pm$ 3.9	32.1 $\pm$ 7.8	6860 $\pm$ 550
Cholate (2 mM)				6.0 $\pm$ 1.0	5.0 $\pm$ 2.3	6490 $\pm$ 420
Glycocholate (2 mM)				7.4 $\pm$ 1.4	5.4 $\pm$ 2.6	—

Values are given as means $\pm$ S.D.

The above results underline the general similarity of trypsin and thrombin, but the latter appears to be rather more specific.

Effect of ionic strength. The effect of ionic strength on $k_{3(\text{app.})}$ for α -N-benzoyl-L-arginine ethyl ester and α -N-toluene-*p*-sulphonyl-L-arginine methyl ester has been determined in solutions of sodium or potassium chloride. The rate of hydrolysis of α -N-benzoyl-L-arginine ethyl ester by thrombin was insensitive to change of ionic strength. On the other hand, the results (Fig. 1) for α -N-toluene-*p*-sulphonyl-L-arginine methyl ester show that $k_{3(\text{app.})}$ decreases with increasing ionic strength (cf. Scheraga & Ehrenpreis, 1958), an effect that is more noticeable in the presence of Na^+ ions than in the presence of K^+ ions. We have obtained evidence above that indicates that deacylation is rate-determining in the hydrolysis of α -N-benzoyl-L-arginine ethyl ester, whereas both acylation and deacylation contribute to $k_{3(\text{app.})}$ in the hydrolysis of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester. It is probable, therefore, that it is the acylation step that is sensitive to ionic strength. However, the value of $k_{3(\text{app.})}$ for the hydrolysis of the latter substrate by trypsin, where deacylation is rate-determining, is insensitive to ionic strength. If the generality of this behaviour can be established, a study of the effect of change of ionic strength on $k_{3(\text{app.})}$ for reactions catalysed by thrombin and trypsin may provide a simple diagnostic test to determine if acylation contributes to $k_{3(\text{app.})}$. Alternatively, it is possible that the different effects of ionic strength on the rate of thrombin-catalysed reactions is in some way dependent on the nature of the α -substituent. A wider range of substrates will have to be examined to decide this point.

Effect of pH. The $\text{pK}_{(\text{app.})}$ values of the group(s) participating in the hydrolysis of α -N-benzoyl-L-arginine ethyl ester and α -N-toluene-*p*-sulphonyl-L-arginine methyl ester by thrombin were 6.59 ± 0.02 and 6.61 ± 0.02 respectively. These values are consistent with the postulate that the imidazole group of a histidine residue is involved in the catalytic process. With the former substrate, deacylation is rate-determining and $\text{pK}_{(\text{app.})}$ is therefore equal to pK_2 . Both acylation and deacylation apparently contribute to the kinetics of hydrolysis of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester and $\text{pK}_{(\text{app.})}$ is intermediate in value between pK_1 and pK_2 .

Effect of temperature. The value of $k_{3(\text{app.})}$ was determined at several temperatures for the hydrolyses of α -N-toluene-*p*-sulphonyl-L-arginine methyl ester and α -N-benzoyl-L-arginine ethyl ester in the absence and presence of cholate. Plots of $\log k_{3(\text{app.})}$ against $1/T$ were non-linear (Fig. 2) and consequently energies of activation could not be calculated. The non-linear Arrhenius plot for the former

substrate is explicable since k_2 and k_3 make comparable contributions to $k_{3(\text{app.})}$. Since deacylation is rate-determining for the hydrolysis of the second substrate, a linear Arrhenius plot would have been expected. Our results could be explained if enzyme denaturation competes seriously with the catalytic process as the temperature is raised. Alternatively, it is possible that more than one esterase activity is present. Marciniak & Seegers (1962) have shown that autoproteolysis of prothrombin C, which is formed simultaneously with thrombin when prothrombin is

Fig. 1. Effect of salt concentration on the value of $k_{3(\text{app.})}$ at 25° and pH 8.4 for α -N-toluene-*p*-sulphonyl-L-arginine methyl ester. ●, KCl; ○, NaCl.

Fig. 2. Variation of $\log k_{3(\text{app.})}$ with $1/T$ for the hydrolysis of esters by thrombin at pH 8.4. ○, α -N-Toluene-*p*-sulphonyl-L-arginine methyl ester; ●, α -N-toluene-*p*-sulphonyl-L-arginine methyl ester + cholate (2 mM); □, α -N-benzoyl-L-arginine ethyl ester; ■, α -N-benzoyl-L-arginine ethyl ester + cholate (2 mM).

activated under suitable conditions, has esterase activity towards α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester.

Effect of bile salts and other kinetic modifiers. We have studied the action of a number of detergents on thrombin-catalysed reactions because bile salts inhibit blood-clotting, probably by modification of the thrombin-fibrinogen reaction (Garagnani & Facchini, 1956), and because various phospholipids accelerate blood-clotting in the presence of Russell's viper venom (Bangham, 1961; Billimoria, Curtis & MacLagan, 1960; Mustard, Medway, Downie & Rowsell, 1962). We have examined the effect of bile salts on $K_{m(\text{app.})}$ and $k_{3(\text{app.})}$ for various synthetic substrates of thrombin (Tables 2 and 3). By using substrates of small molecular size, the action of bile salts can be limited to the enzyme and enzyme-substrate intermediates.

The results in Table 3 and Fig. 2 show that $k_{3(\text{app.})}$ for the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester is decreased by cholate over a range of temperature. Since deacylation is rate-determining, it is evident that cholate diminishes k_3 , an effect that would explain the observed decrease in $K_{m(\text{app.})}$ (Table 2). In contrast, the values of $k_{3(\text{app.})}$ for the hydrolysis of the methyl and ethyl esters of α -*N*-toluene-*p*-sulphonyl-L-arginine are increased by cholate. This could be explained plausibly by supposing that, as in the foregoing reaction, k_3 is decreased by cholate, but that in the present case k_2 is increased sufficiently to give a net increase in $k_{3(\text{app.})}$. Such an effect would also explain the observed decrease in $K_{m(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester in the presence of cholate. This kind of behaviour is not without precedent, since it has been shown that indole, which is a net inhibitor of reactions catalysed by chymotrypsin, inhibits the acylation of chymo-

trypsin by *p*-nitrophenyl acetate but accelerates the deacylation of the resulting acetyl-chymotrypsin (Foster, 1961). Comparison of the values of $k_{3(\text{app.})}$ for the hydrolyses of the methyl and ethyl esters of α -*N*-toluene-*p*-sulphonyl-L-arginine in the absence and presence of cholate indicates that the acylation step of at least one of the reactions is affected by cholate. Thus, let k_2^{Me} and k_2^{Et} be the rate constants for the acylation step with the methyl and ethyl esters respectively and let $k_{3(\text{app.})}^{\text{Me}}$ and $k_{3(\text{app.})}^{\text{Et}}$ be the corresponding zero-order rate constants. For hydrolyses in the presence of cholate, let the corresponding constants be $k_2^{\text{Me}'}$, $k_2^{\text{Et}'}$, $k_{3(\text{app.})}^{\text{Me}'}$ and $k_{3(\text{app.})}^{\text{Et}'}$ respectively. Since a common deacylation step is involved:

$$1/k_{3(\text{app.})}^{\text{Me}} = 1/k_2^{\text{Me}} + 1/k_3$$

and

$$1/k_{3(\text{app.})}^{\text{Et}} = 1/k_2^{\text{Et}} + 1/k_3$$

Subtraction gives:

$$1/k_{3(\text{app.})}^{\text{Et}} - 1/k_{3(\text{app.})}^{\text{Me}} = 1/k_2^{\text{Et}} - 1/k_2^{\text{Me}} = 1.866 \times 10^6 \text{ min. enzyme unit. mole}^{-1}$$

Similarly, for the reactions in the presence of cholate:

$$1/k_{3(\text{app.})}^{\text{Et}'} - 1/k_{3(\text{app.})}^{\text{Me}'} = 1/k_2^{\text{Et}'} - 1/k_2^{\text{Me}'} = 1.190 \times 10^6 \text{ min. enzyme unit. mole}^{-1}$$

Hence:

$$k_2^{\text{Me}} \neq k_2^{\text{Me}'} \quad \text{and/or} \quad k_2^{\text{Et}} \neq k_2^{\text{Et}'}$$

Although the foregoing argument indicates that k_2 is altered in the presence of cholate, an alternative explanation would involve an increase in k_3 . Thus cholate lowers the $pK_{(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester from 6.61 ± 0.02 to 6.37 ± 0.01 . If $K_1 > K_2$ and if both dissociation constants are unaffected by cholate, this observation suggests that cholate

Table 3. *Effect of bile salts on $k_{3(\text{app.})}$ for the hydrolysis of various substrates at pH 8.4 and 25° by thrombin*

Values are given as means $\pm$ S.D., with the numbers of determinations in parentheses.

			$10^7 k_{3(\text{app.})}$ (mole.min. ⁻¹ enzyme unit ⁻¹)				
Substrate ...	...	...	(I; R = O·CH ₃ , <i>n</i> = 4)	(IIa; R = O·C ₂ H ₅ , <i>n</i> = 3)	(IIb; R = O·CH ₃ , <i>n</i> = 3)	(IIb; R = O·C ₂ H ₅ , <i>n</i> = 3)	(IIb; R = NH ₂ , <i>n</i> = 3)
			Bile salt				
None			10.43	3.246	6.754	2.988	0.207
			± 0.60 (6)	± 0.013 (15)	± 0.431 (4)	± 0.051 (10)	± 0.026 (3)
Cholate (2 mM)			14.17	2.370	9.875	4.539	0.209
			± 0.60 (6)	± 0.088 (3)	± 0.337 (5)	± 0.062 (3)	0.037 (3)
Cholate (4 mM)			14.94	1.798	—	—	—
			± 0.16 (3)	± 0.059 (3)			
Cholate (6 mM)			15.96	1.603	—	—	—
			± 0.63 (3)	± 0.069 (3)			
Glycocholate (2 mM)			15.29	2.326	13.98	—	—
			± 0.30 (4)	± 0.025 (4)	± 0.22 (5)		
Glycocholate (4 mM)			17.80	2.202	15.78	—	—
			± 0.46 (3)	± 0.029 (4)	± 0.49 (6)		
Glycocholate (6 mM)			19.29	1.965	16.09	—	—
			± 0.27 (4)	± 0.140 (3)	± 0.19 (4)		

decreases k_2 or increases k_3 or both. If either of these possibilities is true, the decrease in $K_{m(\text{app.})}$ must be attributed to a decrease in k_{-1} or an increase in k_1 or both. In addition, $k_{3(\text{app.})}$ and $K_{m(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine amide by thrombin were insensitive to the presence of 2 mM-cholate. Since acylation is likely to be the predominant rate-determining step for this substrate, $k_{3(\text{app.})} \sim k_2$ and $K_{m(\text{app.})} \sim K_m$, and an increase in k_2 would be expected to increase both $k_{3(\text{app.})}$ and $K_{m(\text{app.})}$.

Cholate accelerates the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester by thrombin and it also has a pronounced effect on the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester at pH 7.0; the kinetics for the latter were changed from first-order with respect to substrate to kinetics of the Michaelis-Menten type

$$[K_{m(\text{app.})} = 3.15 \pm 0.05 \text{ mM}$$

and

$$k_{3(\text{app.})} = 1.69 \times 10^{-7} \pm 0.02 \times 10^{-7} \text{ mole.min.}^{-1} \text{ enzyme unit}^{-1}]$$

This suggests that formation of the enzyme-substrate complex was favoured by either an increase in k_1 or a decrease in k_{-1} .

The effect of cholate on the clotting of fibrinogen by thrombin is shown in Fig. 3. With relatively high concentrations of thrombin cholate inhibits clotting, but with low concentrations of thrombin cholate has a slight accelerating action. The latter effect was reproducible in runs with parallel controls. These results in general confirm the findings of Garagnani & Facchini (1956).

Glycocholate was qualitatively similar to cholate in its action on thrombin-catalysed reactions (Table 3). It was, however, quantitatively a more effective accelerator of the hydrolyses of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester and a less effective inhibitor of the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester. Increase of cholate or glycocholate concentration increased the observed effect, but the relationship between $k_{3(\text{app.})}$ and bile salt concentration was usually non-linear.

β γ -Dipalmitoyl-L- α -kephalin and - α -lecithin had little effect on the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and α -*N*-benzoyl-L-arginine ethyl ester by thrombin. The non-ionic detergents Tween 20, Tween 40 and Tween 80, unlike the bile salts, increased $k_{3(\text{app.})}$ for the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester although the increase in $k_{3(\text{app.})}$ was more noticeable when the substrate was α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester. Tween 80 was the most effective of the non-ionic detergents examined (Table 4).

The modification of the kinetics of enzyme catalysis by detergents may stem from conformational changes in the enzyme or enzyme-substrate intermediates or both. On the basis of optical-rotatory-dispersion measurements, Jirgensons (1961, 1962*a, b*) suggested that detergents increase the helical content of a number of proteins, probably by modifying restrictive hydrophobic interactions between amino acid side chains. It is also likely

Fig. 3. ○, Variation of clotting time of bovine fibrinogen with thrombin concentration; ■, cholate (2 mM) added; ●, α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester (2 mM) added.

Table 4. Effect of non-ionic detergents on $k_{3(\text{app.})}$ for the hydrolysis of arginine derivatives at pH 8.4 and 25° by thrombin

Values are given as means $\pm$ s.d., with the numbers of determinations in parentheses.

Substrate ... Detergent	$10^7 k_{3(\text{app.})}$ (mole.min. ⁻¹ enzyme unit ⁻¹)	
	(IIa; R = O·C ₂ H ₅ , n = 3)	(IIb; R = O·CH ₃ , n = 3)
None	3.246 $\pm$ 0.013 (15)	6.754 $\pm$ 0.431 (4)
Tween 20 (0.3%, w/v)	3.667 $\pm$ 0.096 (4)	9.422 $\pm$ 0.140 (3)
Tween 20 (1.2%, w/v)	—	11.03 $\pm$ 0.49 (3)
Tween 40 (0.3%, w/v)	—	9.219 $\pm$ 0.331 (4)
Tween 40 (1.2%, w/v)	3.505 $\pm$ 0.062 (3)	9.159 $\pm$ 0.097 (4)
Tween 80 (0.3%, w/v)	3.920 $\pm$ 0.116 (4)	11.60 $\pm$ 0.30 (5)

that the presence of detergent affects the degree of binding of solvent molecules by the enzyme. Finally, molecules of ionic detergents that are bound to the enzyme may shield ionic groups of the opposite sign in the enzyme.

Other potential modifiers of the kinetics of reactions catalysed by thrombin have been examined in the present work. The observation that the intravenous administration of 0.01–0.02 mg. of nicotine/kg. to rabbits induced a significant decrease in the time taken for blood to clot (Wenzel & Singh, 1962) prompted us to investigate the effect of the alkaloid on the esterase activity of thrombin. Concentrations of 1–100 mM-nicotine, however, did not alter $k_{3(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester by thrombin.

5-(3-Guanidinopropyl)-2-thiohydantoin (2 mM) and *N*-toluene-*p*-sulphonyl-L-glutamyl- γ -hydrazide α -methyl ester (2 mM), which, as shown above, are not substrates for thrombin, did not affect $k_{3(\text{app.})}$ or $K_{m(\text{app.})}$ for the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester by thrombin. In contrast, α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester, although not a substrate for thrombin, was found to inhibit markedly reactions catalysed by this enzyme. Since the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester was inhibited when the substrate concentration was less than about 0.5 mM but not at higher substrate concentrations, competitive inhibition was indicated. Lineweaver–Burk plots in the presence of 0.05–2.0 mM-inhibitor, however, were non-linear. A possible explanation could be provided if the enzyme preparation contained more than one esterase activity and if these were inhibited to different extents. Nevertheless, the results suggest that α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester is bound at the active site of thrombin but is not a substrate because it fails to acylate the enzyme. At a concentration of about 9 mM, α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester caused a precipitate to separate from solutions of thrombin. After centrifugation and washing, dialysis against 0.15 M-imidazole buffer, pH 7.0, redissolved some of the precipitate, and both this solution and the original supernatant contained esterase activity. The insoluble complex of thrombin and α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester dissolved in 5 M- α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and the latter was hydrolysed, suggesting that the substrate displaced the inhibitor from the active centre. α -*N*-Toluene-*p*-sulphonyl-L-homoarginine methyl ester strongly inhibited the clotting of fibrinogen by thrombin (Fig. 3). At equivalent concentrations, the homoarginine derivative was more effective than the cholate ion. In view of its behaviour in the presence of thrombin, α -*N*-toluene-*p*-sulphonyl-L-

homoarginine methyl ester may be a useful anti-coagulant and a novel reagent for the purification of thrombin.

SUMMARY

1. Three esters of α -*N*-benzoyl-L-arginine are hydrolysed at the same rate by bovine thrombin and it is concluded that deacylation of (α -*N*-benzoyl-L-arginyl)thrombin is rate-determining. Ionic strength does not influence $k_{3(\text{app.})}$.

2. Four esters of α -*N*-toluene-*p*-sulphonyl-L-arginine are hydrolysed at different rates by thrombin and it is concluded that acylation is partly rate-determining. Increase of ionic strength inhibits the reaction, but probably the acylation step only is affected. Thrombin, unlike trypsin, does not catalyse the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-homoarginine methyl ester.

3. α -*N*-Toluene-*p*-sulphonyl-L-lysine methyl ester is an excellent substrate for thrombin, but the ornithine analogue is only slowly hydrolysed and the kinetics are first-order with respect to substrate.

4. The variation of $k_{3(\text{app.})}$ with pH for α -*N*-benzoyl-L-arginine ethyl ester and α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester indicates that a group with $\text{p}K_{(\text{app.})} \sim 6.6$ must be dissociated for hydrolysis to proceed.

5. Cholate and glycocholate ions inhibit the hydrolysis of α -*N*-benzoyl-L-arginine ethyl ester and accelerate the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester and α -*N*-toluene-*p*-sulphonyl-L-lysine methyl ester. Possible mechanisms are discussed to account for these observations. For the hydrolysis of α -*N*-toluene-*p*-sulphonyl-L-ornithine methyl ester in the presence of cholate, the kinetics are of the Michaelis–Menten type. Except at very low enzyme concentrations, cholate inhibits the clotting of fibrinogen by thrombin.

6. Non-ionic detergents accelerate the esterase activity of thrombin.

7. α -*N*-Toluene-*p*-sulphonyl-L-homoarginine methyl ester is a potent inhibitor of the esterase and clotting activities of thrombin.

We are grateful to Imperial Chemical Industries Ltd. and The Chemical Society for grants for equipment and chemicals. This work was supported by the U.S. Department of the Army through its European Research Office and by a research grant (HE-07062) from the U.S. Public Health Service.

REFERENCES

- Baines, N. J., Baird, J. B. & Elmore, D. T. (1964). *Biochem. J.* **90**, 470.
- Bangham, A. D. (1961). *Nature, Lond.*, **192**, 1197.
- Barrass, B. C. & Elmore, D. T. (1957). *J. chem. Soc.* p. 4830.
- Bergmann, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.

- Billimoria, J. D., Curtis, R. G. & MacLagan, N. F. (1960). *Biochem. J.* **78**, 185.
- Curragh, E. F. & Elmore, D. T. (1962). *J. chem. Soc.* p. 2948.
- Curragh, E. F. & Elmore, D. T. (1963). *Biochem. J.* **86**, 9 p.
- Ebata, M. (1961). *J. Biochem., Tokyo*, **49**, 110.
- Ehrenpreis, S. & Scheraga, H. A. (1957). *J. biol. Chem.* **227**, 1043.
- Elmore, D. T., Kingston, A. E. & Shields, D. B. (1963). *J. chem. Soc.* p. 2070.
- Elmore, D. T., Ogle, J. R. & Toseland, P. A. (1956). *J. chem. Soc.* p. 192.
- Foster, R. J. (1961). *J. biol. Chem.* **236**, 2461.
- Garagnani, A. & Facchini, G. (1956). *Arch. Pat. Clin. med.* **33**, 203.
- Gutfreund, H. & Sturtevant, J. M. (1956a). *Proc. nat. Acad. Sci., Wash.*, **42**, 719.
- Gutfreund, H. & Sturtevant, J. M. (1956b). *Biochem. J.* **63**, 656.
- Jirgensons, B. (1961). *Arch. Biochem. Biophys.* **94**, 59.
- Jirgensons, B. (1962a). *Arch. Biochem. Biophys.* **96**, 321.
- Jirgensons, B. (1962b). *Makromol. Chem.* **56**, 138.
- Johnson, J. F. & Seegers, W. H. (1955). In *The Coagulation of Blood*, p. 121. Ed. by L. M. Tocantins. New York: Grune and Stratton Inc.
- Koshland, D. E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 78.
- Marciniak, E. & Seegers, W. H. (1962). *Canad. J. Biochem. Physiol.* **40**, 597.
- Mustard, J. F., Medway, W., Downie, H. G. & Rowsell, H. C. (1962). *Nature, Lond.*, **196**, 1063.
- Ronwin, E. (1959). *Biochim. biophys. Acta*, **33**, 326.
- Scheraga, H. A. & Ehrenpreis, S. (1958). *Symp. 4th int. Congr. Biochem., Vienna*, vol. 10, p. 212.
- Schwert, G. W. & Eisenberg, M. A. (1949). *J. biol. Chem.* **179**, 665.
- Sherry, S. & Troll, W. (1954). *J. biol. Chem.* **208**, 95.
- Trowbridge, C. G., Krehbiel, A. & Laskowski, M. (1963). *Biochemistry*, **2**, 843.
- Wenzel, D. G. & Singh, J. (1962). *J. pharm. Sci.* **51**, 875.

Biochem. J. (1964), **93**, 171

The Purification and Properties of Human Plasminogen

By NORMA ALKJAERSIG

Department of Medicine, Washington University School of Medicine, St Louis, Mo., U.S.A.

(Received 23 December 1963)

The purification of plasminogen has presented problems of special difficulty. Earlier procedures (Milstone, 1941; Remmert & Cohen, 1949; Fletcher, 1954) yielded material that was relatively stable and soluble at neutral pH, but of low specific activity. On the other hand, the acid-extraction method of Christensen & Smith (1950), which has also been used in the work of Kline (1953), Sgouris, Inman, McCall, Hyndman & Anderson (1960), Shulman (1961) and Kline & Fishman (1961), yields material of greater specific activity, but of very limited stability and solubility at neutral pH. These properties of the purified protein are unexpected, since plasminogen occurs in plasma in a form that is both stable and soluble at neutral pH. Consequently it would seem that the acid extraction of Cohn fraction III, which is the first step in the Christensen & Smith (1950) procedure, causes some alteration in the plasminogen molecule, which is reflected in a change of physical but probably not of biochemical properties.

Alkjaersig (1960) presented preliminary results on the purification of plasminogen by DEAE-cellulose chromatography without extreme, especially acid, pH changes. The product possessed approximately equivalent specific activity to that

prepared by acid-extraction procedures, was highly soluble in neutral pH buffers and was relatively stable under these conditions. Wallen & Bergström (1959, 1960) independently reported preliminary findings on a DEAE-cellulose-chromatographic method for plasminogen purification utilizing the solvent effects of lysine for plasminogen elution; several similar reports (Hagan, Ablondi & De Renzo, 1960; Davies & Englert, 1960; Wallen, 1962a, b; Derechin, 1962; Derechin, Johnson & Szuchet, 1962; Robbins & Summaria, 1963; Hink & McDonald, 1962) have recently appeared, but, though the preparations have been reported to be of comparable specific activity, only sparse and somewhat discrepant information has been made available on protein properties.

Since preliminary results suggested that protein-sedimentation parameters might be influenced by treatment with ϵ -aminohexanoic acid, two alternative purification procedures have been developed, one of which utilizes the selective solvation properties of ϵ -aminohexanoic acid and the other does not. The results obtained with these new preparations have confirmed earlier activation (Alkjaersig, Fletcher & Sherry, 1958a, b) and biophysical (Shulman, Alkjaersig & Sherry, 1958) studies with