BBA 65941

PLASMINOGEN–PLASMIN SYSTEM

V A STOICHIOMETRIC EQUILIBRIUM COMPLEX OF PLASMINOGEN AND A SYNTHETIC INHIBITOR*

YASUSHI ABIKO, MASAHIRO IWAMOTO AND MUNEHIRO TOMIKAWA
Central Research Laboratory, Daiichi Seiyaku Co., Ltd., Edogawa-ku, Tokyo (Japan)

(Received March 3rd, 1969)

SUMMARY

The interaction between plasminogen and *trans*-AMCHA (*trans*-4-aminomethylcyclohexanecarboxylic acid) was investigated using ultracentrifugal and gel filtration techniques *trans*-AMCHA was found to alter the sedimentation behavior of plasminogen with a critical concentration of about 0.1 mM at pH 7.5 without dissociation of the plasminogen molecule and to bind to plasminogen with a dissociation constant of 0.085 mM Such effects were specific for *trans*-AMCHA and also for ε-aminocaproic acid

From the comparison of these findings with the inhibitory actions of *trans*-AMCHA and ε-aminocaproic acid on the activation of plasminogen, it was concluded that these compounds inhibited the activation through the formation of a stoichiometric equilibrium complex with plasminogen, accompanying a conformational change of the plasminogen molecule

INTRODUCTION

There have been several reports published on the inhibitory actions of ω-amino acids, such as ε-aminocaproic acid[1], *p*-aminomethylbenzoic acid[2] and 4-aminomethylcyclohexanecarboxylic acid (AMCHA)[3,4] on the plasminogen–plasmin system They demonstrated that these ω-amino acids mainly inhibited the activation of plasminogen to plasmin and that these inhibitors little affected the caseinolytic activity of plasmin Recently, we[5,6] also investigated kinetically the mechanism of inhibition by ε-aminocaproic acid and two stereoisomers of AMCHA, *trans*- and *cis*-AMCHA, as well as the *n*-hexyl ester derivatives of these ω-amino acids which had been found as more potent

Abbreviations AMCHA, 4-aminomethylcyclohexanecarboxylic acid, *trans*-AMCHA-hexyl ester, *n*-hexyl *trans*-4-aminomethylcyclohexanecarboxylate

* Part IV Further kinetic studies on the inhibition of fibrinolysis by synthetic inhibitors (ref 6)

inhibitors than the parent acids by MURAMATSU *et al.*[7–9]. It was demonstrated that all these compounds were competitive inhibitors of plasminogen activation[5]; the acid-form inhibitors were weak noncompetitive inhibitors of the esterolytic and caseinolytic activities of plasmin[5], whereas the esters were strong competitive inhibitors of plasmin[5] (*cf* ref 10). However, the antifibrinolytic actions of the acids were comparable with that of the corresponding esters[5,6] (*cf.* ref 8) The difference in the inhibition patterns between the acids and the esters may suggest that two types of the inhibitors affect the activation of plasminogen through mechanisms different from each other, although they showed the same type of inhibition of competitive nature in graphical analyses

Our preliminary observations on the sedimentation behavior of plasminogen in the presence of the inhibitors indicated that *trans*-AMCHA affected the sedimentation coefficient of plasminogen, whereas the inactive isomer, *cis*-AMCHA, and *trans*-AMCHA-hexyl ester did not This finding has led to the assumption that *trans*-AMCHA, and probably also ε-aminocaproic acid, inhibit the activation by binding to plasminogen. This report demonstrates evidence supporting this assumption Kinetic considerations on the mechanism of inhibition of plasminogen activation by *trans*-AMCHA or ε-aminocaproic acid are also presented here

MATERIALS AND METHODS

Plasminogen was purified from the human euglobulin fraction using Sephadex G-200 gel filtration and DEAE-Sephadex A-50 chromatography as previously described[11] The preparation was homogeneous in the ultracentrifugal and immunochemical criteria

Synthetic inhibitors, *trans*-AMCHA[12], *cis*-AMCHA[12], ε-aminocaproic acid and *trans*-AMCHA-hexyl ester were the preparations of our laboratory.

Ultracentrifuge experiments were kindly performed in Faculty of Pharmaceutical Sciences, University of Tokyo Sedimentation velocity measurements were made with a Spinco model E analytical centrifuge at 59 780 rev /min in 0.02 M sodium phosphate–0 1 M NaCl (pH 7 5) and in 0 025 M Tris–HCl–0 1 M NaCl (pH 8 5) at 10–11° with 0 45% and 0 48% protein solutions, respectively

The binding of an inhibitor to plasminogen was measured using the gel filtration technique introduced by HUMMEL AND DREYER[13] A column of Sephadex G-25 (1 33 cm × 36 cm) was equilibrated with 0 1 M NaCl–0 02 M sodium phosphate buffer (pH 7 5) containing an inhibitor under study Lyophilyzed plasminogen (approx 7 mg protein) was dissolved with 0 6 ml of the same buffer, and 0 5 ml of the solution was passed through the column using the same buffer as an eluant at a flow rate of about 7.5 ml/h Fractions of 1 ml each were collected

Protein was determined by the method of LOWRY *et al* [14] 20 μl of the effluent were used for the determination The inhibitor concentration in the effluent was determined using the trinitrophenylation method of SATAKE *et al* [15] with some modifications The actual procedures were as follows For the determination of the acid-form inhibitors, 0 5 ml of the eluate was mixed with 0 5 ml of water, 1 ml of 4% sodium bicarbonate solution and 1 ml of 0 1% sodium trinitrobenzene sulfonate in methanol The mixture was allowed to stand at room temperature in the dark for 20 min Then, 1 ml of 1 M HCl

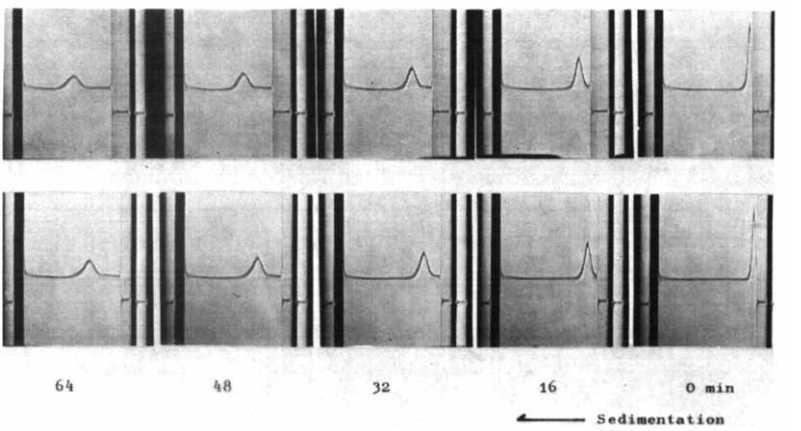

Fig 1 Ultracentrifugal patterns of human plasminogen The ultracentrifuge experiments were performed at pH 8 5 and 10–11° with 0 48% protein solution in the absence (upper) and the presence (lower) of 1 mM *trans*-AMCHA Photographs were taken at 16-min intervals after a maximum speed of 59 780 rev /min was reached

was added to the mixture, and the supernatant solution was read at 340 mμ For the determination of *trans*-AMCHA-hexyl ester, 0 5 ml of the eluate was mixed with 0 5 ml of 4% sodium bicarbonate and 0 5 ml of 0 1% sodium trinitrobenzene sulfonate in methanol After standing in the dark for 20 min, 0 5 ml of 1 M HCl and 2 ml of methanol were added to the mixture The colorimetry was performed at 340 mμ as above

RESULTS

Effects of inhibitors on the sedimentation behavior of human plasminogen

The sedimentation coefficient of plasminogen was found to be 6 23 *S* in the phosphate buffer (pH 7 5) and 6 19 *S* in the Tris buffer (pH 8 5) In the presence of 1 mM *trans*-AMCHA, however, $s_{20,w}$ of plasminogen decreased to 4 58 and 5 27 *S* at pH 7 5 and 8 5, respectively (Table I) Plasminogen behaved as a single component in

TABLE I

EFFECTS OF SYNTHETIC INHIBITORS ON THE SEDIMENTATION BEHAVIOR OF HUMAN PLASMINOGEN

Measurements of the sedimentation coefficient of plasminogen were carried out in the absence and presence of various concentrations of *trans*-AMCHA, *cis*-AMCHA and *trans*-AMCHA-hexyl ester with 0 45% and 0 48% protein solution at pH 7 5 and pH 8 5, respectively

Inhibitor	*Concn (mM)*	$s_{20,w}$ *(S)*	
		pH 7 5	*pH 8 5*
None	—	6 23	6 19
trans-AMCHA	0 01	6 05	—
	0 05	5 82	—
	0 1	5 32	—
	0 5	4 57	—
	1	4 58	5 27
cis-AMCHA	1	5 92	6 10
trans-AMCHA-hexyl ester	1	5 90	6 18

the absence and presence of *trans*-AMCHA (Fig 1) On the other hand, such alteration in the sedimentation behavior of plasminogen was not observable with the inactive isomer, *cis*-AMCHA, or with *trans*-AMCHA-hexyl ester (Table I) which was a strong inhibitor of the same competitive type in plasminogen activation as *trans*-AMCHA[5] These findings suggested that *trans*-AMCHA might specifically interact with the plasminogen molecule As shown in Table I, the effect of *trans*-AMCHA on $s_{20,w}$ of plasminogen was dependent on its concentration At the concentrations below 0 05 mM, *trans*-AMCHA did not alter the $s_{20,w}$, and the critical concentration seemed to be about 0 1 mM

Sephadex G-150 gel filtration of plasminogen indicated that the relative elution volume was slightly decreased when 10^{-3} M *trans*-AMCHA was present (V_e/V_0 = 1 78 in the absence of *trans*-AMCHA and V_e/V_0 = 1 67 in the presence of *trans*-AMCHA) This revealed that *trans*-AMCHA did not cause a dissociation of the plasminogen molecule

Binding of trans-AMCHA by human plasminogen

The result of a typical experiment demonstrating the binding of *trans*-AMCHA to plasminogen is shown in Fig 2 The amount of the inhibitor bound by plasminogen was calculated from the area of the trough in the elution diagram because the peak area in the diagram was the sum of the area due to the bound inhibitor and of that due to trinitrophenylated protein and because this was further complicated by partial acid precipitation of the trinitrophenylated protein Fig 3 shows a double reciprocal plot of the concentration of *trans*-AMCHA used and the amount of the inhibitor bound to plasminogen The dissociation constant determined from the plot was $8\,5 \cdot 10^{-5}$ M (pH 7 5) The intercept on the ordinate indicates maximum binding of 1 mole of *trans*-AMCHA per about 95 000 g of plasminogen

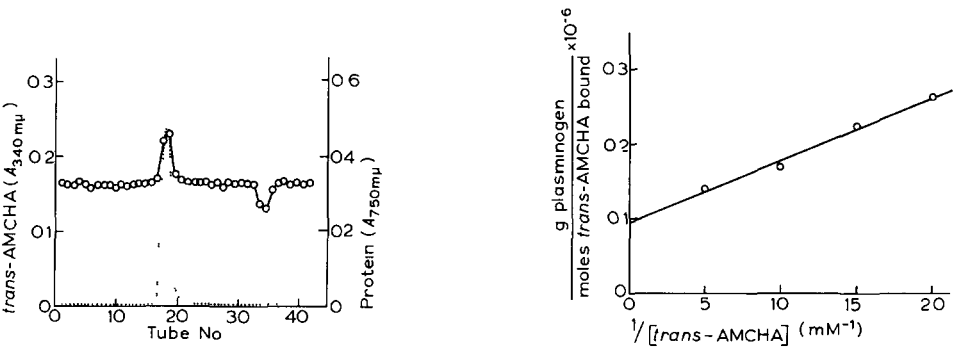

Fig 2 Elution pattern of Sephadex G-25 column (1 33 cm × 36 cm) illustrating the binding of *trans*-AMCHA to plasminogen The column was equilibrated and eluted with 0 1 M NaCl–0 02 M sodium phosphate buffer (pH 7 5), containing 0 1 mM *trans*-AMCHA Plasminogen (7 1 mg) was dissolved in 0 6 ml of the same buffer and 0 5 ml of the solution was used for the gel filtration Fractions of 1 ml were collected at a flow rate of about 7 5 ml/h *trans*-AMCHA and protein in the eluate were determined by the trinitrophenylation method of SATAKE *et al* [15] and the method of LOWRY *et al* [14], respectively ○—○, *trans*-AMCHA, , protein

Fig 3 Reciprocal plot for the binding of *trans*-AMCHA to plasminogen The binding of *trans*-AMCHA to plasminogen was measured by the gel filtration technique as shown in Fig 2 Amount of plasminogen was 6 9 mg, concentration of *trans*-AMCH varied from 0 05 to 0 2 mM Other conditions are described in Fig 2

ε-Aminocaproic acid was also found to bind to plasminogen with a dissociation constant of 0 55 mM, using the same gel filtration technique in which 10 mg of plasminogen and 0 2 mM ε-aminocaproic acid were used The value was obtained from a single experiment according to the following equation[15] on the assumption that the binding is mole to mole

$$K_I = [\text{free } I] \times \frac{[\text{free P}]}{[\text{bound P}]}$$

where K_I is the dissociation constant, I is the inhibitor and P is plasminogen The concentration of free inhibitor was known from the base line absorbance at 340 mμ, and the concentration of bound plasminogen corresponded to the area of the trough, thus the concentration of free plasminogen was obtained as the difference between total and bound plasminogen

On the other hand, *cis*-AMCHA and *trans*-AMCHA-hexyl ester did not bind to plasminogen at the concentration of 0 1 mM Furthermore, no binding was observed with a combination of bovine serum albumin and *trans*-AMCHA

DISCUSSION

ε-Aminocaproic acid and AMCHA were reported to inhibit the activation of plasminogen competitively with plasminogen[1,3,4] Recently, *n*-hexyl ε-aminocaproate and *trans*-AMCHA-hexyl ester were introduced as potent competitive inhibitors of plasmin by MURAMATSU *et al* [7,8,17] Kinetic studies in our laboratory[5] indicated that these hexylester derivatives of ε-aminocaproic acid and *trans*-AMCHA were also competitive inhibitors of the activation of plasminogen, like their parent acids, and that *cis*-AMCHA had little action as compared with the *trans*-isomer The K_i values for these inhibitors in plasminogen activation were 0 96 mM for ε-aminocaproic acid, 0 186 mM for *trans*-AMCHA, 5 mM for *cis*-AMCHA, 0 038 mM for *n*-hexyl ε-aminocaproate and 0 037 mM for *trans*-AMCHA-hexyl ester[5] ε-Aminocaproic acid and *trans*-AMCHA had little effect on the esterolytic and caseinolytic activities of plasmin, whereas the corresponding hexylester derivatives strongly inhibited these plasmin activities[5] (*cf* refs 7, 17) However, ε-aminocaproic acid and *trans*-AMCHA markedly inhibited the fibrinolytic activity of plasmin at almost the same rates as the inhibitors in the esterform[5,6] (*cf* refs 8, 18) The inhibition constants in the plasmin-catalyzed fibrinolysis were 0 24 mM for ε-aminocaproic acid, 0 036 mM for *trans*-AMCHA, 0 053 mM for *n*-hexyl ε-aminocaproate and 0 078 mM for *trans*-AMCHA-hexyl ester[6]

Such a difference in the spectra of inhibition between the acid- and ester-form inhibitors suggests that the mechanism of inhibition of plasminogen activation by ε-aminocaproic acid and *trans*-AMCHA is essentially different from that by their hexyl esters, although they all showed apparently the same type of inhibition of competitive nature in the Lineweaver–Burk plots[5]

As presented in this report, *trans*-AMCHA did markedly decrease the sedimentation coefficient of plasminogen with a critical concentration of about 0 1 mM (Table I) On the other hand, the inactive isomer, *cis*-AMCHA, and *trans*-AMCHA-hexyl ester at the concentration of 1 mM did not alter the sedimentation behavior of plasminogen A similar phenomenon had been observed with ε-aminocaproic acid by ALKJAERSIG[19] who reported that ε-aminocaproic acid decreased $s_{20,w}$ of plasminogen

from 5 0 to 4 3 S at pH 7 6 at the concentration of 1 mM, although the effect of ε-aminocaproic acid was not observable at concentrations below 0 1 mM. Sephadex G-150 gel filtration of plasminogen indicated that *trans*-AMCHA did not cause dissociation of the plasminogen molecule These facts might suggest that *trans*-AMCHA and ε-aminocaproic acid cause a conformational change (probably partial unfolding) of the plasminogen molecule On comparison of the critical concentrations of *trans*-AMCHA and ε-aminocaproic acid with the inhibition constants for these inhibitors in plasminogen activation (Table II), it seems that the interaction of *trans*-AMCHA or ε-aminocaproic acid with plasminogen may result in inhibition of the activation of plasminogen, as postulated by ALKJAERSIG[19] This was further supported by the binding experiments using the gel filtration technique (Fig 2) *trans*-AMCHA and ε-aminocaproic acid were found to bind to plasminogen with the dissociation constants of 0 085 mM and 0.55 mM, respectively, and with a maximum binding of 1 mole/95 000 g of plasminogen in the case of *trans*-AMCHA (Fig 3) *cis*-AMCHA and *trans*-AMCHA-hexyl ester were inactive for the binding, and *trans*-AMCHA did not interact with bovine serum albumin Since the molecular weight of plasminogen is known to be about 90 000 (ref 20), these findings indicate the existence of a single binding site on plasminogen that is highly specific for *trans*-AMCHA and ε-aminocaproic acid and again strongly suggest a close relation between the interaction of these inhibitors with plasminogen and their inhibitory actions (Table II) From these results, it may be concluded that *trans*-AMCHA and ε-aminocaproic acid inhibit the activation of plasminogen through the formation of an inhibitor–plasminogen complex, probably accompanying a conformational change of the plasminogen molecule *N*-Acetyl-3,5-dibromotyrosine was reported to inhibit the trypsin-catalyzed activation of chymotrypsinogen in a similar manner by forming a stoichiometric equilibrium complex with the zymogen[21] On the other hand, *trans*-AMCHA-hexyl ester and *n*-hexyl ε-aminocaproate may inhibit the activation of plasminogen through the formation of an usual inhibitor–enzyme (activator) complex

From this conclusion the question arises whether an inhibitor which binds to its substrate behaves as a competitive inhibitor in the Lineweaver–Burk plot The following considerations might answer this question

TABLE II

RELATIONSHIP BETWEEN THE INHIBITORY EFFECTS ON PLASMINOGEN ACTIVATION, THE EFFECTS ON THE SEDIMENTATION BEHAVIOR OF PLASMINOGEN AND THE BINDING TO PLASMINOGEN OF THE SYNTHETIC INHIBITORS

Inhibitor	*Inhibition constant in plasminogen activation** K_i *(mM)*	*Critical concn for alteration of* $s_{20,w}$ *of plasminogen (mM)*	*Dissociation constant for binding to plasminogen* K_I *(mM)*
ε-Aminocaproic acid	0 96	0 1–1**	0 55
cis-AMCHA	5	No effect	No binding
trans-AMCHA	0 19	approx 0 1	0 085
trans-AMCHA-hexyl ester	0 037	No effect	No binding

* Data from IWAMOTO *et al* [5]
** Data from ALKJAERSIG[19]

The activation of plasminogen proceeds in the Michaelis–Menten type reaction[1,3–5] Thus,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P, \quad \frac{[E][S]}{[ES]} = \frac{k_2 + k_3}{k_1} = K_m$$

where E is an activator, S is plasminogen and P is plasmin When total activator is $\bar{E}$,

$$v = k_3[ES] = \frac{k_3[\bar{E}][S]}{[S] + K_m} = \frac{v_{max}[S]}{[S] + K_m} \tag{1}$$

Since *trans*-AMCHA or ε-aminocaproic acid (I) forms a stoichiometric equilibrium complex with plasminogen, as demonstrated above, then

$$I + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} IS, \quad \frac{[I][S]}{[IS]} = \frac{k_5}{k_4} = K_I$$

where K_I is the dissociation constant On the other hand, if these inhibitors to a lesser extent inhibited the esterolytic activity of an activator such as a complex of streptokinase and plasminogen, as demonstrated by ROBERTS AND BURKAT[23] with ε-aminocaproic acid, it could be possible to neglect the interaction between the inhibitors and the activator at the concentrations under study (1–0 1 mM)

When $\bar{S}$ is total plasminogen, then

$$[\bar{S}] = [S] + [IS] + [ES] = [S] + \frac{[I][S]}{K_I} + [ES] = [S]\left\{1 + \frac{[I]}{K_I}\right\} + [ES]$$

If $\bar{S} \gg \bar{E}$, then

$$[S] = \frac{[\bar{S}] - [ES]}{1 + [I]/K_I} = \frac{[\bar{S}]}{1 + [I]/K_I} \tag{2}$$

Introduction of Eqn 2 into Eqn 1 leads to

$$v = \frac{\dfrac{v_{max}[\bar{S}]}{1 + [I]/K_I}}{\dfrac{[\bar{S}]}{1 + [I]/K_I} + K_m} = \frac{v_{max}[\bar{S}]}{[\bar{S}] + K_m\{1 + [I]/K_I\}}$$

which can be rewritten as

$$\frac{1}{v} = \frac{K_m}{v_{max}}\left\{1 + \frac{[I]}{K_I}\right\}\frac{1}{[\bar{S}]} + \frac{1}{v_{max}} \tag{3}$$

This is the Lineweaver–Burk equation for the inhibition of competitive type, and it is indicated that the dissociation constant obtained from the binding experiments corresponds to the inhibition constant in the kinetic experiments for inhibition However, for confirmation the above considerations require further investigations on their applicability to other activators such as urokinase and tissue activator(s) because urokinase was reported to be affected to some extent by ε-aminocaproic acid and *trans*-AMCHA[22]

The similar mechanism might be expected for the inhibition of the activation of trypsinogen by these ω-amino acids because the autocatalytic or enterokinase-activation of trypsinogen was reported to be inhibited by these ω-amino acids[2,3,24–26], which little affected the proteolytic activity of trypsin[1,3].

ACKNOWLEDGMENTS

The authors wish to thank Dr M Shimizu, Director of this Laboratory, for his valuable advice Thanks are also due to Dr T Ishiguro, President of this company, for his encouragement

REFERENCES

1 N Alkjaersig, A P Fletcher and S Sherry, *J Biol Chem*, 234 (1959) 832
2 F Markwardt, H Landmann and A Hoffmann, *Z Physiol Chem*, 340 (1965) 174
3 A H C Dubber, G D McNicol and A S Douglas, *Brit J Haematol*, 11 (1965) 237.
4 M Maki and F K Beller, *Thromb Diath Haemorrhag*, 16 (1966) 668
5 M Iwamoto, Y Abiko and M Shimizu, *J Biochem Tokyo*, 64 (1968) 759
6 M Iwamoto and Y Abiko, *J Biochem Tokyo*, 65 (1969) 821
7 M Muramatsu, T Onishi, S Makino, S Fujii and Y Yamamura, *J Biochem Tokyo*, 57 (1965) 402
8 M Muramatsu, T Onishi, S Makino, S Fujii and Y Yamamura, *J Biochem Tokyo*, 57 (1965) 450
9 M Muramatsu, Y Hayakumo and S Fujii, *J Biochem Tokyo*, 62 (1967) 408.
10 T Onishi, *Shikoku Acta Med*, 23 (1967) 52
11 Y Abiko, M Iwamoto and M Shimizu, *J Biochem Tokyo*, 64 (1968) 743
12 T Naito, A Okano, S Kadoya, T Miki, M Inaoka, R Moroi and M Shimizu, *Chem. Pharm Bull Tokyo*, 16 (1968) 728
13 J P Hummel and W J Dreyer, *Biochim Biophys Acta*, 63 (1962) 530
14 O H Lowry, N J Rosebrough, A L Farr and R J Randall, *J Biol Chem*, 193 (1951) 265
15 K Satake, T Okuyama, M Ohashi and T Shinoda, *J Biochem Tokyo*, 47 (1960) 654
16 P Cuatrecasas, S Fuchs and C B Anfinsen, *J Biol Chem*, 242 (1967) 3063
17 M Muramatsu and S Fujii, *J Biochem Tokyo*, 65 (1969) 17
18 H Lukasiewicz, S Niewiarowski, K Worowski and B Lipinski, *Biochim Biophys Acta*, 159 (1968) 503
19 N Alkjaersig, *Biochem J*, 93 (1964) 171
20 K C Robbins, L Summaria, D Elwyn and G H Barlow, *J Biol Chem*, 240 (1965) 541.
21 F J Kezdy, *Biochim Biophys Acta*, 132 (1967) 197
22 P L Walton, *Biochim Biophys Acta*, 132 (1967) 104
23 P S Roberts and R K Burkat, *Biochim Biophys Acta*, 118 (1966) 592
24 H -J Trettin and H Mix, *Z Physiol Chem*, 340 (1965) 24
25 M Von Gulzow, H Mix and H -J Trettin, *Z Physiol Chem*, 348 (1967) 285
26 H Von Landmann and F Markwardt, *Z Physiol Chem*, 348 (1967) 745