Table I. The effect of histones and protamine sulphate on fibrinolysis and proteolysis of fibrin by plasmin

Histones, protamine (final concentration mg/ml)	Fibrinolysis time (min)		Proteolysis (tyrosine μg/ml)	
	Histones	Protamine	Histones	Protamine
2.5	120.0	26.0	25.0	24.0
2.0	75.0	26.0	25.0	23.0
1.5	55.0	20.0	24.0	23.2
1.0	35.0	14.0	23.0	24.0
0.5	18.0	8.0	23.5	22.0
0.25	6.0	8.0	22.0	22.0
0.125	5.0	5.0	21.9	22.0
0.0	5.0		23.0	

Fibrinolysis test system: 0.2 ml of histones or protamine + 0.2 ml of plasmin 0.1% + 0.2 ml of FSF-free fibrinogen[9] 0.35% + 0.2 ml of thrombin 10 U/ml. Proteolysis test system: 0.5 ml of plasmin 0.1% + 0.5 ml of histones or protamine + 0.5 ml of FSF-free fibrinogen 0.35% + 0.5 ml of thrombin 10 U/ml. After 15 min incubation at 37°C, 1 ml of TCA was added and the amount of TCA-soluble tyrosine in the supernatant was estimated[10].

Table II. Built-up histones and protamine sulphate to the structure thrombin-clotted fibrin evaluated with arginine and tyrosine

Histones, protamine (final concentration mg/ml)	Arginine (μg/ml)		Tyrosine (μg/ml)	
	Histones	Protamine	Histones	Protamine
2.5	333.0	390.0	37.0	30.0
2.0	315.0	372.0	35.5	29.5
1.5	298.0	361.0	32.9	30.0
1.0	292.0	329.0	31.0	29.8
0.5	281.0	293.0	30.5	28.5
0.25	262.0	285.0	29.8	29.0
0.125	241.0	250.0	28.4	28.0
0.0	240.0		29.0	

Test system: 1 ml of FSF-free fibrinogen 0.33% + 1 ml histones or protamine + 2 ml of thrombin 5 U/ml was incubated at 37°C for 30 min. The clot after separation was solubilized by heating in 2 ml of 2 *N* NaOH at 100°C for 5 min and amount of arginine[11] and tyrosine was estimated.

amine, whereas the increase in tyrosine was observed only when histones were added (Table II). The increase of arginine was closely related to the concentration of these proteins and was higher when protamine was added. This can be explained by the fact that protamine contains more arginine than histones. When only protamine was added, no increase in tyrosine was found because protamine does not contain this aminoacid.

The results of the present report have shown that though histones and protamine exhibit high antifibrinolytic properties, they do not inhibit the proteolytic activity of plasmin. The antifibrinolytic action of histones and protamine seems to be brought about by building-up these proteins into the fibrin clot and by modification of its structure making the clot resistant to the fibrinolytic action of plasmin.

Recently it has been suggested that basic proteins which are widely distributed in mammalian tissues, and which might be released into circulation, may be an important factor contributing to the formation and deposition of fibrin-like material in clinical conditions[7]. It may be connected with the destruction of tissues from the disintegrated tumor cells, necrotic cells, damage of granulocytes and intravascular formation of platelet aggregates[1,3,8]. In such cases, basic proteins by building-up into the structure of clot, exhibit the antifibrinolytic properties.

Zusammenfassung. In-vitro-Versuche über basische Eiweisskörper zeigen, dass Protamine und Histone in die Struktur des unter Thrombin-Einfluss entstehenden Fibrins eingebaut werden. Einbau basischer Proteine führt zur Änderung der Fibrinstruktur und zur Widerstandserhöhung auf die Plasminwirkung. Es scheint, dass die basischen Proteine die fibrinolytische Aktivität des Plasmins spezifisch hemmen.

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The Effect of Antamanide and Perhydroantamanide on the Permeability of Model and Biological Membranes

The cyclic decapeptide antamanide from *Amanita phalloides*[1] is attracting considerable attention as an inhibitor of phalloidine and amanitins (the toxic principles of the toadstool *Amanita phalloides*) and as a K^+ and Na^+ complexone with marked preference for Na^+[2]. This complexing peculiarity of antamanide indicated its possible use as selective promoter of Na^+-flow through membranes, an application of considerable interest as no substances have as yet been found that could serve as such an instrument in membrane study. Accordingly we investigated the action of antamanide on a variety of model membranes, monolayers, bilayers, liposomes and on mitochondria and also determined its antimicrobial activity. A similar study was also carried out of perhydroantamanide, a compound obtained from antamanide on hydrogenation over an Adams catalyst[3] so that it possessed L-α-cyclohexyl-

alanyl instead of L-phenylalanyl residues. The complexing properties of perhydroantamanide proved to be very similar to those of antamanide (according to a personal communication by TH. WIELAND, perhydroantamanide does not inhibit toxins from *Amantia phalloides*), whereas its solubility in non-polar organic solvents is somewhat higher[3].

Experiments on bilayer phospholipid membranes, however, showed no marked changes in their conductivity on addition of either antamanide or perhydroantamanide. For instance, the membrane resistance in 0.1 M NaCl or 0.1 M KCl containing 10^{-7}–10^{-6} M antamanide or perhydroantamanide was practically the same as that measured in solutions not containing the decapeptide ($R_m = 1 \times 10^8$ ohm $\times$ cm^2 for membranes from ox brain lipids and 1×10^7 ohm $\times$ cm^2 for membranes from egg lecithin).

We next compared the penetration of antamanide and perhydroantamanide into lipid monolayers and studied their effect on the cation permeability of liposomes. On addition of antamanide to the underlying solution, the surface pressure of the lecithin monolayer changed but slightly, and antamanide did not penetrate the monolayers at pressures above 20 dyne/cm. Addition of perhydroantamanide, on the contrary, resulted in a significant increase of $\Delta\pi$ (Figure 1), showing that it penetrates the monolayers far more easily than the parent decapeptide. With respect to liposomes, Table I clearly shows that antamanide does not increase their cation permeability, not only in the presence of chloride but even in the presence of nitrate ions, although the latter are known as promoters of cation transport through membranes treated with valinomycin[4]. On the other hand, the addition of perhydroantamanide produced a marked increase in liposome permeability, particularly in the presence of nitrate ions. In should be noted, however, that no difference is observed between the K^+ and Na^+ leakage from the 'nitrate' liposomes. Such loss of K^+/Na^+ discrimination might be due to the specific properties of the nitrate ions, since a similar decrease in discriminating ability has been also found with the valinomycin-treated liposomes[4].

From the above, it follows that, although antamanide selectively binds Na^+ ions, it exhibits no valinomycin type of membranal aclivity[5]. The more lipophilic perhydroantamanide, however, exerts a pronounced if not selective effect on the cation permeability of artificial membranes.

Further to assess the membrane activity of antamanide and its hydrogenated analogue, we studied their behaviour in a series of standard bio-assays used for macrocyclic complexones of alkali metals. It was shown that antamanide affects although weakly K^+-ion transport in the rat liver mitochondria. At 10^{-6} M the induced K^+ ion flux was about 5% of that obtained with valinomycin at 10^{-7} M, the effect of perhydroantamanide was twice as strong as that of antamanide (Figure 2). It is noteworthy that both cyclic decapeptides were found to be strong decouplers of oxidative phosphorylation in mitochondria, considerably enhancing the oxygen uptake at concentration 10^{-6} M. Antimicrobial tests revealed no activity in

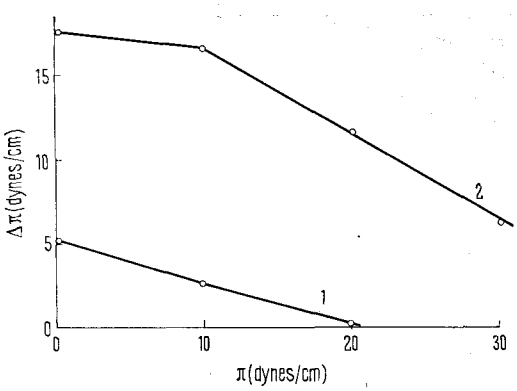

Fig. 1. Penetration of antamanide and perhydroantamanide into egg lecithin monolayers. π, initial surface pressure; $\Delta\pi$, surface pressure increase after addition of an ethanolic solution of the decapeptide (0.02 ml, $10^{-4}M$); 1. Antamanide; 2. Perhydroantamanide. Cell surface area equals 200 cm^2; cell volume 250 cm^3.

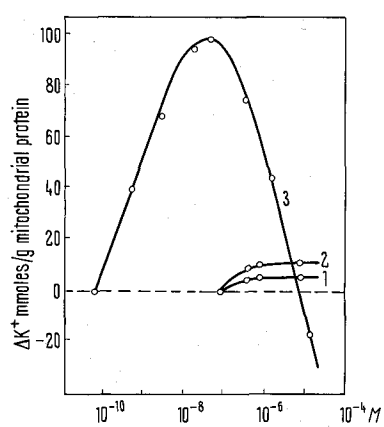

Fig. 2. The effect of antamanide (1), perhydroantamanide (2) and valinomycin (3) on K^+-ion transport in rat liver mitochondria. Incubation medium: 250 mmole sucrose, 8 mmole *tris*-HCl, K^+ 0,5 · $10^{-3}M$, Na^+ 0,15 · $10^{-3}M$, pH 7.0, –27 °C, 5 mg mitochondrial protein per ml (oxidative phosphorylation was measured at K^+ 2 · $10^{-2}M$).

Table I. Leakage of Na^+ and K^+ from lecithin liposomes in the presence of antamanide and perhydroantamanide[a]

Salt	Leakage of cations, as % of initial salt content, after 10 min	
	Antamanide	Perhydroantamanide
NaCl	<1	<1
KCl	<1	3
$NaNO_3$	<1	18
KNO_3	<1	14

[a] Liposomes were prepared by conventional technique[4] in 0.15M salt solutions. Na^+ and K^+ leakage was measured with cation sensitive glass electrodes. Concentrations of cyclic depsipeptide in the cell was $5 \times 10^{-5}M$/l.

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Table II. Antimicrobial activity of antamanide and perhydroantamanide

Compound	Minimal growth inhibiting concentration (γ/ml)							
	Staph. aureus 209P	*Staph. aureus* UV-3	*Strept. faecalis*	*Sarcina lutea*	*B. subtilis*	*C. albicans*	*Mycob. phlei*	*E. coli*
Antamanide	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
Perhydroantamanide	> 50	18	6–9	> 50	> 50	> 50	> 50	> 50

antamanide while perhydroantamanide was found to be active against some gram-positive strains (Table II).

The above results indicate that the biological action of antamanide is probably not connected with increase in cation transport through membranes. As for perhydroantamanide, its comparatively high lipophilic properties and marked effect on the transmembranal transport provides a clue for the preparation of new antamanide analogues for membrane studies. The rational search for such analogues has been greatly facilitated now that the three dimensional structure of the antamanide-sodium complex has been proposed[6].

Zusammenfassung. Untersuchungen mit Antamanid und Perhydroantamanid deuten darauf hin, dass für letzteres die Beeinflussung des Transportes von Na^+- und K^+-Ionen durch Membranen von Bedeutung sein kann.

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Altersbedingte Abnahme von Kreatinphosphat und Änderungen der Adeninnukleotide in der Skelettmuskulatur von Ratten

In früheren Untersuchungen konnte gezeigt werden, dass mit zunehmendem Alter der Gehalt an Kreatinphosphat (KP) in der Skelettmuskulatur von Ratten stark absinkt[1], ohne dass sich dabei die Menge des Gesamtkreatins ändert. Die Aktivität der Kreatinphosphokinase (E.C. 2.7.3.2.) blieb unverändert[2,3]. Diese Befunde deuten darauf hin, dass die Reaktion Kreatin + ATP ⇢ KP + ADP (Lohmann[4]) im Alter gestört ist. Da die Störung nicht auf eine Abnahme der Kreatinphosphokinase zurückgeführt werden kann, haben wir in Muskeln von Ratten verschiedenen Alters ausser KP auch die Adeninnucleotide Adenosinmono-, -di- und -triphosphat (AMP, ADP, ATP) bestimmt.

Methoden. Junge (5–6 Monate) und alte (20–30 Monate) Wistar-Ratten der Alterszucht des Institutes wurden verwendet. Da keine Geschlechtsunterschiede im KP- und Nukleotidgehalt der Muskulatur gefunden wurden, benützten wir männliche und weibliche Tiere zusammen. Die Ratten wurden mit Nembutal (i.p. 2.5 mg/100 g Körpergewicht) narkotisiert und meist nach 40–45 Min. durch Herzschnitt und Ausbluten getötet (abweichende Zeitdauer der Narkose s. Tabelle II).

Die weissen Muskeln (M. rectus femoris, M. glutaeus maximus, peripherer Teil[5]) wurden sogleich in Aethanol-Trockeneis gefroren, gewogen und anschliessend in Perchlorsäure (PCA) während 30 Sek. homogenisiert (Ultraturax). Die Suspension wurde 10 Min. bei $3000 \times g$ zentrifugiert und der Überstand mit 5*N* KOH auf pH 7.5 gebracht. Der $KClO_4$-Niederschlag wurde während 5 Min. bei $3000 \times g$ abzentrifugiert.

Die Kreatin- und KP-Bestimmung erfolgte nach den Methoden von Eggleton et al.[5], Ennor und Stocken[6] und Ennor[7]. Dabei wird als «totales Kreatin» die Summe des freien plus des im KP vorliegenden Kreatins bezeichnet. Im PCA-Extrakt wird das freie und, nach Säurehydrolyse, das totale Kreatin bestimmt; durch Subtraktion errechnet sich der Anteil des als KP vorliegenden Kreatins. Bei der Berechnung des als KP vorliegenden Kreatins in Prozenten des totalen Kreatins müssen natürlich die unterschiedlichen Molekulargewichte von Kreatin und KP berücksichtigt werden, falls Kreatin und KP, hier in mg pro g Muskel ausgedrückt, direkt miteinander verglichen werden (Tabelle I). Die KP-Werte in Tabelle I müssen deshalb immer zuerst durch 1.62 dividiert werden, will man sie mit den Werten für das totale Kreatin in Beziehung bringen.

Die Bestimmung der Adeninnukleotide wurde nach Säulenchromatographie entsprechend der Methode von

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