such diverse dust treatment, with dust-cell ratio capable of affecting macrophage respiration and releasing enzymes from lysosomal granules and the cell sap[7]. By rising this ratio, the $^{86}Rb^+$ efflux from silica-treated cells might increase significantly. However, severe toxic effects are produced by silica in this condition, with extracellular release of mitochondrial and microsomal enzymes[22] possibly indicating complete cell disruption. Nevertheless, the $^{86}Rb^+$ efflux from macrophages might also be quantitatively different from that by K^+.

Present results suggest that silica does not cause alterations in plasma membrane permeability and ion shifts not mediated by failure of the metabolic activity of cells. Release of lysosomal enzymes from silica-treated macrophages[7] might be due to lytic actions on the membranes of intracellular vacuoles[6]. However, phagocytosis in vitro occurs in 2 discrete stages[23], the first or attachment phase not requiring serum or cations for its accomplishment, the subsequent or ingestion phase requiring serum and being chiefly inhibited by the absence of Ca^{2+}. When silica is incubated in Ca^{2+}-free Ringer, lysosomal enzymes are released by cells[22] in rates similar to those found with normal Ringer[7]. This possibly poses some questions on the in vitro mechanism of silica action on phagocytes. During incubation in normal Ringer not supplemented with serum or serum factors, as usually done in this laboratory, attachment of dust particles on the cell surface occurs readily[7]. However, it is very probable that no significant true ingestion takes place in this condition, as is suggested also by lack[7,12] of the modifications of cell metabolism usually implied by phagocytosis[24]. Still more difficult dust ingestion is to be expected when a Ca^{2+} free medium is used. It should be supposed that penetration of (mono-)silicic acid into the cell[25] from the attached dusts might occur, with resultant intracellular slight polymerization and eventual inhibition of the cytochrome oxidase or succinic oxidase systems[26,27]. This might cause loss of lysosomal enzymes from cells, as occurs also when metabolic inhibitors are used[22].

Recent in vivo experiments suggest that the cytotoxic action of fibrogenic silica might primarily be due to an influence on the cytochrome oxidase system of the cells[28]. This should agree with the present suggestions and with those put forward years ago by JAMES and MARKS[29] in in vitro experiments[30].

Riassunto. Viene studiata la possibilità che precoci modificazioni della permeabilità cellulare si producano nei fagociti trattati sperimentalmente con polveri silicee. A tale scopo vengono compiute determinazioni dell'efflusso di $^{86}Rb^+$ da cellule macrofagiche peritoneali trattate in vitro con forme diverse di questo minerale. I risultati appaiono contrari a tale ipotesi. Essi vengono discussi con particolare riferimento all'aumentato efflusso enzimatico che si riscontra in tali condizioni.

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Über die Hemmung des Gerinnungsfermentes Thrombin durch Benzamidinderivate

Im Rahmen von Untersuchungen über Struktur-Wirkungs-Beziehungen synthetischer Proteasenhemmstoffe[1,2] fanden wir, dass einige Benzylamino-, Phenylguanidin- und Benzamidin-Derivate die Aktivität des Gerinnungsfermentes Thrombin zu hemmen vermögen. Die Hemmwirkung der Verbindungen wurde durch Messung der hydrolytischen Spaltung von Benzoyl-D,L-arginin-4-nitroanilid (BANI) durch Thrombin (gereinigtes Rinder-Thrombin, Aktivität 1000 NIH-E/mg) bestimmt. Wie aus den in der Tabelle zusammengefassten Versuchsergebnissen ersichtlich ist, erwiesen sich unter den geprüften Verbindungen insbesondere die Benzamidinderivate als starke kompetitive Thrombininhibitoren, unter denen die 4-Amidinophenylbrenztraubensäure, welche bereits von GERATZ[3] als Trypsininhibitor gekennzeichnet wurde, die stärkste Hemmwirkung zeigte.

Die Aktivität des Thrombins (50 NIH-E Rinder-Thrombin/ml) wurde durch spektrophotometrische Messung der BANI-Spaltung (bei 405 nm) in 0,1 *M Tris*-HCl-Puffer pH 8,4 bei 25 °C nach 30 min langer Inkubation bestimmt. Die Inhibitorkonstanten wurden nach der Methode von DIXON[4] ermittelt.

In weiteren Versuchen wurde der Einfluss dieser Verbindung auf die Blutgerinnungsvorgänge geprüft. Dabei zeigte sich, dass auch die Wirkung des Thrombins auf das natürliche Substrat Fibrinogen bereits in 10^{-5} *M* 4-Amidinophenylbrenztraubensäure-Lösung gehemmt wird (Figur). Übereinstimmend damit wurde die Gerinnungszeit von Humanblut wie auch die Rekalzifizierungszeit von Humanzitratplasma durch Zusatz von 0,1 µMol Hemmstoff/ml auf das Doppelte verlängert. Die antikoagulierende Wirkung von 4-Amidinophenylbrenztraubensäure übertrifft die Hemmwirkung des synthetischen Thrombinsubstrates TAME auf die durch Thrombin ausgelöste Fibrinogengerinnung sowie die Wirkung des durch Modifikation des synthetischen Substrates gewon-

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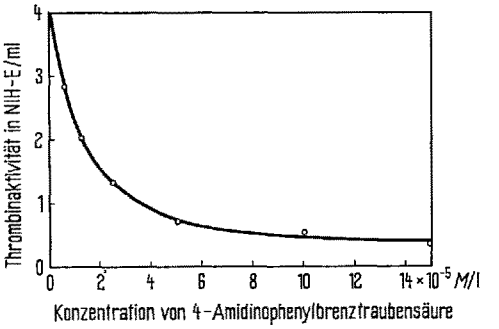
Antithrombinwirkung von Benzylamin-, Phenylguanidin- und Benzamidin-Derivaten

Hemmstoff	$Ki\,(M)$
Benzylamin	$1{,}2 \times 10^{-2}$
4-Aminomethylbenzoesäure	$> 2{,}0 \times 10^{-2}$
4-Methoxybenzylamin	$4{,}0 \times 10^{-3}$
4-Aminobenzylamin	$3{,}8 \times 10^{-3}$
4-Chlorbenzylamin	$2{,}2 \times 10^{-4}$
4-Aminomethylbenzoesäurebenzylester	$1{,}4 \times 10^{-3}$
Phenylguanidin	$9{,}0 \times 10^{-3}$
4-Guanidinobenzoesäure	$4{,}0 \times 10^{-2}$
Benzamidin	$2{,}0 \times 10^{-4}$
4-Aminobenzamidin	$8{,}0 \times 10^{-5}$
4-Amidinobenzoesäure	$> 2{,}0 \times 10^{-3}$
4-Amidinobenzoesäurebenzylester	$1{,}5 \times 10^{-4}$
4-Amidinophenylbrenztraubensäure	$3{,}0 \times 10^{-5}$

nenen kompetitiven Thrombinhemmstoffes Tosylagmatin[5,6] bei weitem.

Die Feststellung, dass die bisher gefundenen kompetitiven Thrombinhemmstoffe zugleich Hemmstoffe des Trypsins sind[7,8], weist auf eine enge Verwandtschaft der beiden Fermente in ihrem Wirkungsmechanismus und im Aufbau ihres aktiven Zentrums hin[9].

Summary. A series of aminomethyl, guanidino and amidino derivatives of benzene were investigated for their inhibitory effect on the activity of thrombin. Amidines were generally more potent than the other compounds. *p*-Amidinophenylpyruvic acid, a strong trypsin inhibitor, was found also to be the strongest small-molecular inhibitor on the activity of thrombin.



Hemmung der Thrombinwirkung auf Fibrinogen durch 4-Amidinophenylbrenztraubensäure (gemessen an der Gerinnungszeit von 1%igen Rinderfibrinogen-Lösungen in *Tris*-HCl-Puffer pH 7,4 bei 37 °C).

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Reaction of Lysozyme with Dithiothreitol and with Other Mercaptans

Most proteins contain disulfide bonds and these bonds have a unique structural role because they can link together otherwise separate polypeptide chains or tie noncontiguous parts of a single chain into loops. Scission of the disulfide bonds has frequently been used in studies of protein structure, of their function, and of the relationship between them. Disulfides may be cleaved by reaction with mercaptans, and various mercaptans have been used for this purpose with several proteins. However, little information is as yet available about the kinetics of the reaction and about the factors that determine reactivity[1].

In the present study, the rates have been measured for the reaction of 4 mercaptans with hen's egg-white lysozyme (E.C. 3.2.1.17)[2]. The rates vary widely and even the kinetic law may be different. The reactivity of lysozyme is of particular interest because its complete molecular structure is now known. The molecule consists of a single chain of 129 amino acid residues, folded to form a compact, rough ellipsoid, about $45 \times 30 \times 30$ Å. Four cystine residues are present, linking positions 6–127, 30–115, 64–80 and 76–94[3].

Two samples of lysozyme were examined, from Worthington Biochemicals (twice crystallized, salt-free) and from Calbiochem (grade A), with the same results. 2-Aminoethanethiol hydrochloride from Evans Chemetics was recrystallized from ethanol-ether to remove impurities that absorbed at 280 nm. Dithiothreitol (DTT) from Aldrich and all other chemicals were used without further purification.

Lysozyme was dissolved in 0.025 M borate buffer and mixed with a freshly prepared solution of thiol in the same medium. As the reaction proceeded, a precipitate formed. At appropriate intervals, a 0.2 ml aliquot of the reaction solution was withdrawn and was added to 5.00 ml of 0.1 M hydrochloric acid; the mixture was centrifuged at 2000 rpm for 3 min to remove any precipitate; and the absorbance of the supernatant was determined at 280 nm. The difference between the absorbance at any time and that at zero time gave the fraction of lysozyme that had reacted and precipitated.

A plot of log $[L]$ versus time ($[L]$ = lysozyme concentration) gave sensibly straight lines, i.e. first-order kinetics were followed. The Figure shows some representative results. Since the mercaptans were taken in large excess, the order does not reflect the molecularity of the reaction; this was investigated by varying the mercaptan concentration. The Table reports the half-reaction times obtained with the various mercaptans and in different conditions. The product obtained in the reaction with 2-mercaptoethanol was characterized in the

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