in the range of 6.2 (Table II). Enzyme preparations A and B were so active that it was impossible to follow the reaction course by the method used; DIF was reduced to its leuco-form in a few seconds.

In contrast to the TTC reductase, the activity of the DIF reductase was not influenced by the addition of menadione. Our results revealed that the enzymatic reduction of TTC depends on the presence of catalytic amounts of menadione (0.006 μmoles) which evidently serves as an intermediary electron transferring agent. The other differences of the respective enzyme from the one reducing DIF include its substantially lower activity, optimal pH in the region of 7.4 (Table III) and the lack of evidence for its elution from the acetone powder.

Table II. Effect of pH on the $NADH_2$-dichlorophenol-indophenol reductase activity in *O. mucida*

Enzyme preparation (0.2 ml)	pH	μmoles of reduced DIF/mg protein after 2 min incubation
C	6.2	12.9
	7.4	8.0
	7.9	4.1

Table III. Effect of pH and menadione on the $NADH_2$-triphenyltetrazolium chloride reductase activity in *O. mucida*

Enzyme preparation (0.5 ml)	Menadione 0.006 μmoles	pH	μmoles of reduced TTC/mg protein after the incubation period (min)	
			30	60
A	+	6.2	0	0.02
	+	7.4	0.05	0.15
	+	7.9	0.006	0.06
	−	7.4	0	0

These results suggest that the reduction of DIF and TTC in the basidiomycete *O. mucida* is mediated by 2 enzyme systems differing in their activity, optimal pH, requirements for menadione and by their solubility. The fact that the dehydrogenation of $NADH_2$ with both artificial electron acceptors was faster than its oxidation with aerial oxygen as terminal acceptor supports our view about the removal of some lipophilic cofactors, necessary for the transfer of electrons to oxygen, during the acetone treatment of the mycelium. This view is in accord with the results of LESTER and FLEISCHER[2], KLUGE et al.[3], DOWNEY[4] and other authors.

The low activity of the $NADH_2$-TTC reductase even in the presence of menadione could be caused by the not fully adequate function of this compound in the respective enzymatic process in *O. mucida*; other compounds of the quinone type (e.g. CoQ, other K-vitamins etc.) were not tested. On the other hand, the positive effect of menadione on enzymatic dehydrogenation of $NADH_2$ was observed also in *Puccinia graminis*[5].

Zusammenfassung. In der Basidiomycete *Oudemansiella mucida* (Schr. ex Fr.) Höhnel wurde eine $NADH_2$-Dichlorophenolindophenol-Dehydrogenase und eine $NADH_2$-Menadion-Triphenyltetrazoliumchlorid-Dehydrogenase gefunden. Beide Enzyme unterscheiden sich durch ihre pH-Abhängigkeit, Aktivität und Eluierbarkeit.

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Effect of Silica Dusts on Macrophage Permeability Studied with $^{86}RbCl$

The intracellular ion environment seems to influence greatly the activity of many apparently regulatory reactions in the cell[1]. Alterations in ion distribution in cells by impaired plasma membrane permeability have been suggested to imbalance the cell metabolism and to underlie the initial stages of cell necrosis[2,3]. Movements of Na^+ in and K^+ out occur in injured cells, and increased intracellular Ca^{2+} concentrations interfere with Na^+ and K^+ transport[4,5]. Evidence for altered plasma membrane permeability is possibly the appearance of cell enzymes in the extracellular fluids[2,3].

This study investigates whether increased permeability phenomena may occur in the toxic action of silica on macrophages. Silica has been shown to release enzymes from phagocytes[6,7] and to cause erythrocyte lysis[8–10]. This might render silica very similar in its action to a fat-active or surface-active agent. Electron microscopy indicates defects in the osmotic control by phagocytes in this condition[11]. Inhibition of macrophage metabolism[12] might so be initiated by ion shifts[13,14].

The $^{86}Rb^+$ efflux from peritoneal macrophages previously loaded with this isotope and incubated with different preparations of silica dust[7] has been studied. Rb^+ is known to behave like K^+, being electrochemically similar and having similar affinities to its carrier[15–19]. Peritoneal macrophages were obtained and handled as previously indicated[7]. Cells were counted and incubated in siliconized flasks with $^{86}RbCl$ $3.7 \times 10^{-6} M$ (Sorin, Saluggia, Italy: specific activity 0.16 mc/mg Rb^+) for 2.5 h at 37 °C in a modified Ringer-*Tris* medium[7] containing $1.5 \times 10^{-3} M$ glucose. Following centrifugation at 1800 *g* for 10 min at 2 °C, washing and recentrifugation, the final cell pellet was suspended to the appropriate concentration in Ringer-*Tris* medium[7] and incubated in 25 ml siliconized flasks in metabolic shaking incubator at 37 °C. Portions of the incubate were withdrawn from

flasks before the commencement (zero time) and at 15, 30, 60 and 120 min incubation. When necessary, dusts with a mean diameter $< 3\,\mu$[7] were added at the zero time (1 mg/10^7 cells initially present). Samples were chilled and immediately centrifuged at 12,000 g for 10 min at 2 °C. Aliquots of the supernatant were plated on stainless steel planchets for determination of medium radioactivity. After blotting of the tubes, cell pellets were dissolved in 1 ml 5% TCA, centrifuged and radioactivity determined on the resulting supernatant in the same way. Radioactivity was measured on dried samples using a thin mica-end window G.M. counter, with 2% standard error. Cell radioactivity was referred to 10^6 cells initially present, medium radioactivity to 1 ml.

Figures 1 and 2 show respectively the $^{86}Rb^+$ efflux from cells and the increase into the extracellular medium. Best fitting lines were drawn according to the method of least squares, after their linearity had been tested[20]. Efflux of $^{86}Rb^+$ from control cells after every 15 min incubation averages 11%. As the experiments were performed without synthetic substrate or serum, minor percentual losses might occur using supplemented media[21]. Crystalline silicas (tridymite T67-70-72M treated with hydrofluoric acid and T51M untreated) modify the 15 min efflux vs. control rates approximately to the same extent, + 3%. With fused quartz (hydrofluoric acid-treated vitreous silica VS74M), the mean percentual increase amounts to + 1.5%. When inert dusts (carbon A88M) are used, the extracellular efflux averages + 1%. The rate constant of the increase of $^{86}Rb^+$ concentration into the incubation medium remains unmodified by dust treatment. The statistical evaluation[20] of the regression coefficients of the lines demonstrates no significant evidence against their parallelism. This indicates absence of significant action on $^{86}Rb^+$ efflux from macrophages in

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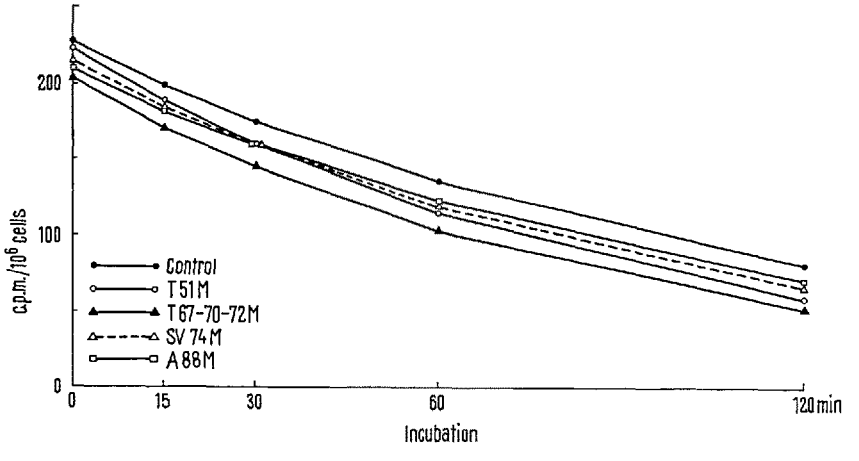

Fig. 1. Rate of disappearance of $^{86}Rb^+$ from peritoneal macrophages. Each point represents the results of 6 experiments. Lines are drawn according to the method of least squares.

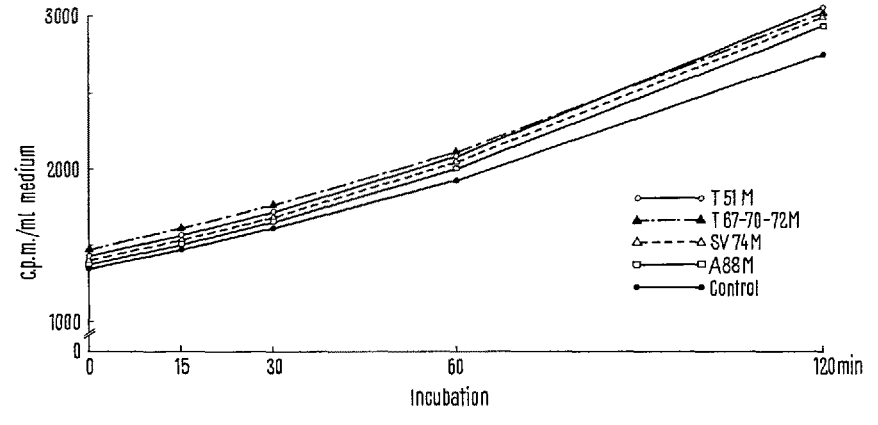

Fig. 2. Rate of increase of $^{86}Rb^+$ concentration into the incubation medium. Each point represents the results of 6 experiments. Lines are drawn according to the method of least squares.

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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