Chromatographically purified preparations of prochymosin are activated very rapidly at pH 2 and no specific activating agent has been found. To explain this, the hypothesis has been advanced that the zymogen is stabilized in its inactive form by electrostatic interactions. At low pH the carboxylic groups become uncharged and the prochymosin may rearrange into an active conformation. Having undergone such a change in conformation the zymogen molecules undergo irreversible activation by splitting off the activation peptide.

By this limited proteolysis the molecular weight is decreased from approx. 36000 for the zymogen to approx. 31000 for the active enzyme. Simultaneously the isoelectric point is shifted from approx. pH 5.0 to pH 4.6.

Analysis of the primary structure of chymosin is in progress. The sequences of 21 residues from the *N*-terminus and 28 residues from the *C*-terminus are known. Three disulphide bridges are present, two of these forming relatively short loops. 45 residues around the disulphide bridges have been located. Analyses of methionine-containing fragments have shown that four methionine residues are clustered in a sequence of 38 residues. From these investigations it is possible to account for 132 amino acid residues in chymosin. The *C*-terminal sequence and the sequence around the two short disulphide loops show a pronounced homology with hog pepsin (Foltmann, 1969). The *N*-terminal sequence has no resemblance to any known sequence from pepsin.

The proteolytic activity of chymosin has optimum pH about pH 3.5. The proteolytic specificity is somewhat similar to that of pepsin.

The well-known milk-clotting activity of chymosin is due to a limited proteolysis of the κ -fraction of the casein. The κ -casein consists of a hydrophobic and a hydrophilic part (Hill & Wake, 1969). In milk this protein stabilizes the casein micelles against aggregation. During milk-clotting, a Phe-Met bond is hydrolysed, the hydrophilic moiety of the κ -casein is liberated and aggregation occurs. The Phe-Met bond seems to be particularly exposed in casein, since the same bond is not hydrolysed by chymosin in a tryptic digest of κ -casein (Jollès, Alais & Jollès, 1968).

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Kallikreins

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The central property of the kallikreins (EC 3.4.4.21) is the hydrolysis of certain serum proteins, the kininogens, whereby pharmacologically highly active hypotensive peptides, the kinins, are liberated. Kininogenase activity, however, is also shown by certain other proteolytic enzymes, and this makes a precise definition of which enzymes should be called kallikreins very difficult, particularly as kallikreins from different sources show very different properties with regard to unspecific proteolysis, activity against synthetic substrates or sensitivity against inhibitors. Most of these studies, however, have been conducted with materials of doubtful purity, and this might in part explain the diversified results. It has been proposed that the name of kallikrein should be restricted to those kininogenases so named by their discoverers, and this proposal will be adhered to here. This limitation, however, is in contrast with the view of the Commission on Enzymes, which gave to kallikrein a system number of its own. The final clarification will need much more experimentation with pure enzymes.

Serum kallikrein from the pig was isolated by Habermann & Klett (1966). It had a molecular weight of 97000, liberated bradykinin from kininogen, and hydrolysed α -*N*-benzoyl-L-arginine ethyl ester, α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester, α -*N*-benzoyl-DL-arginine *p*-nitroanilide and casein. The isolation of bovine serum kallikreinogen (prekallikrein) was reported by Nagasawa, Takahashi, Koida, Suzuki & Schoenmakers (1968). The proenzyme was activated by Hageman factor or trypsin to an enzyme hydrolysing α -*N*-toluene-*p*-sulphonyl-L-arginine methyl ester but not casein. Rabbit serum kallikrein is inhibited by L-7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one (Paskhina *et al.* 1968). Guinea-pig serum kallikrein was shown to hydrolyse, among others, *N*-acylated lysine esters with *N*-acetylated amides of serine and tyrosine, but not threonine, as alcoholic components (Holman, Lowe, Morley & Smithers, 1968).

Simplified purification procedures for porcine submandibular-gland and urinary kallikreins are being worked out by the group of Fritz at our institute, but not much work has been done on the properties of these preparations as yet. Four different isoenzymes, having a molecular weight of about 25000 and able to hydrolyse α -*N*-benzoyl-L-arginine ethyl ester, α -*N*-benzoyl-DL-arginine *p*-nitroanilide and α -*N*-benzoyl-L-arginine β -naphthylamide, were isolated from submandibular glands of rats by Ekfors, Riekinen, Malmiharju &

Hopsu-Havu (1967). The enzymes slowly attacked casein and haemoglobin and are reported, strangely enough, not to be inhibited by di-isopropyl phosphorofluoridate. Specificity experiments have been carried out with rather crude preparations of urinary kallikreins. The rat enzyme splits α -N-benzoyl-L-arginine ethyl ester and α -N-benzoyl-DL-arginine *p*-nitroanilide, and is not inhibited by L-7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one but is inhibited competitively by certain amidines and guanidines (Mares-Guia & Diniz, 1967). The horse enzyme has similar properties. Preparations of the human enzyme have been described that showed no esterase activity against α -N-benzoyl-L-arginine ethyl ester (Diniz, Pereira, Barroso & Mares-Guia, 1965). Horse urinary kallikrein is reported to hydrolyse salmine (Brandi, Mendes, Paiva & Prado, 1965) and poly-L-arginine.

Our own interest centred on porcine pancreas kallikrein, for which newer purification methods have been described also by Moriya, Kato & Fukushima (1969) and by Sach & Thely (1968), who isolated a double peak of an enzyme that is most probably kallikrein. Our material was purified by the method of Fritz, Eckert & Werle (1967) from prepurified preparations generously supplied by Farbenfabriken Bayer. It contains 7% sugars, among them sialic acid, and has, according to the composition, a molecular weight of about 33600. Although homogeneous on ultracentrifugation, it is separated into two about equally active components A and B on electrophoresis at about pH 8 (Habermann, 1962). Both kallikreins A and B, separated on DEAE-cellulose by the method of Schmidt-Kastner, show on electrophoresis at about pH 4.5 up to six components, which are also seen in Ampholine pH-gradient electrophoresis, where the most basic main fraction shows an isoelectric point of 4.0. After treatment with neuraminidase, the more acid components merge into this main peak. The prekallikrein of porcine pancreas, for which a purification procedure was developed, also has two components A and B. The most prominent subfraction of the proenzyme B has an isoelectric point of 4.55. On treatment with trypsin it is activated to kallikrein B' with an isoelectric point of 4.3. Evidently in the usual isolation procedure, which begins with an autolysis step of pancreas homogenates, the prekallikrein is degraded by the combined attack of several enzymes.

The further studies have been conducted with neuraminidase-treated kallikrein B. The rate of inhibition by di-isopropyl phosphorofluoridate is quite low (Fiedler, Müller & Werle, 1968) and might be used for the characterization and differentiation of the enzyme. A single residue is incorporated

into a completely inhibited kallikrein molecule and is found in phosphoserine after hydrolysis, characterizing the enzyme as a 'serine hydrolase'. Porcine pancreas kallikrein reacts also with cinnamoylimidazole, forming a derivative with an absorption spectrum similar to that of cinnamoyl-trypsin. The procedure can be used for the titration of the active site of kallikrein. The enzyme is inhibited by heavy-metal ions. In the hydrolysis of *N*-acylated amino acid esters it exhibits the phenomena of substrate activation and inhibition (Fiedler & Werle, 1968). Kinetic constants have been determined for the hydrolysis of some methyl esters (Fiedler, 1968), recently also of α -N-benzoyl-L-citrulline (K_m 11 mM; k_{cat} 8 sec.⁻¹). Although the α -N-benzoyl-L-lysine ester is hydrolysed, pancreas kallikrein is not inhibited by L-7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one. The low efficiency of the hydrolysis of esters of neutral amino acids, as compared with arginine compounds, throws some doubt on kallidin being the primary product from kininogen containing the amino acid sequence determined by Hochstrasser & Werle (1967). A series of α -N-benzoyl-L-arginine esters with different alcohols show different V_{max} values, indicating that acylation of the enzyme is the rate-limiting step in ester hydrolysis. α -N-Toluene-*p*-sulphonyl-L-arginine esters are hydrolysed much more slowly than the α -N-benzoyl compounds. In the toluene-*p*-sulphonyl series, the rate of the hydrolysis of the methyl ester is three to four times that of the ethyl ester. This ratio resembles the ratio of the alkaline-hydrolysis velocities of these two compounds and suggests that kallikrein catalysis begins with a nucleophilic attack on the substrate. The participation in catalysis of a group of the enzyme with a pK_a about 6.9, most probably histidine, was derived from the pH-dependence of the rate of the inhibition of porcine pancreas kallikrein by di-isopropyl phosphorofluoridate, and of V_{max} in the hydrolysis of α -N-benzoyl-L-arginine ethyl ester as well, where the acylation reaction presumably is also the rate-limiting step.

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COMMUNICATIONS

Purification and Characterization of Bovine Enteroviruses

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Bovine enteroviruses are widely distributed in the cattle population of Ireland. Recent reports (McFerran, Nelson, McCracken & Ross, 1969) indicate their economic importance in relation to enteritis.

Serologically distinct bovine enteroviruses have been shown to grow readily in baby-hamster kidney (BHK) cells. This system has been used to grow virus in sufficient quantity for the investigation of their nucleic acid and protein components.

Overnight incubation of virus with monolayers of BHK cells disrupts the cell sheet, releasing over 99% of the virus formed into the medium. The bulk of the cell debris is removed by centrifugation at 2000 rev./min. Remaining cellular contamination is then removed by differential centrifugation, gel filtration through Sepharose 2B and sedimentation in 15–40% sucrose gradients. The purity of the virus has been demonstrated by double-labelling experiments and detergent treatment. This purification scheme is readily adaptable to large-scale zonal-centrifugation procedures.

The nucleic acid of the viruses has been identified as RNA by labelling with [³H]uridine. On sucrose gradients viral RNA sediments at 35s relative to 28s ribosomal RNA. The base compositions of RNA from several serologically distinct bovine enteroviruses are similar. The viruses have a sedimentation value of 165s relative to 80s ribosomes extracted from BHK cells. Electron-microscope studies have shown the virus particles to be spherical with a diameter of 27nm. Their density is 1.34g./ml. as determined by density of the isopycnic centrifugation in CsCl gradients run in angle heads (Flamm, Bond & Burr, 1966).

Work is now in progress to study the capsid proteins of a number of bovine enteroviruses.

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The Reaction Catalysed by a Partially Purified Nucleoside Triphosphate-Dependent Deoxy-ribonucleic Acid-Breakdown System from *Mycobacterium smegmatis*

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Streptomycin-treated extracts of *Mycobacterium smegmatis* that had been grown for 72hr. in iron-limited medium were prepared as described by Winder & Coughlan (1969). Protamine sulphate (0.1%) was added, and the precipitate was dissolved in 0.1M-phosphate buffer, pH 7.5, containing 1mM-dithiothreitol, concentrated on a Diaflo UM-10 ultrafilter, transferred to a column of Sephadex G-200 and eluted with 0.1M-phosphate buffer, pH 8.0, containing 1mM-dithiothreitol. The enzyme was eluted immediately after the void volume, with a 15–20-fold increase in specific activity over the crude extract. Attempts at further purification were unsuccessful owing to low stability.

Incubation mixtures contained usually 0.2ml. of enzyme, 0.4ml. of the latter buffer, 1mg. of the sodium salt of DNA, 2 μ moles of ATP and 20 μ moles of MgCl₂ in a total volume of 1ml., or was appropriately scaled up. It was incubated at 37°, usually for 30min. Assays were as described by Winder & Coughlan (1969).

The enzyme strongly preferred native to heat-denatured DNA. However, it could degrade more than half of the DNA, probably as the result of a slow attack on single-stranded DNA. The kinetics of loss of viscosity of the DNA and of appearance of acid-soluble deoxyribose were similar, and extraction of DNA remaining after a period of