TABLE II

DIFFUSION CONSTANT OF TETANUS TOXOID

Time of diffusion (seconds)	*Diffusion constant (Inflection method) cm^2/sec*	*Diffusion constant (Method of moments) cm^2/sec*	*Average diffusion constant*
178,920	$5.07 \cdot 10^{-7}$	$5.17 \cdot 10^{-7}$	$5.09 \cdot 10^{-7}$
246,840	$4.85 \cdot 10^{-7}$	$5.00 \cdot 10^{-7}$	

has approximately the same molecular size as the parent toxin. This similarity in molecular size has also been shown for diphtheria toxin and formol toxoid, which have approximately the same sedimentation constants (4.6 Svedberg units)[6,7].

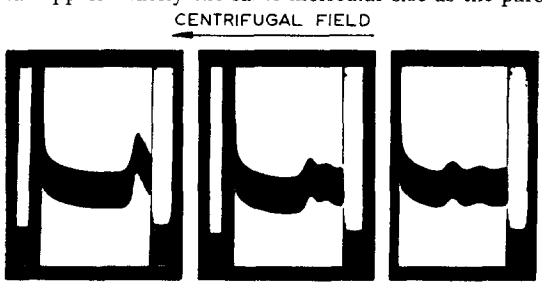

Fig. 2. Sedimentation diagrams of the formol tetanus toxoid after 12, 32 and 56 minutes at 60,000 r.p.m. The two components, which are presumably the formol toxoid and the spontaneously formed toxoid, have sedimentation constants of 4.2 and 7.6 Svedberg units respectively.

It would appear that de-toxification of tetanus toxin occurs as a result of two reactions: (i) Spontaneous conversion of the toxin (3.9 Svedberg units) to the toxoid (7.6 Svedberg units), and (ii) Interaction of formaldehyde to form a toxoid (4.2 Svedberg units). This latter toxoid is devoid of any tendency to dimerise and hence shows up in a reasonable concentration in the sedimentation diagram. The spontaneous formation of toxoid is both far slower and less complete than that induced by formaldehyde.

Acknowledgements. The authors wish to express their thanks to Dr. A. POLSON for his guidance and assistance and to Professor M. VAN DEN ENDE for his interest and advice during the course of this work. This communication is published with the permission of the South African Council for Scientific and Industrial Research.

C.S.I.R. and U.C.T. Virus Research Unit, Department of Pathology; and Department of Chemistry, University of Cape Town (South Africa) J. F. LARGIER*

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria (South Africa) F. J. JOUBERT

1 L. PILLEMER, R. G. WITTLER, J. I. BURRELL AND D. B. GROSSBERG, *J. Exptl. Med.*, 88 (1948) 205.
2 L. PILLEMER AND D. H. MOORE, *J. Biol. Chem.*, 173 (1948) 427.
3 A. POLSON, *Biochim. Biophys. Acta*, 11 (1953) 315.
4 J. F. LARGIER, *J. Immunol.* (in the press).
5 O. LAMM AND A. POLSON, *Biochem. J.*, 30 (1936) 528.
6 M. L. PETERMANN AND A. M. PAPPENHEIMER, *J. Phys. Chem.*, 45 (1941) 1.
7 L. PILLEMER, D. TOLL AND S. J. BADGER, *J. Biol. Chem.*, 170 (1947) 571.

Received February 1st, 1956

* Present address: Seravac Laboratories, Epping, Cape Town.

The activation of chymotrypsinogen by subtilisin

The tryptic activation of chymotrypsinogen has long been a subject of great interest in the protein field. With the background provided by the observations of NORTHROP AND KUNITZ[1], and the investigations of JACOBSEN[2] concerning the mechanism of activation of this zymogen, the recent work of NEURATH *et al.*[3], and of the DESNUELLE group[4] has provided continuing progress in the understanding of the activation process.

Although trypsin is used as the initial catalyst in all of these studies, and further seems to be responsible for the activation of chymotrypsinogen in physiological systems, it is not the only enzyme capable of initiating the conversion of the zymogen to its active form. KUNITZ[5], in 1938, noted that culture media of the mold *Penicillium* contained a "kinase" capable of slowly activating acid solutions of chymotrypsinogen, and in 1951, ABRAMS AND JACOBSEN[6] observed that crude enzyme preparations from *Bacillus subtilis*, which had previously been shown to convert

ovalbumin to plakalbumin[7], were able, at low temperatures and pH's near neutrality, to bring about a slow activation of chymotrypsinogen.

The present work was accordingly undertaken with the idea that further study of the effect of the *B. subtilis* proteinase, now obtainable in crystalline form[8], upon chymotrypsinogen might provide new insight into the essential processes involved in the conversion of this inactive zymogen to an active enzyme.

As a means of attempting demonstration of intermediate compounds and degradation products which might be formed during the activation, reaction mixtures of chymotrypsinogen and crystalline subtilisin, incubated at 0° C, pH 7.3 for varying lengths of time, were adjusted to pH 3 with 1 *N* HCl to destroy the enzyme and prevent autolysis of chymotrypsin during storage. These samples were subsequently subjected to chromatography on columns of the carboxylic acid ion exchange resin IRC-50 (XE-64), according to the procedure described by HIRS[9] for the chromatography of chymotrypsinogen, using 0.9 × 30 cm columns of the resin and *M*/6 phosphate buffer, pH 6.02. Under these conditions the undegraded substrate, Worthington crystalline salt-free chymotrypsinogen, emerged as a sharp symmetrical peak at 30 effluent ml, with only small amounts of impurities. Control runs of chymotrypsinogen incubated for 24 hours with subtilisin previously inactivated by standing at pH 3 for three hours at 5° C showed no discernable chromatographic differences, and α-chymotrypsin (Worthington crystalline salt-free), while appearing less homogeneous chromatographically, showed a main peak at the same effluent volume. Protein content of all effluent fractions was determined on the basis of optical density at 280 mμ, and ninhydrin analysis was performed according to the method of MOORE AND STEIN[10]. Assays of proteolytic activity of selected effluent samples and total reaction mixtures before and after activation with trypsin were carried out by a modification of the general procedure of NORTHROP *et al.*[1,9], measuring the optical density at 280 mμ of TCA soluble products of casein hydrolysis. Considerable difficulty was encountered in obtaining uniform blanks and standard curves with this method, but despite obvious limitations in accuracy, it still provides a roughly quantitative means of comparing activities.

Fig. 1 shows a typical series of chromatograms obtained from reaction mixtures containing 1 % chymotrypsinogen substrate and 0.04 % crystalline subtilisin, incubated at pH 7.3 and 0° C.

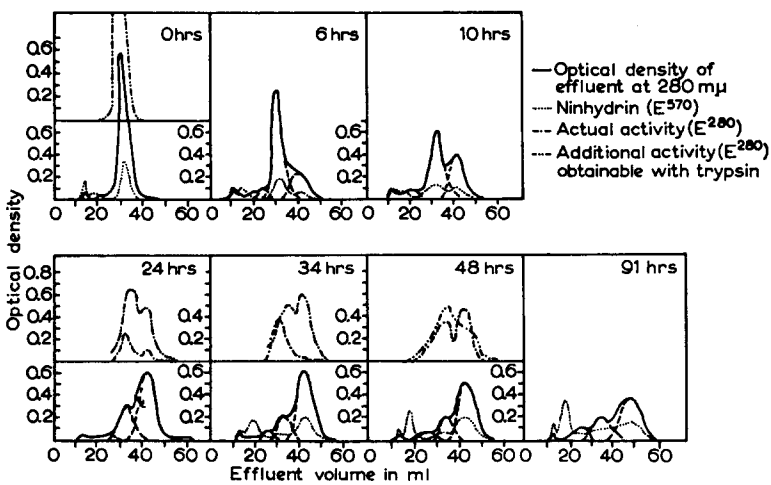

Fig. 1.

As the 30 ml protein peak of chymotrypsinogen diminishes, a new peak appears at 42 ml, increases during the first 24 hours, and then slowly recedes. During the initial period of its formation the new peak, like chymotrypsinogen, is itself inactive, but can be activated by trypsin, thereby suggesting the formation of an altered, but still activatable chymotrypsinogen rather than a new chymotrypsin. The relatively late appearance of the peak in the effluent indicates that it is not identical with chymotrypsinogen-B (as described by BROWN, SHUPE AND LASKOWSKI[11]) which would pass through columns of this type with the effluent front[9]. The late increase of the protein peak at 30 ml, and the gradual appearance of actual activity at this location concurrent with the diminution of the 42 ml peak after the first 24 hours, suggest that this altered chymotrypsinogen may actually be an intermediate in the formation of a second 30 ml peak containing an active chymotrypsin. The development of a new peak of actual activity at 42 ml in the 48 hour

sample points to the additional possibility that the altered zymogen or the active chymotrypsin from the 30 ml peak is further degraded to a material which is active, but which emerges at the same effluent volume as does the altered zymogen.

Fig. 2a shows the increase in actual activity and ninhydrin color of the total reaction mixture with time, and Fig. 2b the variation in amount of material with maximum absorption at 280 mμ in the 30 and 42 ml peaks. The steady rise in total ninhydrin color suggests the likelihood that peptide or amino acid liberation may be involved in the activation process. The initial close correspondence between the disappearance of protein material from the 30 ml peak, and the increase in the new 42 ml peak, points strongly to a direct conversion from chymotrypsinogen to the altered zymogen, and the fact that most of the starting material has already undergone this conversion at a time when the overall activation still shows a steady rise, makes it likely that the altered zymogen of the 42 ml peak is actually an intermediate which subsequently undergoes further degradation to the active enzyme appearing at 30 effluent ml.

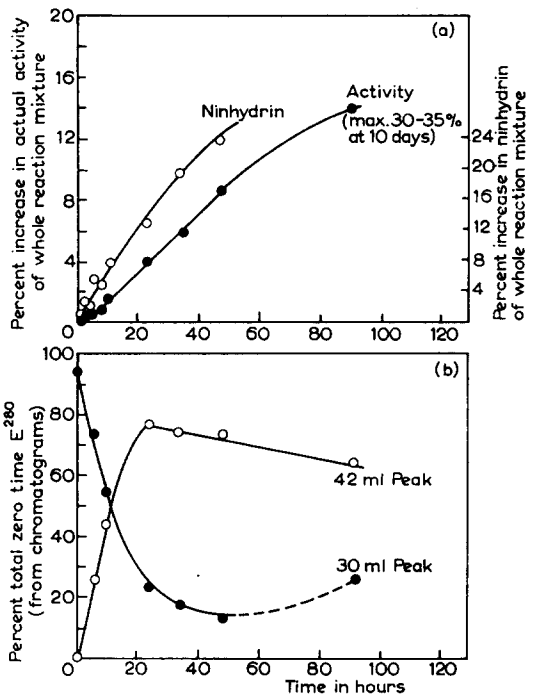

Fig. 2.

Following exhaustive dialysis at pH 3 and subsequent lyophilization, the material obtained from the 42 ml chromatographic peak of 30 hour reaction mixtures shows a relatively homogenous peak at the same location when rechromatographed. Preliminary experiments using solutions of this lyophilized intermediate indicate that it exhibits no appreciable actual activity initially, nor does it appear to undergo autoactivation when incubated for several hours at pH 7.3. It is not activated by addition of α-chymotrypsin, but addition of trypsin or of subtilisin in amounts corresponding to previous experimental conditions causes significant activation, so that both of the first two postulated steps in the activation process under study appear to be catalyzed by subtilisin.

Carlsberg Laboratorium, Copenhagen (Denmark)

ALICE BRONFENBRENNER*
K. LINDERSTRØM-LANG
M. OTTESEN

[1] J. H. NORTHROP, M. KUNITZ AND R. M. HERRIOTT, *Crystalline Enzymes*, 2nd ed., Columbia Univ. Press, New York, 1948.
[2] C. F. JACOBSEN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 25 (1947) 325.
[3] See *e.g.*: J. A. GLADNER AND H. NEURATH, *J. Biol. Chem.*, 205 (1953) 345. W. J. DREYER AND H. NEURATH, *J. Biol. Chem.*, 217 (1955) 527.
[4] See *e.g.*: M. ROVERY, C. FABRE AND P. DESNUELLE, *Biochim. Biophys. Acta*, 12 (1953) 547. M. ROVERY, M. POILROUX, A. CURNIER AND P. DESNUELLE, *Biochim. Biophys. Acta*, 17 (1955) 565.
[5] M. KUNITZ, *J. Gen. Physiol.*, 21 (1938) 601.
[6] A. ABRAMS AND C. F. JACOBSEN, *Compt. rend. lab. Carlsberg, Sér. chim.*, 27 (1951) 447.
[7] K. LINDERSTRØM-LANG AND M. OTTESEN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 26 (1949) 403.
[8] A. GÜNTELBERG AND M. OTTESEN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1954) 27.
[9] C. H. W. HIRS, *J. Biol. Chem.*, 205 (1953) 93.
[10] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
[11] K. D. BROWN, R. E. SHUPE AND M. LASKOWSKI, *J. Biol. Chem.*, 173 (1948) 99.

Received January 31st, 1956

* U.S. National Research Council fellow in the Medical Sciences 1952–1954.