centrifuged for 10 min at 17,000 *g*. The sediment was resuspended and centrifuged in 6 *N* HCl 3 times and thereafter hydrolyzed in 6 *N* HCl for 7 h. The last supernatant and the hydrolysate were examined for the following substances: A) Dopa, according to ANTON and SAYRE[10]. B) Adrenaline and noradrenaline, according to BERTLER et al.[11]. C) Dopamine, according to CARLSSON and WALDECK[12]. D) 5-hydroxytryptamine, according to BERTLER and ROSENGREN[13].

Results and comments. The determination of dopa and the various biogenic amines showed that dopa and dopamine could be recovered in the hydrolysate of Substantia nigra melanin. When hydrolysis was repeated 3 times, only dopa was found. No dopa, dopamine or other amines were present in the last supernatant after repeated washing of melanin in 6 *N* HCl.

Dopamine has not previously been found in naturally occurring melanin, but under experimental conditions dopamine may form melanin, and knowledge of this melanin is to a considerable extent thanks to the work of SWAN[14]. According to the classic Raper-Mason concept, melanin formed from dopa or dopamine should contain only indole-quinones, but it is now thought that dopa or dopamine can be incorporated in the melanin[14–17].

The presence of dopamine in Substantia nigra melanin is of great interest, since dopamine has been demonstrated in considerable amounts in this structure[18]. Dopamine is believed to have a special function in the extra-pyramidal system, and the dopamine-containing nerve terminals of the putamen and of the caudate nucleus probably originate in Substantia nigra[19]. It is noteworthy that pigment forms only in this structure, while the dopamine concentration is 10-fold higher in the caudate nucleus and the putamen[18,20].

Various explanations may be offered for the presence of dopa and dopamine in Substantia nigra pigment. Pigment granules may be formed by oxidation of tyrosin and dopa and polymerization of the oxidation products. Then dopa may be copolymerized with the polymerized indole-quinones. Dopamine available in Substantia nigra cells might then also be copolymerized. It is also possible that pigment granules are formed by oxidation of dopamine and polymerization of the formed indole-quinones. Dopamine, but also some still undecarboxylated dopa, may then be copolymerized.

Finally, it cannot be excluded that the Substantia nigra melanin is a mixture of dopa melanin and dopamine melanin in which case dopa and dopamine should be present in different granules.

The finding of dopa only, after 3 hydrolyses of 7 h each, favours the view that the primary nucleus of melanin formed is dopa melanin, while the dopamine melanin forms a shell on the melanin particles[21].

Zusammenfassung. Hydrolysate der Pigmentpartikel der Substantia nigra enthalten Dopa und Dopamin, jedoch kein Adrenalin, Noradrenalin oder Serotonin.

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1 R. D. LILLIE, J. Histochem. Cytochem. *5*, 325 (1957).
2 P. E. DUFFY and V. M. TENNYSON, J. Neuropath. exp. Neurol. *24*, 398 (1965).
3 H. L. MOSES, C. E. GANOTE, D. L. BEAVER and S. S. SCHUFFMAN, Anat. Rec. *155*, 167 (1966).
4 M. H. VAN WOERT, K. N. PRASAD and D. C. BORG, J. Neurochem. *14*, 707 (1967).
5 T. MAEDA and R. WEGMANN, Brain Res. *14*, 673 (1969).
6 J. H. FELLMAN, J. Neurol. Neurosurg. Psychiat. *21*, 58 (1958).
7 C. C. MARSDEN, Q. Jl microsc. Sci. *102*, 407 (1961).
8 C. V. WENDE and M. T. SPOERLEIN, Life Sci. *6*, 386 (1963).
9 R. A. NICOLAUS, *Melanins* (Hermann, Paris 1968).
10 A. H. ANTON and D. F. SAYRE, J. Pharmac. exp. Ther. *145*, 326 (1964).
11 Å. BERTLER, A. CARSLSSON and E. ROSENGREN, Acta physiol. scand. *44*, 273 (1958).
12 A. CARLSSON and B. WALDECK, Acta physiol. scand. *44*, 293 (1958).
13 Å. BERTLER and E. ROSENGREN, Experientia *15*, 382 (1959).
14 G. A. SWAN, Rend. Acc. Sci. Fis. Mat. Napoli *31*, 212 (1964).
15 M. PIATTELLI, E. FATTORUSSO, S. MAGNO and R. A. NICOLAUS, Tetrahedron *18*, 941 (1962).
16 G. W. KIRBY and L. OGUNKOYA, Chem. Comm. *21*, 546 (1965).
17 K. HEMPEL, in *Structure and Control of the Melanocyte* (Eds. G. DELLA PORTA and O. MÜHLBOCK; Springer-Verlag, Berlin 1966), p. 162.
18 Å. BERTLER, Acta physiol. scand. *51*, 97 (1961).
19 Å. BERTLER and E. ROSENGREN, Pharmac. Rev. *18*, 769 (1966).
20 Å. BERTLER and E. ROSENGREN, Experientia *15*, 10 (1959).
21 Supported by grants from The Swedish Medical Research Council No. B71-14X-712-06A and No. B71-14X-56-07A and from The Swedish Cancer Society No. 67-111.
22 Reprint requests to: Dr. H. Rorsman, Dept. of Dermatology, Lasarettet, S-221 85 Lund (Sweden).

Inhibitory Effect of Proteolytic Enzymes on Platelet Aggregation Induced by ADP or Thrombin

Although a number of substances have been reported to inhibit platelet aggregation or adhesion, most of them should be inquired more to be approved as reliable and available in clinical use on thromboembolism. One of these categories includes proteolytic enzymes. The present study describes alterations in the sensitivity of unwashed and washed platelets with ADP and thrombin when platelets were treated with proteolytic enzymes.

Materials and methods. Human venous blood anticoagulated with 10% by volume of 8% trisodium citrate was centrifuged at 170*g* for 30 min at room temperature to obtain platelet-rich plasma (PRP). The platelet concentration in PRP was adjusted by using platelet poor plasma (PPP) as a diluent to $25 \times 10^4/mm^3$ employing BRECHER-CRONKITE method[1]. All glasswares were siliconized. 50 mg of each proteolytic enzyme: protease (1,900,000 U/g), by courtesy of Pacific Lab., Inc., Honolulu, HI.; papain (1000 GDU (gelatin digesting unit)/g, and bromelain (1200 GDU/g), provided by Dr. S. TAUSIK) Director of Research, Dole Co., Honolulu, HI.; ficin (200–800 U/g), obtained from Sigma Chemical Co. (St. Louis, Mo.), was dissolved in phosphate buffered saline (PBS) pH 7.2 to yield the concentration of 20 and 200 µg/ml. PRP was incubated with an equal volume of each enzyme solution in a plastic tube at 37 °C for 30 min. The mixture of PRP and PBS was used as a control.

The ADP sensitivity test of platelets was performed by method of YAMAKIDO et al.[2] as modified by SANO et al.[3,4]. The principle of the method is to obtain the

minimum concentration of ADP required to induce platelet aggregation by mixing equal volumes of PRP and serially two-fold diluted ADP. The sensitivity was expressed by the absolute value of the exponent of the concentration (2^{-n} mg/ml) of ADP required. For instance, when the endpoint was 2^{-13} mg/ml, the value of sensitivity was expressed as '13'.

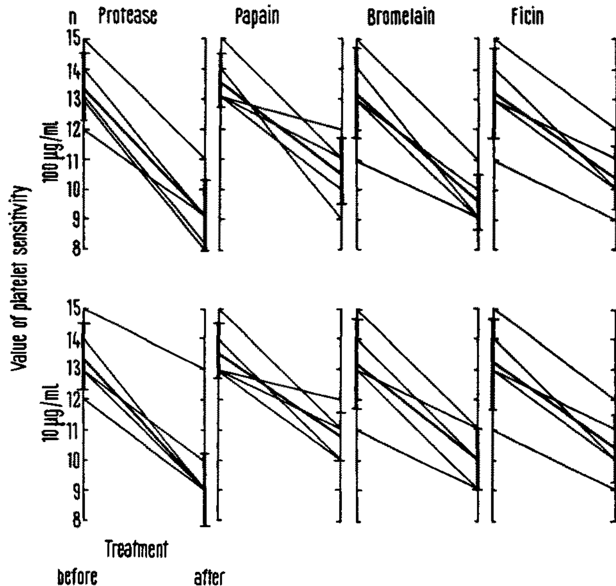

Fig. 1. Effect of proteolytic enzymes on ADP-induced aggregation of unwashed platelets.

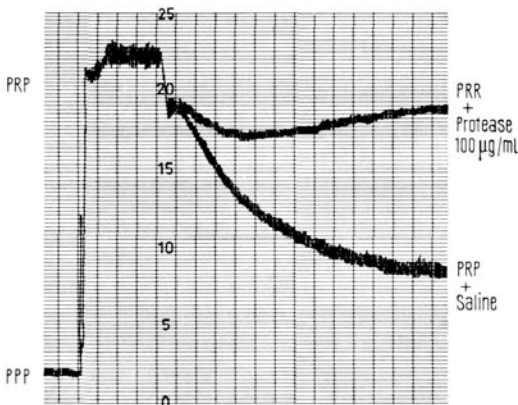

Fig. 2. Aggregometric curve of platelet aggregation after treatment with protease (ADP 5 μg/ml).

Five specimens were tested before and after treatment with each of the 4 enzyme preparations at each of 2 different concentrations. Changes in platelet aggregation were tested for significance using a *t*-test on the difference between the paired observations. Turbidimetric observation of platelet aggregation[5,6] was also performed on the effect of protease by an aggregometer (Chrono-Log Corp., Broomall, Pa.).

In comparison with PRP analysis, experiments were carried out using washed platelets. In this case, 15% by volume of hyperacid ACD (0.085 M trisodium citrate, 0.065 M citric acid and 2% dextrose)[7] was employed as an anticoagulant. Red blood cells in PRP was eliminated by an additional spinning at 260 g for 5 min. Washing and resuspension of platelet, obtained by centrifuging PRP at 1000 g for 30 min, was performed using 5.4 mM trisodium citrate in PBS pH 7.2. After washing 3 times, platelet suspension with a concentration of 25×10^4/mm^3 was treated by 200 μg/ml solution of protease for 5 min in the same manner described above. Then the samples were washed and resuspended in PBS to restore the above concentration.

Human thrombin ('Fibrindex' Ortho Diagnostic, Raritan, N.J.) was employed as an aggregating agent for the washed platelet suspension. The thrombin was dissolved in physiologic saline immediately before use in a concentration of 50 U/ml and diluted serially two-fold with PBS yielding 50×2^{-1} through 50×2^{-18} U/ml. The procedure for aggregation study was followed by the previous description.

Results. The sensitivity of unwashed platelets against ADP decreased significantly at the 0.01–0.05 confidence level by treatment with 10 and 100 μg/ml of protease, papain, bromelain and ficin (Table and Figure 1).

An example of turbidimetric observation of ADP-induced platelet aggregation is shown in Figure 2. The decrease of optical density was minimized by treatment with 10 μg/ml solution of protease.

Figure 3 shows the effect of protease on thrombin-induced aggregation of washed platelets. The mean and S.D. of the absolute values of the exponent in this concentration of thrombin (50×2^{-n} U/ml) required to induce platelet aggregation in control and treated samples were

[1] G. BRECHER and E. P. CRONKITE, in *The Coagulation of Blood* (Ed. L. M. TOCANTIS; Bruen and Stratton, Inc., New York 1955), p. 41.
[2] M. YAMAKIDO, N. OISHI and M. YOKOYAMA, Hawaii med. J. *29*, 446 (1970).
[3] T. SANO, L. A. BOXER and M. YOKOYAMA, 13th Annual Meeting of American Society of Hematology, San Juan, Puerto Rico, Dec. 5–8, 1970 (Abstr.).
[4] T. SANO, M. G. J. BOXER, L. A. BOXER and M. YOKOYAMA, Thromb. Diath. haemorrh., in press (1971).
[5] G. V. R. BORN, Nature, Lond. *194*, 927 (1962).
[6] J. R. O'BRIEN, J. clin. Path. *15*, 452 (1962).
[7] R. H. ASTER and J. H. JANDL, J. clin. Invest. *43*, 843 (1964).

Effet of proteolytic enzymes on ADP induced platelet aggregation

Enzyme	Protease		Papain		Bromelain		Ficin	
μg/ml (final)	100	10	100	10	100	10	100	10
Control	13.4 ± 1.1	13.4 ± 1.1	13.6 ± 0.9	13.6 ± 0.9	13.2 ± 1.5	13.2 ± 1.5	13.2 ± 1.5	13.2 ± 1.5
Treated	9.0 ± 1.2[b]	10.0 ± 1.7[b]	10.6 ± 1.1[a]	10.8 ± 0.8[b]	9.6 ± 0.9[b]	10.0 ± 1.0[b]	10.4 ± 1.1[b]	10.4 ± 1.1[b]

Value indicates mean ± 1 S.D. [a] $p < 0.05$. [b] $p < 0.01$.

8.5–1.3 and 5.5–1.7, respectively. The decrease by treatment was statistically significant at the 0.05 confidence level.

Discussion. The inhibition of ADP-induced platelet aggregation by addition of *Aspergillus* enzyme to PRP in vitro was reported by BYGDEMAN[8] and DE NICOLA et al.[9]. The latter investigators also, observed this action of the enzyme in vivo by i.v. infusion[9].

Our results show that proteolytic enzyme counteracted the aggregation of unwashed as well as washed platelets, induced by ADP or thrombin.

Hyperacid ACD[7] used as an anticoagulant, exclusion of red cells by respinning and washing of platelets with 5.4 mM sodium citrate[10], promoted the preparation of washed platelets without spontaneous aggregation maintaining their proper aggregability.

Although the deprivation of platelet sensitivity to ADP by washing had been reported[11], restoration of aggregability by inclusion of calcium, magnesium, glucose and albumin in the suspending fluid was described by ARDLIE et al.[12]. But, because of unsuitability of providing extraneous protein as a component of platelet suspending solution in the present study, thrombin was chosen as an aggregating agent instead of ADP. The enzymatically treated platelets were rewashed prior to mixing with thrombin to avoid further enzymatic action of thrombin.

The effectiveness of protease on washed platelets would at least partially suggest that the inhibitory effect of proteolytic enzymes on platelet aggregation could be an effect of enzymes on platelet membrane.

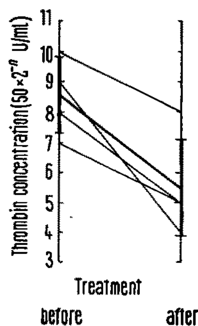

Fig. 3. Effect of protease on thrombin-induced aggregation of washed platelets.

Zusammenfassung. Behandlung ungewaschener und gewaschener Thrombozyten mit proteolytischen Enzymen (Protease, Bromelain und Ficin) bewirkt eine Abnahme der Aggregationsfähigkeit von Thrombozyten mit ADP' oder Thrombin.

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[8] S. BYGDEMAN, Life Sci. *6*, 499 (1967).
[9] P. DE NICOLA, A. GIBELLI, G. TURAZZA and P. GIAROLA, Life Sci. *6*, 1233 (1967).
[10] C. W. ROBINSON JR., R. G. MASON and R. H. WAGNER, Proc. Soc. exp. Biol. Med. *113*, 857 (1963).
[11] J. R. MCLEAN, R. E. MAXWELL and D. HERTLER, Nature, Lond. *202*, 605 (1964).
[12] N. G. ARDLIE, M. A. PACKHAM and J. F. MUSTARD, Br. J. Haemat. *19*, 7 (1970).

Influences of Nystagmic and Spontaneous Oculomotor Activity on Superior Colliculus Neurons in Curarized Cat

The influence of eye movement on neurons in primary visual structures of cat has recently been studied by several authors. Modifications of neuronal activity related to eye movements have been described at the level of lateral geniculate body[1] and tectum opticum[2]. The aim of the present study was to investigate the influence of nystagmic and spontaneous oculomotor activity on superior colliculus (SC) neurons in absence of actual eye movements, thus avoiding possible retinal image shift or proprioceptive input.

Material and methods. Cats were prepared under ether anesthesia. A high cervical transsection was performed ('encéphale isolé'); expiratory CO_2 and body temperature were monitored. After recovering from the surgical procedure, animals usually exhibited a satisfactory amount of spontaneous and pursuit eye movement. Nystagmus was induced by polarization of labyrinths. Horizontal eye movements were recorded by electrooculography. A concentric bipolar macroelectrode was then introduced stereotactically in or in the close neighborhood of nucleus abducens. Correlation between eye movement and synchronous oculomotor discharges was established for each animal. Glass microelectrodes were introduced through the cortex into the SC and the animal curarized with Flaxédil. Visual stimuli consisted in switching on and off a diffuse light, or hand-moving bright or dark objects. In absence of these stimuli, the animal was facing a structureless background (photopic conditions). Both eyes were left open. Microelectrode tip, at the end of a penetration, was localized by electrophoresis of pontamine[3]. The brain was perfused with formalin, frozen and cut serially. Blue spots were used to reconstruct the electrode track and the location of the units within the collicular layers.

Results and discussion. The influence of nystagmic oculomotor activity on spontaneous firing pattern of 82 collicular neurons was studied. Their visual characteristics were determined by methods described above: 46 units (56%) were thus found to be unresponsive to visual stimulation.

The effects of nystagmic oculomotor activity may be roughly divided into 2 categories. Spontaneous activity of 9 collicular neurons (11%; group I) was phasically modified in synchrony with nystagmic oculomotor discharges recorded at the level of nucleus abducens (Figure, I). 2 cells were inhibited, the others were activated. 3 units were found to be visually driven. A second set of 7 neurons (9%; group II) showed 'tonic' modifications of activity during sequences of nystagmic oculomotor discharges. These effects are rather complex and, most frequently, consist of an increase of spike frequency (Figure, II). 2 of these units were sensitive to movement of a visual stimulus, others could not be driven by visual stimulation.

[1] M. JEANNEROD and P. T. S. PUTKONEN, Brain Res. *24*, 125 (1970).
[2] M. STRASCHILL and K. P. HOFFMANN, Expl Brain Res. *11*, 318 (1970).
[3] J. M. GODFRAIND, J. Physiol., Paris *61*, 436 (1969).