red cell integrity was not necessary for inactivation because the enzyme of cell-free membrane preparations behaved like that of intact erythrocytes. Furthermore, the percent reduction in ACHE activity in membranes prepared from TPB-treated cells was the same as that observed with whole erythrocytes. The possibility that the effect on ACHE was due to the interaction of TPB with components of the assay system was ruled out by mixing experiments and by adding increasing amounts of the agent to complete assay systems. Enzyme activity in mixtures of various proportions of treated cells and controls was additive, indicating absence of inhibition. Membrane preparations suspended in 0.1 *M* *tris*-HCl, pH 8.0 were used to assess the effect on the enzyme reaction. No inhibition of ACHE activity was noted with up to 10^{-4} *M* TPB (Table II). However, considerable precipitation occurred at higher concentrations.

Enzyme inactivation was irreversible, repeated washing of TPB-treated cells did not restore ACHE activity and none was detected in the supernatant liquid after incubation with TPB. Prolonged dialysis of hemolysates and of membrane preparations did not reverse enzyme inactivation. Reduction in activity was also noted when whole blood was exposed to TPB. Additional information was obtained by studying other parameters which influence enzyme inactivation. Thus, the effect of TPB was dependent on temperature. With 3×10^{-4} *M*, 12% of the activity was lost at 4 °C as compared with a reduction of 39% and 52% at 37 °C and 45 °C, respectively. Essentially the same proportion of activity was destroyed when cells were incubated with TPB dissolved in 0.1 *M* sodium phosphate buffers adjusted to pH's varying between 6.5 and 8.5. Inactivation was a relatively fast process and the percent of activity lost did not significantly increase with the length of incubation; e.g., with 2.4×10^{-4} *M* and at 37 °C, 30% of the activity was destroyed after 10 min and 35% after 180 min. The action of TPB was related to the amount of erythrocyte in the incubation mixture. Thus, reduction in ACHE activity was inversely proportional to the amount of cells present when the concentration and volume of TPB were kept constant (Table III).

Our results show that the exposure of human erythrocytes to TPB resulted in the reduction of ACHE activity. That this loss represents irreversible enzyme inactivation was substantiated by the failure to augment or restore ACHE activity by repeated washing or prolonged dialysis of TPB-treated cells or membranes. These findings resemble the effect seen with other ACHE-inactivating agents[10, 14, 15] but contrast the partial restoration of Mg^{2+}-ATPase by dialysis of thyroid cell membrane preparations treated with TPB[9]. Several additional lines of evidence indicate that this agent altered the enzyme rather than interacted with components of the assay system. These include: 1. The absence of inhibition when ACHE activity was measured in the presence of TPB; 2. Enzyme activity was assayed on 0.1% dilutions of cells or membranes and any remaining TPB would have been diluted by the same factor and 3. ACHE activity in mixtures of treated cells and controls was additive.

Since ACHE does not require potassium for activity, the present results cannot be attributed to chelation. Although the hemolysis noted at concentrations greater than 6×10^{-4} *M* is probably due to the lipid solubility of TPB[16], it appears unlikely that enzyme inactivation is related to this characteristic, since ACHE activity is not affected by hypotonic lysis or by hemolytic, polyene antibiotics such as amphotericin B and filipin[14]. A more likely possibility is that TPB reacts directly with the ACHE of the red cell membrane causing molecular changes which render the enzyme inactive. Such an interpretation would be in accord with the notion of a TPB-protein interaction, as has been postulated to explain the inhibition of other enzymes by this agent[9]. Although the present investigation was restricted to a constituent of the erythrocyte membrane, the study of the effects of TPB on intracellular components could yield additional information which may explain the unusual alterations observed when other types of cells and their organelles are exposed to this substance[17].

Zusammenfassung. Menschliche Erythrozyten wurden mit Tetraphenylbor (TPB) behandelt, wobei die TPB-Konzentrationen über 6×10^{-4} *M* hämolytisch waren. Die Acetylcholinesterase der Erythrozytenmembranen wurde durch nicht-hämolytische TPB-Konzentrationen irreversibel, temperatur- und konzentrationsabhängig inaktiviert.

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[14] F. Herz, Nature, Lond. *214*, 497 (1967).
[15] F. Herz, Proc. Soc. exp. Biol. Med. *127*, 1240 (1968).
[16] O. H. LeBlanc Jr., Biochim. biophys. Acta *193*, 350 (1969).
[17] This work was supported by NIH Grant No. HD 01461 and by the Baltimore Rh Typing Laboratory, Inc.

Activation of Subtilisin by Luteotropic and Luteinizing Hormone

The aim of the present paper is to provide information about a specific effect exerted by mammalian peptide hormones on the action of subtilisin. The rate of the hydrolysis of *N*-L-leucyl-2-naphthylamine catalyzed by subtilisin was 10–11 times higher in the presence of sheep luteotropic hormone (0.014 m*M*) and 7–8 times in the presence of equine luteinizing hormone (0.011 m*M*), than without any hormones. No other enzyme-hormone system investigated in this laboratory was found to display this type of effect, which was termed competitive activation.

Luteinizing hormone and luteotropic hormone, as well as *N*-L-leucyl-2-naphtylamine, were obtained from Mann Research Laboratories, Inc. (New York, N.Y., USA). Subtilisin of *Bacillus subtilis* was purchased from Sigma Chemical Company (St. Louis, Mo., USA). All other reagents were products of E. Merck AG (Darmstadt, Germany). The assay of subtilisin was in principle carried out with *N*-L-leucyl-2-naphthylamine as substrate, as described earlier[1]. It may be mentioned, however, that all reactions were performed in 0.025 *M* borate buffer, pH 8.0, at 30 °C. The amount of subtilisin was 30 µg in all reaction mixtures (0.6 ml)[1], unless otherwise mentioned. The experimental data were treated by a numerical method which involved regression analysis of weighted data by the method of least squares. Details about the computer calculations and about the use of Hanes' equation in the determination of the kinetic parameters have been

described earlier[2]. The values of 24,000 and 30,000, respectively[3], were used as molecular weight of sheep luteotropic and equine luteinizing hormone. It is evident that the preparations contained low amounts of foreign proteins. Hence the molarities used may not be completely correct.

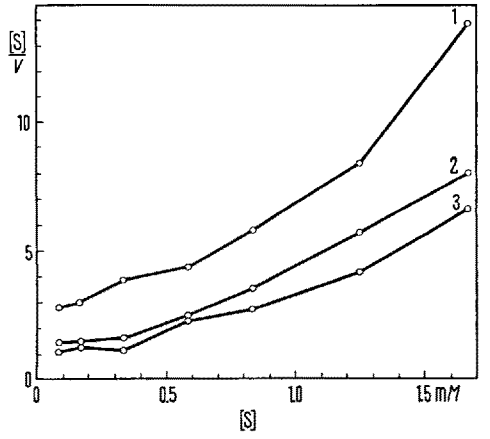
Preliminary experiments showed that, of reactions catalyzed by several different proteolytic and other enzymes, only the rates of those catalyzed by subtilisin were potently increased by luteotropic and luteinizing hormone (Table I). The less enzyme present, the more pronounced was the activation caused by these hormones. Enzyme amounts 10 times higher than that indicated in Table I led to much less noticeable effects. The hormone preparations themselves did not exert any enzyme activity towards the substrate used and they were found to be homogenous in gel permeation chromatography on Sephadex G-100 Superfine columns. The effect of the 2 hormones on the maximum velocity (V) and substrate constant (K_s) is presented in Table II. V was increased in both cases with increasing concentration of the activators. K_s was first increased with an increasing concentration of the hormones, but higher concentrations decreased the value of K_s. Preincubation of the enzyme (from 5 to 60 min at 30 °C without substrate) in the presence of the hormones did not significantly alter the pattern of activation: the same increase in the rate of the hydrolysis was obtained regardless of the length of the preincubation. The Figure shows the effect of 3 different concentrations of luteotropic hormone. In the conditions involved and in the total absence of activator, the ratio of [S] to v (where v is the initial velocity) becomes very large and the corresponding curve did not fit into the Figure. At infinite concentrations of luteotropic hormone $[S]/v$ became almost constant at different substrate concentrations. There was pronounced curvature in the plots which indicates that the degree of activation was not linear with hormone concentration.

The results showed that both sheep luteotropic and equine luteinizing hormone specifically activated subtilisin. An increasing concentration of luteotropic and luteinizing hormone first increased K_s and then decreased its value, an indication of stronger interaction between substrate and enzyme at low and high hormone concentrations. With both hormones, the curve of K_s versus hormone concentration passed through a maximum. Luteotropic and luteinizing hormone may act as competitive activators[4].

Table I. Effect of various peptide hormones on the rate of the hydrolysis of *N*-L-leucyl-2-naphthylamine catalyzed by subtilisin

Luteotropic hormone (sheep)	Luteinizing hormone (equine)	Vasopressin (synthetic)	Oxytocin (synthetic)
+1000	+700	—60	—95

Hormone concentrations: vasopressin and oxytocin, 0.16 mM, luteotropic hormone, 0.014 mM (200 µg of the commercial preparation in the reaction mixture of 0.6 ml); luteinizing hormone, 0.011 mM (200 µg per 0.6 ml). Substrate concentration: 0.166 mM. The effect of the hormones is given in inhibition % (designated as (—) or activation % (+).



Effect of sheep luteotropic hormone on the rate of the hydrolysis of *N*-L-leucyl-2-naphthylamine catalyzed by subtilisin. Hane's plot of substrate concentration divided by the initial velocity ([S]/v in $10^3 \times$ min) versus the substrate concentration. Concentrations of luteinizing hormone: curve 1, $3.5 \times 10^{-6} M$; curve 2, $7.0 \times 10^{-6} M$; curve 3, $10.05 \times 10^{-6} M$.

Table II. Calculated values of the maximum velocity (V) and substrate constant (K_s) for the subtilisin-catalyzed hydrolysis of N-L-leucyl-2-naphthylamine, activated by luteotropic hormone (sheep) and luteinizing hormone (equine)

Hormone concentration ($10^6 \times M$)	$10^7 \times V$ M min^{-1}	$10^4 \times K_s$ M
Luteotropic hormone		
10.05	6.86 ± 1.79	4.53 ± 2.20
8.75	5.55 ± 1.02	4.32 ± 1.43
7.00	5.75 ± 1.04	5.50 ± 2.09
5.25	5.82 ± 1.27	7.86 ± 2.13
3.50	1.05 ± 0.10	0.95 ± 0.31
1.75	0.52 ± 0.08	0.50 ± 0.15
0	0.38 ± 0.05	0.50 ± 0.18
Luteinizing hormone		
8.31	3.50 ± 0.18	3.88 ± 0.57
6.92	3.09 ± 0.11	3.74 ± 0.41
5.54	2.93 ± 0.18	5.18 ± 0.85
4.15	2.41 ± 0.23	4.48 ± 1.19
2.77	1.56 ± 0.13	3.73 ± 0.90
1.38	1.13 ± 0.12	3.80 ± 0.91
0	0.34 ± 0.04	2.15 ± 0.60

The calculations were based on Hanes' equation[2].

Zusammenfassung. Die Katalysegeschwindigkeit der Hydrolyse von *N*-L-Leucyl-2-naphthylamin durch Subtilisin war grösser in Gegenwart von luteotrophem Schafhormon und etwas geringer in Gegenwart von luteinisierendem Pferdehormon. Dieses Verhalten wird als kompetitive Aktivierung erklärt.

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[1] K. K. MÄKINEN and P.-L. MÄKINEN, Biochim. biophys. Acta *206*, 143 (1970).

[2] K. K. MÄKINEN, E. K. EURANTO and J. J. KANKARE, Acta chem. fenn. *B42*, 129 (1969).

[3] S. M. RAPAPORT, *Medizinische Biochemie*, 3rd Edn. (VEB Verlag Volk und Gesundheit, Berlin 1965), p. 745.

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