and 50 µg/l of phenylmethane sulfonyl fluoride. The purity of the preparation was checked by phase contrast microscopy and by biochemical analysis (protein/DNA/RNA ratio).

In vitro phosphorylation and dephosphorylation of nucleoli. Nucleoli (0.3 mg protein/ml) were incubated at 30 °C in a reaction medium containing 250 m*M* sucrose, 100 m*M* *Tris*-HCl, pH 7.5, 20 m*M* $MgCl_2$, 100 m*M* NaCl, 6 m*M* NaF, 10 m*M* DTT and 0.005 m*M* (γ-^{32}P) ATP (1–1.5 Ci/mmole)[10]. At appropriate time intervals, aliquots were used for protein-bound ^{32}P determination according to the method B of REIMANN et al.[11]. The dephosphorylation rate due to phosphatase activity was determined by diluting the (γ-^{32}P)ATP 10-fold with unlabelled ATP in the reaction mixture after 10 min of incubation and measuring the radioactivity retained in the nucleolar proteins during the following 20 min. Radioactivity was measured in a Beckman LS 100 liquid scintillation spectrometer. All assays were performed at least in duplicate.

Analytical methods. Proteins were determined by the technique of LOWRY et al.[12] with bovine serum albumin as a standard. DNA and RNA were estimated according to the methods of BURTON[13] and CERIOTTI[14], respectively, by using appropriate standards.

Results and discussion. The effect of T_3 administration on in vitro net ^{32}P uptake into liver nucleolar proteins is shown in the Table. The results indicate a marked increase in ^{32}P incorporation into liver nucleolar proteins (to about 74%) which takes place 12 h after T_3 administration to thyroidectomized rats. A larger increase has been found after 24 and 48 h of treatment. The time course of in vitro phosphorylation and dephosphorylation of liver nucleolar proteins from control and T_3-treated rats is illustrated in the Figure 1. The results obtained in these experiments are in agreement with the observation of GRUMMT[10] who demonstrated that the *in vitro* turnover of phosphate groups under these conditions is very low. In addition, it can be seen that T_3 administration does not induce appreciable changes in the phosphatase activity of nucleoli incubated in vitro. This excludes the possibility that the increased amount of ^{32}P incorporated into liver nucleolar proteins from T_3-treated rats may depend on a lower dephosphorylation rate. Therefore, it can be assumed that the increased in vitro ^{32}P uptake into nucleolar proteins is due to a stimulation of the nucleolus-associated protein kinase activity induced by T_3 administration.

In vitro net ^{32}P uptake into liver nucleolar proteins

Time of treatment (h)	No. of experiments	Net ^{32}P uptake	Increase (%)	Student's *t*-test (*p*)
—	16	405 ± 195		
12	6	705 ± 248	+ 74	0.001
24	6	810 ± 328	+ 100	0.01
48[a]	4	778 ± 307	+ 92	0.01

The net ^{32}P uptake is expressed as pmoles ^{32}P incorporated into 1 mg nucleolar proteins /10 min. The values represent the mean ± SD. In each experiment, nucleoli pooled from 3 to 4 rats were used. [a]The rats received a second dose of T_3 24 h before sacrifice.

In another set of experiments (manuscript in preparation), the substrate specificity of the nucleolus-associated protein kinase activity was investigated. It has been shown that this kinase activity is able to phosphorylate casein and phosvitin, does not utilize to a significant extent histones or protamine as substrate, and is not stimulated by cyclic AMP.

So far the physiological role of the phosphorylation of nucleolar proteins has not yet been defined, although studies with isolated nucleoli support the suggestion that several highly phosphorylated proteins are involved in the assembly and processing of preribosomal particles[8]. According to this hypothesis, the increased phosphorylation of liver nucleolar proteins induced by T_3 administration to thyroidectomized rats may result in a stimulated maturation of preribosomal particles and, eventually, in the well known accumulation of newly formed ribosomes in the cytoplasm of liver cells.

Studies are in progress to investigate whether the phosphorylation pattern of liver nucleolar proteins is altered by T_3 administration.

[10] I. GRUMMT, FEBS Lett. *39*, 125 (1974).
[11] E. M. REIMANN, D. A. WALSH and E. G. KREBS, J. biol. Chem. *246*, 1986 (1971).
[12] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).
[13] K. BURTON, Biochem. J. *62*, 315 (1956).
[14] G. CERIOTTI, J. biol. Chem. *214*, 59 (1955).

Influence on the Trypsin Activity by the Side Chain of Arginine Homologues

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Summary. The N-α-tosyl-*p*-nitroanilides of homoarginine and of the two shorter arginine homologues were synthesized. These compounds behave as specific, chromogenic substrates for trypsin.

In previous studies[1,2] the question arose to what extent longer or shorter homologues could replace L-arginine in its function as a part of biologically active molecules. In addition to other systems, the behaviour of trypsin toward suitable Arg-analogues presented itself as a simple model for this purpose.

In this study, use was made of *N*-α-tosyl-*p*-nitroanilides of homoarginine and the Arg-homologues shortened by 1 and 2 methylene groups, because, on the one hand, the tosyl group causes no racemization during its attachment as in the case of the benzoyl residue[3]. On the other hand, the *p*-nitroanilides are the more specific substrates than the corresponding esters[4].

Materials and methods. The shorter homologues with the tosyl group were synthesized in accordance with the method of RUDINGER[5], tosyl-homoarginine as described

elsewhere[6,7]. KASAFIREK's method[8] was selected for the subsequent reaction to the following *p*-nitroanilides: *N*-α-tosyl-L-2-NH_2-3-guanidinopropionic acid-*p*-nitroanilide (TPA). $C_{17}H_{20}N_6O_5S \cdot HCl$ (456.9) mp: 228–230 °C, calc.: C 44.69 H 4.63 N 18.39. Found: C 44.90 H 4.56 N 18.29. *N*-α-tosyl-L-2-NH_2-4-guanidinobutyric acid-*p*-nitroanilide (TBA). $C_{18}H_{22}N_6O_5S \cdot HCl \cdot H_2O$ (488.9) mp: 200–204 °C, Calc.: C 44.22 H 5.13 N 17.19. Found: C 44.47 H 5.13 N 17.14. *N*-α-tosyl-L-homoarginine-*p*-nitroanilide (THA). $C_{20}H_{26}N_6O_5S \cdot HCl$ (499.0) mp: 134–136 °C, calc.: C 48.14 H 5.45 N 16.84 Found: C 48.22 H 5.78 N 16.92.

All substances were chromatographically pure (solvent system $CH_3OH/CHCl_3/NH_4OH_{conc.}$ = 10:10:5 on cellulose-TLC-plates, Riedel de Haen, Germany, detection Sakaguchi reagent). The measurement of the enzymatic hydrolysis was made at 25 °C. The conditions, substances and concentrations were chosen as described[8].

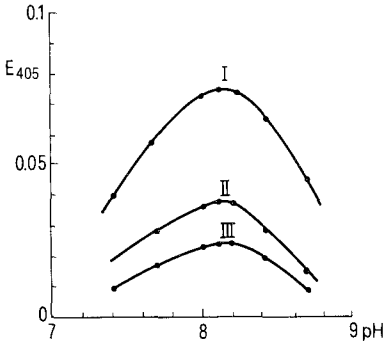

Fig. 1. Tryptic hydrolysis of THA (I), TBA (II) and TPA (III) as a function of pH. Trypsin concentration 7.5 μg/ml, incubation period 10 min, substrate concentration $0.83 \cdot 10^{-3}$ *M*, 0.042 *M Tris* buffer (0.017 *M* $CaCl_2$) pH 8.2, temperature 25 °C.

Results and discussion. As in the case of the Arg-*p*-nitroanilides, the cleavage of the substances by trypsin gives rise to an increase of the extinction at 405 nm. The investigation of the initial velocity of the trypsin digestion as a function of the pH shows a bell-shaped curve for all 3 substrates (Figure 1). The maximum is around pH 8.2 and therefore in the range of the Arg-derivatives reported before[3,8].

Figure 2 shows the rate of the hydrolysis as a function of the concentration of trypsin. For a correct estimation of the extent of the cleavage, the purchasable benzoyl-Arg-*p*-nitroanilide (BAPA) was determined in parallel measurements, which is in a similar order as the tosyl derivative of Arg[8]. Considering the hydrolysis of the homologous substances and BAPA, there is a low rate of digestion of the compounds with an altered side chain. Nevertheless there is, up to a range of 8 μg/ml trypsin, a linear dependence of the rate of hydrolysis on the enzyme concentration. This fact suggests that there is a specifity of trypsin for these chromogenic substrates. The longer the side chain, the better can the Arg-homologues be attacked by trypsin. The reaction conditions used in this study caused no detectable self-hydrolysis, which could occur in the investigation of the corresponding esters[4].

Due to the mean solubility in the systems employed, the measurement of the rate of the hydrolysis as a function of the concentration of the substrate was carried out only for the homoarginine derivative. The data obtained resulted in the K_m (app) = $0.87 \cdot 10^{-2}$ *M* for tosyl-har-*p*-nitroanilide. This value is considerably greater than that found for the corresponding Arg compound[8].

The results presented here corroborate and extend the findings pointed out by other groups[9–11], wherein it has been established that suitable Arg-homologous compounds can be split by trypsin. Still open to question is how to correlate this fact with SHIELDS et al.[12] where it is reported that *N*-α-benzoyl-L-har amide is not cleaved by trypsin.

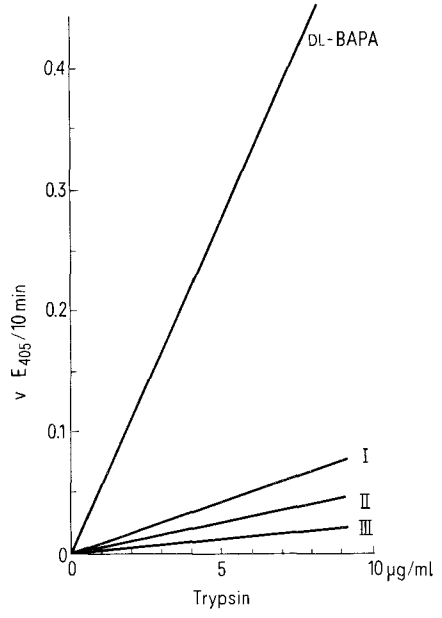

Fig. 2. Rate of hydrolysis of THA (I), TBA (II) and TPA (III) as a function of the trypsin concentration. Reaction conditions see Figure 1.

[1] G. WEITZEL, K. EISELE and W. STOCK, Hoppe-Seylers Z. physiol. Chem. *356*, 583 (1975).
[2] F. SCHWEGLER and W. STOCK, Hoppe-Seylers Z. physiol. Chem. *356*, 839 (1975).
[3] B. F. ERLANGER, N. KOKOWSKI and W. COHEN, Arch. Biochem. Biophys. *95*, 271 (1961).
[4] H. TUPPY, U. WIESBAUER and E. WINTERSBERGER, Hoppe-Seylers Z. physiol. Chem. *329*, 278 (1962).
[5] J. RUDINGER, K. PODUSKA and M. ZAORAL, Colln. Czech. chem. Commun. *25*, 2022 (1960).
[6] B. C. W. HUMMEL, Can. J. Biochem. Physiol. *37*, 1393 (1959).
[7] K. EISELE and F. SCHWEGLER, Justus Liebigs Annln. Chem. *1975*, 2033.
[8] E. KASAFIREK, M. CHAVKO and M. BARTIK, Colln. Czech. chem. Commun. *36*, 4070 (1971).
[9] J. B. BAIRD, E. F. CURRAGH and D. T. ELMORE, Biochem. J. *96*, 733 (1965).
[10] J. CHAUVET and R. ACHER, FEBS Lett. *18*, 265 (1971).
[11] D. S. SEIDL and I. E. LIENER, Biochem. biophys. Res. Commun. *42*, 1101 (1971).
[12] G. S. SHIELDS, R. L. HILL and E. L. SMITH, J. biol. Chem. *234*, 1747 (1957).