Table 2. Effect of L-lysine administration on the biosynthesis and degradation of L-ascorbic acid by the liver (the values are means ± S. E. M.)

Group of animals	L-ascorbic acid synthesized from D-glucuronolactone (μmoles/g microsomal protein)	from L-gulonolactone (μmoles/g microsomal protein)	Dehydroascorbatase activity (μmoles of 2–3 diketogulonic acid formed/g soluble supernatant protein)
Pair-fed control	97.4 ± 9.9 (4)	280 ± 19 (4)	639 ± 27 (5)
Treated	57.6 ± 4.9 (5) $p < 0.01$	188 ± 12 (5) $p < 0.01$	485 ± 29 (5) $p < 0.01$

The figures in the parentheses indicate the number of animals.

ascorbic acid content of the plasma and the kidney remained unaltered, whilst the liver total ascorbic acid level was markedly reduced following lysine treatment. The in vitro biosynthesis of L-ascorbic acid from D-glucuronolactone as well as from L-gulonolactone, as demonstrated in table 2, was found to be significantly reduced after lysine administration. This suggests that the fall in liver total ascorbic acid level after L-lysine administration might arise from the diminished synthesis of L-ascorbic acid. L-tyrosine at 5% level in the diet has also been found to depress the synthesis of L-ascorbic acid from D-glucuronolactone[11]. Administration of phenylalanine was reported to result in an inhibition of protein synthesis[12]. The diminution in the activities of D-glucuronoreductase and L-gulonooxidase after L-lysine administration may likewise be attributed to the reduced synthesis of the enzyme protein resulting from a depression of synthesis of protein as a whole.

Administration of L-lysine also produced a marked depression in the liver dehydroascorbatase activity (table 2). This suggests that the animal tries to compensate the loss in tissue ascorbic acid produced by the reduction in the rate of its biosynthesis. But the fact that the liver total ascorbic acid level still remained diminished signifies that the magnitude of reduction in the rate of ascorbic acid biosynthesis was more than that of the reduced rate of its degradation.

The absence of alteration in the kidney total ascorbic acid level after L-lysine administration may be ascribed to the unchanged plasma total ascorbic acid level under the same condition. Inspite of diminished synthesis of L-ascorbic acid by the liver, the plasma total ascorbic acid level remained unaltered after L-lysine administration. This absence of alteration in the plasma total ascorbic acid level might be due to supposedly diminished excretion of ascorbic acid in the urine. The possibility that the changes in ascorbic acid metabolism following repeated injections of L-lysine might be an artefact may be ruled out by the fact that the pair-fed control rats also received repeated injections of the medium used to dissolve the amino acid.

In the course of this investigation, it was also noted that food intake by the treated animals did not differ significantly from those of the control groups, and their body weight too remained unchanged. It therefore suggests that L-lysine administration at the present dose appears to have no effect on the general growth pattern or on the growth of different organs of the animal.

Hydrolysis of histones by horse urinary kallikreins

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Summary. Horse urinary kallikrein was shown to hydrolyze histones from calf thymus and chicken nuclei erythrocytes. The hydrolysis is inhibited by benzamidine and hyposulfite but not by soy-bean trypsin inhibitor; horse plasma kallikrein also produces this hydrolysis.

Kallikrein (EC 3.4. 21.8) is the general designation for serine proteases which liberate hypotensive peptides (kinins) from a plasma protein, kininogen. These enzymes have been found in several tissues and biological fluids such as plasma, glandular secretions and urine. Although they have some properties similar to those of trypsin, mainly in their ability to hydrolyze N-α-substituted-arginine esters, they show a much higher proteolytic specificity towards its natural substrate, kininogen[3]. Horse urinary kallikrein (HoUK), a glandular kallikrein which liberates lysyl-bradykinin from horse kininogen, does not cleave either casein or hemoglobin[4,5] but it was reported to hydrolyze salmine[5] and polyarginine[6]. These findings raised some doubts whether kallikreins or contaminants were responsible for this hydrolysis[7], but the results on specific inhibition are in favour of hydrolysis by kallikrein itself; hog pancreatic kallikrein was also said to hydrolyze salmine[8].

The studies reported in this communication were undertaken as an attempt to confirm the observation of non-specific proteolysis of salmine and to extend these studies to the action of HoUK on histones, proteins which have also a large basic amino acid content.

Material and methods. Horse urinary kallikrein (HoUK) was prepared by procedures already described[4,9]. The enzyme activity was followed by its esterase activity on N-α-tosyl-L-arginine-methyl ester (TAME), under the conditions described for human plasma kallikrein[10]. An enzyme preparation with specific activity 10.1 units/mg (μmoles TAME hydrolyzed per min) was used throughout

the experiments. The actual concentration of the enzyme solution used was calculated to be 3.0×10^{-6} M by the titration of its active site by p'-nitro-phenyl-p-guanidine-benzoate (NPGB)[11]. This preparation had no detectable activity on casein, aminoacyl-β-naphthylamides (aryl-amidase activity) and lysyl-bradykinin (LBK) (amino-peptidase activity) which could not be converted to the more potent polypeptide bradykinin (BK)[12]. 10fold higher concentrations of HoUK did not inactivate BK or LBK under conditions in which other enzymes easily hydrolyze both peptides[13], showing that the HoUK preparation was free of kininase activity. Horse plasma kallikrein (HoPK) (1.3 TAME units/mg) was prepared by affinity chromatography with soy-bean-trypsin-inhibitor-sepharose, as described for the human enzyme[10]. Hog pancreatic kallikrein (HPK)[14] (10 TAME units/ml) was a kind gift from Bayer AC. The kinin liberating activity of HoUK was determined by the bioassay on the isolated guinea-pig ileum of LBK released from heat-treated horse plasma[4]. Chicken erythrocyte histones were prepared from purified nuclei[15]; the nucleoproteins were separated by the method previously described[16], and the histones extracted with 0.05 M sulfuric acid and 0.05 M sodium hyposulfite. They were precipitated with 20% trichloroacetic acid and then with 85% acetone. The histones were free from the proteolytic activities described above and no intrinsic hydrolysis occurred after prolonged incubations.

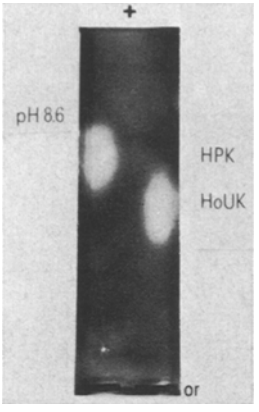

Fig. 1. Electrophoresis of HoUK (0.01 TAME units) and HPK (hog pancreatic kallikrein, 0.05 TAME units) on 1% agarose gel (100 volts, 4 °C, 0.05 M barbital buffer, pH 8.6). Following electrophoresis, the gel was incubated with a cellulose acetate strip saturated with 1% salmine, for 15 min, 37 °C. The protein was stained with 0.1% amido black (for details see text).

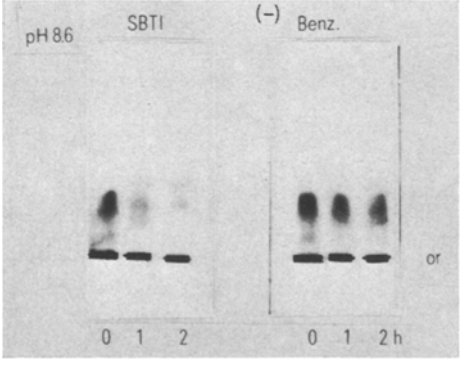

Fig. 2. Agarose electrophoresis of chicken erythrocytes histones; 100 μg of histone incubated with 0.1 TAME units of HoUK, 0.05 M Tris-HCl, pH 8.0 30 °C, at different times. The reaction was stopped by 0.05 M H_2SO_4 0.04 M $NaHSO_3$. The electrophoresis of histones was carried out at pH 8.6, 0.05 M barbital buffer, 100 volts, 1 h, 4 °C. SBTI; 100 μg/ml final concentration, and 10^{-2} M benzamidine.

Horse urinary and hog pancreatic kallikreins were subjected to electrophoresis in 1% agarose gel at pH 8.6, with 0.05 M barbital buffer[17]. After electrophoresis, the gel was overlaid by a cellulose acetate strip previously wetted with 1% of one of the following protein substrates: casein, salmine, calf thymus and chicken erythrocyte histones. After incubation, the agarose gel was fixed and stained with amidoblack; the enzyme activity showed as a negative spot against a blue background[18]. For the determination of the initial rate of histone hydrolysis, the histones either from chicken erythrocytes or calf thymus (10 μg) were incubated with kallikrein (0.1 TAME/unit) in 100 μl of 0.05 M Tris-HCl, pH 8.0 at 37 °C for different time intervals, the reaction was stopped by 0.05 M H_2SO_4, 0.04 M $NaHSO_3$. The incubates were applied to an agarose gel plate and the electrophoresis carried out for 1 h in 0.05 M barbital buffer, pH 8.6[17], 100 volts. The fixed and stained remaining histones were estimated by densitometry, and the initial rate calculated from the first order hydrolysis curves.

Results and discussion. The visualization of proteolytic activity after gel electrophoresis of HoUK showed that both salmine (figure 1) and histones are substrates for kallikrein, while no proteolytic activity could be detected when casein or azocasein were used as substrates. The enzyme eluted from the gel had both kinin-releasing and esterolytic activities, indicating that kallikrein is hydrolyzing the histones and salmine. In order to confirm the action of kallikreins upon histones, specific inhibitors were used (figure 2). Soy-bean trypsin inhibitor, which is known as an inhibitor of plasma kallikrein but not of glandular kallikreins, had no effect, while benzamidine,

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2 Fellow from CNPq, from Instituto Biológico (São Paulo).
3 M. E. Webster, in: Handbuch der experimentellen Pharmakologie, vol. 25, p. 659. Ed. E. G. Erdös. Springer-Verlag, New York 1970.
4 E. S. Prado, J. L. Prado and C. M. W. Brandi, Archs int. Pharmacodyn. *137*, 358 (1962).
5 C. M. W. Brandi, J. Mendes, A. C. M. Paiva and E. S. Prado, Biochem. Pharmac. *14*, 1665 (1965).
6 E. S. Prado, R. C. R. Stella, M. J. Roncada and J. L. Prado, in: International Symposium on Vaso-Active Polypeptides: Bradykinin and Related Kinins, p. 141. Ed. M. Rocha e Silva and H. A. Rothschild. Edart, São Paulo 1966.
7 J. V. Pierce, in: Handbuch der experimentellen Pharmakologie, vol. 25, p. 21. Ed. E. G. Erdös. Springer-Verlag, New York 1970.
8 M. Zuber and E. Sache, Biochemistry *13*, 3098 (1974).
9 M. E. Webster and E. S. Prado, in: Methods in Enzymology, vol. 19, p. 681. Ed. G. E. Perlmann and L. Lorand. Academic Press, New York 1970.
10 C. Sampaio, S. C. Wong and E. Shaw, Archs Biochem. Biophys. *165*, 133 (1974).
11 C. A. M. Sampaio and E. S. Prado, Gen. Pharmac. *7*, 163 (1976).
12 J. A. Guimarães, D. R. Borges, E. S. Prado and J. L. Prado, Biochem. Pharmac. *22*, 3157 (1973).
13 C. A. M. Sampaio, S. T. Nuñez, M. G. N. Mazzacoratti and J. L. Prado, Biochem. Pharmac., *25*, 2391 (1976).
14 H. Fritz, I. Eckert and E. Werle, Hoppe Seylers Z. Physiol. Chem. *348*, 1120 (1967).
15 V. Seliagy and M. Miyagi, Exp. Cell Res. *58*, 27 (1969).
16 C. M. Mauritzen, W. C. Starbuck, I. S. Saroja, C. W. Taylor and H. Busch, J. biol. Chem. *242*, 2240 (1967).
17 C. P. Dietrich, in: Plasma Protein Turnover, p. 17. Ed. R. Bianchi, G. Mariani and A. S. McFarlane. The MacMillan Press, London 1976.
18 C. A. M. Sampaio, in: International Symposium on Macromolecules (abstr.), p. 259. Rio de Janeiro 1974.

Inhibition of kallikrein (HoUK) using histones as substrates

Incubations	Half times of hydrolysis* (min)	Per cent Inhibition**
HoUK	80	–
HoUK + SBTI	81	0
HoUK + 0.1 M benzamidine	155	48
HoUK + 0.05 M $NaHSO_3$	No hydrolysis	100

* Hydrolysis of 50% of the histones in the conditions described in the text. ** The inhibition was compared by the half times of hydrolysis.

which is a competitive inhibitor for both enzymes[3], did inhibit the histones hydrolysis. In the table the effectiveness of inhibition of HoUK hydrolysis of chicken erythrocyte histones by both inhibitors is indicated. The hydrolysis of histones by trypsin was inhibited by both soybean-trypsin-inhibitor (SBTI) and benzamidine as would be expected. The table shows that hyposulfite is a potent inhibitor of kallikrein; this inhibition is similar to that promoted by this agent towards proteases contaminating crude preparations of histones[19].

Calf thymus histones were also cleaved by horse plasma kallikrein (HoPK). It is known that this preparation of histone can be fractionated, at least, into 5 fractions by electrophoresis in polyacrylamide gel[20]; those fractions were prepared and all of them were hydrolyzed by HoPK, forming initially large fragments stained with amidoblack and finally soluble peptide chains.

The proteolysis of proteins other than kininogen by kallikreins is unusual, because these enzymes have a very strict specificity; arginine-rich substrates, however, were expected to be cleaved unless the susceptible bonds were not accessible to the protease. The primary structures of some histones are known[21], and this group of proteins may be used as model compounds to study the specificity of kallikreins in the same way as the the B-chain of insulin is used for several proteolytic enzymes.

19 S. Panyim, R. H. Jensen and R. Chalkley, Biochim. biophys. Acta *160*, 252 (1968).
20 S. Panyim and R. Chalkley, Archs Biochem. Biophys. *130*, 337 (1969).
21 Y. Ogawa, G. Quagliarotti, J. Jordan, C. W. Taylor, W. C. Starbuck and H. Busch, J. biol. Chem. *244*, 4387 (1969).

The effect of bovine myelin basic protein on uptake and release of H^3-labelled 5-hydroxytryptamine, L-noradrenalin and γ-aminobutyric acid in rat cortex slices

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Summary. At concentrations above 10^{-5} M myelin basic protein (MBP) induced a small inhibition of the uptake of H^3-5HT and H^3-NA into rat cortex slices. Release of 5HT, NA and Gaba was not affected by 10^{-5} M MBP.

The first indication that myelin basic protein (MBP) could have electrophysiological activity came from Jankovic et al.[2], who reported that MBP, injected intraventricularly into the rabbit, caused major bioelectric and behavioural changes. Interference of MBP with the action of 5-hydroxytryptamine (5HT) in particular has been suggested by Carnegie[3,4]; he proposed that the encephalitogenic region of MBP has a structure similar to that of the natural 5HT receptor, and that interaction of MBP with 5HT could be the chemical basis of its electrophysiological action. Honegger et al.[5] have provided evidence that MBP has an inhibitory effect on the bioelectric activity of mouse cerebellum cultures and of rat spinal cord in situ, but the biochemical correlate of this electrophysiological activity is not yet known. Direct activation of L-noradrenalin (NA), L-dopamine (DA) or γ-aminobutyrate (Gaba) receptors does not seem to be responsible[5]. We have therefore investigated the presynaptic mechanisms, by testing the action of MBP on the uptake and release of 3 H^3-labelled neurotransmitters in rat cortex slices. Some of these results have been published as an abstract[6].

Materials and methods. Myelin basic protein (MBP) was prepared from fresh bovine spinal cord by a slight modification of the standard methods[7,8]. The solutions of purified MBP were not older than $^1/_2$ h when utilized for the experiments.

Transmitter uptake and subcellular distribution. Male adult SIV 50 rats (150–270 g) were used. 100 mg cerebral cortex slices were pre-incubated (5 min/25 °C) in Krebs-Henseleit medium under oxycarbon. H^3-labelled 5HT or NA (20 µl, 10^{-5} M) and 20–50 µl MBP solution were added to give final concentrations of 10^{-7} M for 5HT and NA, and 10^{-4} to 10^{-7} M for MBP, in 2 ml incubation volume. After 10 min incubation at 25 °C, the reaction was stopped by chilling, and the slices rapidly washed with cold medium. 0.9 ml 0.32 M sucrose was added to 100 mg slices for homogenization. The cell nuclei were removed by

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2 B. D. Janković, Lj. Rakić, M. Janjić, K. Mitrović and J. Ivanuš, Path. eur. *2*, 87 (1966).
3 P. R. Carnegie, Nature, Lond. *229*, 25 (1971).
4 P. R. Carnegie, in: The Neurosciences, Third Study Program, p. 925. Ed. F. O. Schmitt and F. G. Worden. MIT Press, Cambridge, Mass. 1974.
5 C. G. Honegger, B. Gähwiler and H. Isler, in preparation.
6 C. G. Honegger, L. M. Krepelka and H. P. von Hahn, Experientia *32*, 770 (1976).
7 G. E. Deibler, R. E. Martenson and M. W. Kies, Preparat. Biochem. *2*, 139 (1972).
8 P. R. Dunkley and P. R. Carnegie, in: Research Methods in Neurochemistry, vol. 2, p. 219. Ed. N. Marks and R. Rodnight. Plenum Press, New York 1974.