Molecular weights of buffalo αS_1-casein calculated separately on the basis of tryptophan and arginine contents were found to be 26,300 and 26,100 respectively. Here buffalo αS_1-casein was assumed to be a single polypeptide chain[22].

Discussion. Different techniques showed that the carboxyl terminal amino acid residue of buffalo αS_1-casein is leucine-tryptophan. It was also observed that tryptophan was completely released from the C-terminal end under present experimental conditions. It is assumed that 1 mole of amino acid is released per mole of protein and thus the molecular weight of buffalo αS_1-casein was calculated from the data on terminal amino acids. Molecular weights determined on the basis of tryptophan and arginine contents were in reasonable agreement. The molecular weight of buffalo αS_1-casein by ultracentrifugal analysis[23] is reported to be 25,900 which agrees quite well with our value.

Primarily we identified tryptophan, leucine and arginine as their PAPTH derivatives. As amino acid PAPTHs are colored[5], visual identification of these compounds on thin layer chromatograms were convenient. Moreover, the percentage recoveries of these 3 amino acid derivatives were higher than those of others, particularly DNP (2,4 dinitrophenyl)[24] or DNS derivatives.

Although the C-terminal amino acid residues of both buffalo αS_1 and bovine αS_1-caseins are - leucine-tryptophan, the rates of release of these 2 amino acids from the carboxypeptidase-A treated casein differed. It is reported[25] that the concentration of leucine released from bovine αS_1-casein was higher than that of released tryptophan after 5 h, and traces of tyrosine, phenylalanine and valine were also released within 22 h. On the other hand, the concentration of leucine released from buffalo αS_1-casein did not overlap the tryptophan concentration within 30 h, and also no other amino acid was released during this period. The amino acid composition of buffalo αS_1-casein was in reasonable agreement with that of bovine αS_1-casein. Amino acid residues per mole of bovine αS_1-casein was calculated on the basis of a mol. wt value of 28,000[26]. The red color of PAPTH-arginine[5] was intensified by reaction with Sakaguchi reagent[7]. The procedure was also useful to distinguish PAPTH-arginine from PAPTH-lysine, which has the same mobility in some solvent systems.

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Detection of gamma-carboxy glutamic acid residues in an extract of cock spermatozoa

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Summary. In a dialyzed extract of cock spermatozoa - containing proacrosin, a precursor of a trypsin-like endopeptidase - gamma-carboxy glutamic acid residues (Gla) were detected. Such residues are known to exist in several zymogens of vitamin K-dependent serine proteases.

Although the biological activity of vitamin K in the blood clotting system has been known for more than 40 years[3], its molecular function in this process was elucidated only recently.

With prothrombin as an example it was shown that in a post-translational step, which is dependent on the presence of vitamin K and bicarbonate ions, specific glutamic acid residues positioned in the N-terminal part of this protein are converted to gamma-carboxy glutamic acid residues (Gla residues) (Suttie and Jackson[4]). Later on, Gla was detected in factors VII, IX and X[5,6] as well as in other proteins not related to blood coagulation[6,7].

All vitamin K-dependent blood clotting factors are zymogens of proteolytic enzymes belonging to the class of serine-proteases. In addition a vitamin K dependence could be demonstrated for the activities of other serine-proteases such as trypsin, chymotrypsin[8] and collagenase and elastase[9].

Acrosin, a trypsin-like enzyme occuring in sperm cells, where it is stored as an inactive precursor called proacrosin, belongs to the same class of endopeptidases. Its activity in cock sperms was shown to be reduced after chronic marcoumar treatment and subsequently to be restored after application of vitamin K_1[10].

All these experimental facts led to the hypothesis that Gla residues could occur in proacrosin, too, and therefore the present study was undertaken to determine whether in an extract of cock sperms, containing proacrosin, this special amino acid is detectable.

Methods. Cocks (Hubbard strain, 7-9 months old) were supplied by an ordinary chicken breeding farm (SEG, Zell, Switzerland). The animals had free access to water and normal grains (NAFAG, Gossau, Switzerland) during the whole experimental period.

Sperms were taken every day by the method of abdominal massage. Fresh spermatozoa were separated from seminal plasma by immediate centrifugation at 4°C. After having washed the cells with 0.15 M NaCl/ 0.015 M Na-citrate, proacrosin was extracted from the spermatozoa by incubation with 2% acetic acid. The combined extracts were centrifuged (30 min, 45000 rpm, Sorvall ultracentrifuge) and the resulting supernatant was stored at 4°C.

Sperms of 5 animals collected during 5 consecutive days were extracted daily. All supernatants were pooled and dialyzed against a 2% acetic acid solution. Prior to hydrolysis this pool was reduced in volume by Aquacid III (Calbiochem).

For basic hydrolysis 1 portion of the acetic acid extract was mixed with NaOH to a final concentration of 5 M, sealed in a glass ampoule (Durobax glass) and heated for 24 h at 110°C. The hydrolyzed product was desalted on a cationic column (Dowex 50 W X8, NH_4^+-form, elution with 1 M ammonium hydroxide solution) and the solvent was evaporated.

Acidic hydrolysis was performed with HCl (final concentration 6 M) for 24 h at 110°C. The solvent was again removed by evaporation. For a partial acidic hydrolysis (selective destruction of Gla) diluted HCl was added to 1 portion of the basic hydrolytic product to a final concentration of 50 mM. This 2nd hydrolysis was performed at 95°C and lasted 150 min. All hydrolytic products were dissolved in 10 mM HCl prior to being applied to the amino acid analyzer.

Amino acid analyses were run on an automatic aminoacid analyser (BC 200, Bio Cal Instruments). A 1 column/ 3 buffer system was used for separation:

Buffer A: pH 2.85	0-65 min
Buffer B: pH 4.25	65-125 min
Buffer C: pH 6.25	125-240 min

Resin: Animex A 5 (Bio Rad)

Elution of amino acids was visualized by reaction with ninhydrin. L-Gla used as a reference was synthesized and kindly made available by M. Oppliger (Institut für Molekularbiologie, ETH Zürich).

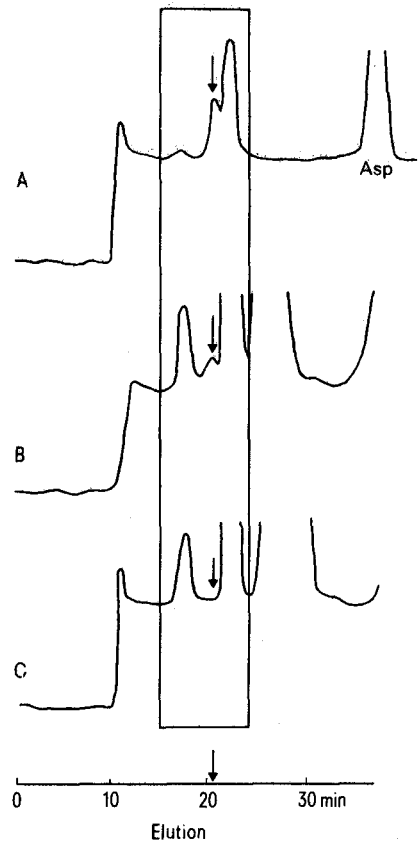
Results. The presence of Gla residues in acetic acid extracts of cock sperms is illustrated in the figure.

As this special amino acid occurs in a very small quantity, its detection was only possible after the extracts of several ejaculates had been pooled. The biological material derived from 5 animals over 5 consecutive days (corresponding to approximatly $7 \cdot 10^{10}$ spermatozoa) was used for a parallel basic and partial acidic hydrolysis. Using such a large amount of material it is not surprising that the more frequently occurring amino acids are no longer well separated and eluted as symmetrical peaks; therefore, a smaller sample was always run in parallel to confirm normal elution.

As expected from the chemical stability of this carboxylated amino acid no Gla was detectable after a hydrolysis in 6 M HCl (results not shown). The disappearance of the Gla peak after a partial acidic hydrolysis may be taken as a proof of the existence of Gla residues in this spermatozoal extract. Synthetic L-Gla added to the product of a partial acidic hydrolysis was eluted at the expected position and the presence of Gla in this biological material was thus verified. An increase in the concentration of glutamic acid after a partial acidic hydrolysis (due to the decarboxylation of Gla) - normally taken as a conclusive indication for the presence of Gla residues - was not observable in these experiments; this may be explained by the unfavorable proportion between Gla and Glu residues in the present extract (about 1 Gla/1000 Glu residues).

Discussion. The presence of a Gla peak after a basic but not after an acidic hydrolysis and the disappearance of this ninhydrin-positive peak after a partial acidic hydrolysis in the amino acid analysis of a spermatozoal extract demonstrates the existence of this carboxylated amino acid in this biological material.

To our knowledge the only previous attempt to demonstrate Gla residues in ejaculates was described by Hauschka[6]. His inability to find Gla is not longer surprising if one takes into consideration that in the present study Gla was only detectable after the material of several ejaculates had been pooled. Additionally, Hauschka did his experiments on semen which was not purified prior to analysis; in such a fluid the spermatozoal proteins are diluted by seminal plasma proteins and therefore, in our experience, Gla residues are extremly unlikely to be found in such material. The following experimental findings seem to be in line with



Amino acid analyses (ninhydrin elution diagram) of differently hydrolyzed portions of a cock sperm extract. *A* After a 24-h hydrolysis in 5 M NaOH (110°C) prior to a partial acidic hydrolysis in 50 mM HCl (150 min, 95°C); after the addition of 25 µg synthetic L-Gla. 50 µl were analyzed. *B* Direct analysis after a 24-h basic hydrolysis; volume 500 µl. *C* After a 24-h basic hydrolysis this portion was additionally hydrolyzed in 50 mM HCl (150 min, 95°C); volume again 500 µl. ↓ position of Gla elution.

a functional connection between vitamin K, Gla residues and the enzymatic activity of acrosin:
a) As previously reported[10], chronic treatment of cocks with marcoumar leads to a significant reduction (around 50%) of acrosin activity in a spermatozoal extract. To corroborate an effect of vitamin K on acrosin activity, it was further shown[10] that this enzymatic activity, when it had been reduced by the administration of marcoumar, could be fully restored by oral application of vitamin K_1[10].
b) After a single oral application of highly labelled ^{3}H-synkavit (tetrasodium-2-methyl-(5,6,7,8-^{3}H)-naphtho-1,4-chinol-diphosphate) the existence of ^{3}H-vitamin $K_{2(20)}$ was observed in spermatozoa[10]. Since ejaculated sperms are not able to convert synkavit to vitamin $K_{2(20)}$ in an in vitro system, in which most other tissues synthesize it[10], all spermatozoal vitamin $K_{2(20)}$ seems to be packed into these cells during developmental stages.
c) The similarities in temporal appearance of labelled spermatozoa after an oral dose of ^{3}H-synkavit and ^{3}H-acetate suggest that vitamin $K_{2(20)}$ is present in membraneous structures. Additionally, after fractionation of sperm cells or partial solubilisation with Triton X-100 of these cells, marked parallels between subcellular- and subfraction-partition of vitamin $K_{2(20)}$ and phospholipids were observed[10], which further supports the localization of vitamin K in membranes. Such a finding indicates that vitamin $K_{2(20)}$ is located in that intracellular region where the activation (carboxylation) of initially inactive proacrosin is thought to take place.
d) According to the time-course of the appearance of labelled sperm cells after a single dose of ^{3}H-leucine (a marker of protein synthesis)[10] it can be stated that maximal synthesis of these macromolecules takes place in round spermatides. At this developmental stage of the sperm cell vitamin K is found in spermatozoa and it can be speculated that a carboxylation of certain Glu residues to Gla would be possible, provided that the carboxylating enzyme can be shown to be present in spermatozoa (to our knowledge this has not yet been shown).

Whether the Gla residues found in an extract of cock sperms are actually derived from proacrosin molecules, and whether they are located in the N-terminal portion of the molecule, as in zymogens of other vitamin K dependent proteases, is not conclusively shown by the present experiments, although many arguments do support such an assumption. It can, however, be stated that the Gla residues characterized here are parts of macromolecules derived from spermatozoa, as they are found in an amino acid analysis after the cells have been freed from seminal fluids and the extract has been dialyzed. Unfortunately, preliminary attempts to purify the zymogen were not successful enough to encourage us to do experiments on such fractions. Until determinations on the purified zymogen, or even better on defined fragments of this molecule are possible it can only be presumed that Gla residues are found in another precursor of a vitamin K-dependent serine protease.

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Inhibition of molybdenum blue formation by ATP

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Summary. Molybdenum blue formation was not affected by the presence of ATP up to a concentration of 1.2 mM/l. At higher concentrations the color development was inhibited relative to ATP concentration, finally reaching complete inhibition. Auto-hydrolysis of ATP was found at a rate of 1.4%/h. An exact determination of inorganic phosphate in the presence of easily hydrolyzed phosphate esters requires the measurement of extinction at fixed time intervals and extrapolation back to time zero.

Most conventional techniques for the determination of membrane-associated ATPase activity involve a timed incubation of enzyme with excess ATP followed by subsequent measurement of released inorganic phosphate by the molybdenum blue method. This procedure depends on the reaction of ammonium molybdate with inorganic phosphate in an acid milieu and subsequent reduction of the complex to molybdenum blue, the optical density of which is directly related to the concentration of orthophosphate. As reducing agents hydroquinone[2], aminonaphthol sulfonic acid[3], stannous chloride[4,5], 2,4-diaminophenol dihydrochloride[6], ferrous sulfate[7], ascorbic acid[8] and other reducing agents have been used. In some instances certain compounds have been observed to curtail the reaction of ammonium molybdate with inorganic phosphate or the subsequent reduction to the blue complex[5,9-12]. An effect of ATP on the color development has not yet been reported. On the other hand an apparent inhibition of Na-K-ATPase by high ATP concentrations has been observed[13] when inorganic phosphate was determined by the molybdenum