ches sur la croissance des cultures bactériennes', Hermann and Cie., Paris, 1942). The present discussion is limited to the former, stoichiometric concept of limitation.
[3] J. von Liebig, 'Organic Chemistry and its Application to Agriculture and Physiology' (English translation by L. Playfair), Taylor and Walton, London, 1840.
[4] a) D.E.F. Harrison, *J. Appl. Chem. Biotechnol.* **1972**, *22*, 417; b) C. Cooney, D.C.I. Wang, R.I. Mateles, *Appl. Environ. Microbiol.* **1976**, *31*, 91; c) S. Hueting, D.W. Tempest, *Arch. Microbiol.* **1979**, *123*, 189; d) T. Egli, *ibid.* **1982**, *131*, 95.
[5] a) T. Egli, J.R. Quayle, *J. Gen. Microbiol.* **1986**, *132*, 1779; b) S. Gräzer-Lampert, T. Egli, G. Hamer, *ibid.* **1986**, *132*, 3337; c) I.G. Minkevich, A.Y. Krynitskaya, V.K. Eroshin, in 'Continuous Culture', Eds. P. Kyslik, E.A. Dawes, V. Krumphanzl, M. Novak, Academic Press, London, 1988, p. 171; d) M.G. Duchars, M.M. Attwood, *J. Gen. Microbiol.* **1989**, *135*, 787; e) M. Rutgers, P.A. Balk, K. van Dam, *Arch. Microbiol.* **1990**, *153*, 487.
[6] D. Herbert, in 'Continuous culture 6: application and new fields', Eds. A.C.R. Dean, D.C. Ellwood, C.G.T. Evans, J. Melling, Ellis Horwood, Chichester, 1976, p. 1.
[7] T. Egli, *Ant. van Leeuwenhoek* **1991**, *60*, 225.
[8] a) O. Hao, C. Chang, *Biotechnol. Bioengin.* **1987**, *29*, 819; b) C. Bonting, H. van Veen, A. Taverne, G. Kortstee, A.J.B. Zehnder, *Arch. Microbiol.* **1992**, *158*, 139; c) S. Frank, T. Egli, A.J.B. Zehnder, *Internat. Symp. Microb. Ecol.*, Halifax, Canada, Abstract book, 1988, p. 155.
[9] a) B. Pengerud, E. Skjoldal, F. Thingstad, *Marine Ecol. Prog. Ser.* **1987**, *35*, 111; b) R.A. Durner, Swiss Federal Institute of Technology, Zürich, Thesis, ETH 1998, Nr. 12591; c) M.S. Zinn, Swiss Federal Institute of Technology, Zürich, Thesis, ETH 1998, Nr. 12987.
[10] G. Piñar, K. Kováčová, T. Egli, J.L. Ramos, *Appl. Environ. Microbiol.* **1998**, *64*, 2970.
[11] N. Al-Awadhi, T. Egli, G. Hamer, C.A. Mason, *Biotechnol. Bioeng.* **1990**, *36*, 816.
[12] a) M. Lucca, M. Romero, J. Diaz, O. Garro, D. Callieri, *World J. Microbiol., Biotechnol.* **1991**, *7*, 359; b) U. von Stockar, C. Larson, I. Marison, M. Cooney, *Thermochim. Acta* **1995**, *250*, 247.
[13] R.A. Durner, Swiss Federal Institute of Technology, Zürich, Thesis, ETH 1998, Nr. 12591.
[14] C.M. Brown, in 'Continuous culture 6: application and new fields', Eds. A.C.R. Dean, D.C. Ellwood, C.G.T. Evans, J Melling, Ellis Horwood, Chichester, 1976, p. 170.
[15] a) Y. Poirier, C. Nawrath, C. Sumerville, *Biotechnology* **1991**, *13*, 142; b) A. Steinbüchel, in 'Biomaterials, novel materials from biological sources', Ed. D.Byrom, Macmillan, Basingstoke, 1991, p. 125.

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1. Introduction

Suitable derivatives of phosphorylcholine that allow labelling are needed for the detection and the characterization of binding properties of proteins interacting with this ligand. A number of different types of phosphorylcholine-binding proteins exist that differ in their properties and functions. C-reactive protein (CRP) [1], frequently employed as a clinical index of acute inflammation, represents the most studied phosphorylcholine-binding protein. CRP is also found in the invertebrate *Limulus polyphemus* as a constitutive and major component of horseshoe crab hemolymph [2].

Another type of proteins possessing the ability to interact with phosphorylcholine was found among proteins isolated

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[4] a) D.E.F. Harrison, *J. Appl. Chem. Biotechnol.* **1972**, *22*, 417; b) C. Cooney, D.C.I. Wang, R.I. Mateles, *Appl. Environ. Microbiol.* **1976**, *31*, 91; c) S. Hueting, D.W. Tempest, *Arch. Microbiol.* **1979**, *123*, 189; d) T. Egli, *ibid.* **1982**, *131*, 95.
[5] a) T. Egli, J.R. Quayle, *J. Gen. Microbiol.* **1986**, *132*, 1779; b) S. Gräzer-Lampert, T. Egli, G. Hamer, *ibid.* **1986**, *132*, 3337; c) I.G. Minkevich, A.Y. Krynitskaya, V.K. Eroshin, in 'Continuous Culture', Eds. P. Kyslik, E.A. Dawes, V. Krumphanzl, M. Novak, Academic Press, London, 1988, p. 171; d) M.G. Duchars, M.M. Attwood, *J. Gen. Microbiol.* **1989**, *135*, 787; e) M. Rutgers, P.A. Balk, K. van Dam, *Arch. Microbiol.* **1990**, *153*, 487.
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[9] a) B. Pengerud, E. Skjoldal, F. Thingstad, *Marine Ecol. Prog. Ser.* **1987**, *35*, 111; b) R.A. Durner, Swiss Federal Institute of Technology, Zürich, Thesis, ETH 1998, Nr. 12591; c) M.S. Zinn, Swiss Federal Institute of Technology, Zürich, Thesis, ETH 1998, Nr. 12987.
[10] G. Piñar, K. Kováčová, T. Egli, J.L. Ramos, *Appl. Environ. Microbiol.* **1998**, *64*, 2970.
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[12] a) M. Lucca, M. Romero, J. Diaz, O. Garro, D. Callieri, *World J. Microbiol., Biotechnol.* **1991**, *7*, 359; b) U. von Stockar, C. Larson, I. Marison, M. Cooney, *Thermochim. Acta* **1995**, *250*, 247.
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[14] C.M. Brown, in 'Continuous culture 6: application and new fields', Eds. A.C.R. Dean, D.C. Ellwood, C.G.T. Evans, J Melling, Ellis Horwood, Chichester, 1976, p. 170.
[15] a) Y. Poirier, C. Nawrath, C. Sumerville, *Biotechnology* **1991**, *13*, 142; b) A. Steinbüchel, in 'Biomaterials, novel materials from biological sources', Ed. D.Byrom, Macmillan, Basingstoke, 1991, p. 125.

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Abstract. Biotin- and FITC-labelled water-soluble poly(acrylamide) derivatives of phosphorylcholine were prepared by coupling either maleinylated (*a*) or periodate-oxidized (*b*) L-glyceryl phosphorylcholine to poly-(acrylamide-allylamine) copolymer. Biotinylated phosphorylcholine poly(acrylamide) derivatives of both types were tested with *Limulus polyphemus C*-reactive protein and were used for the study of the phosphorylcholine-binding properties of boar seminal plasma proteins. Binding sites for phosphorylcholine on the surface of bull sperms were visualized using a FITC-labelled derivative of the ligand.

1. Introduction

Suitable derivatives of phosphorylcholine that allow labelling are needed for the detection and the characterization of binding properties of proteins interacting with this ligand. A number of different types of phosphorylcholine-binding proteins exist that differ in their properties and functions. C-reactive protein (CRP) [1], frequently employed as a clinical index of acute inflammation, represents the most studied phosphorylcholine-binding protein. CRP is also found in the invertebrate *Limulus polyphemus* as a constitutive and major component of horseshoe crab hemolymph [2].

Another type of proteins possessing the ability to interact with phosphorylcholine was found among proteins isolated

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from seminal plasma of different species. Some of seminal plasma proteins are known to bind specifically to the sperm surface; in this case, an interaction of surface sperm proteins with phosphorylcholine-containing components of the sperm membrane is involved. This type of interaction was studied in detail in the case of bull seminal plasma proteins [3], which bind to phosphorylcholine-containing lipids of spermatozoa at ejaculation and play an active role in sperm capacitation. Much less is known about the phospholipid-binding properties of seminal plasma proteins of other species, as well as about the function of these proteins [4].

Different approaches have been used for the preparation of suitable phosphorylcholine derivatives in studies on the binding characteristics of CRP.The methods involved either coupling of phosphorylcholine-caproyl-*p*-nitrophenyl esters [5], periodate-oxidized L-glyceryl phosphorylcholine [6], or isocyanatophosphorylcholine [7] to bovine serum albumin.

In our previous studies [8–11], biotinylated poly(acrylamide) derivatives of various types of saccharides and enzyme linked binding assay (ELBA) were successfully applied in studies of the saccharide-binding properties of different proteins. In this report, we present new, simple methods for the preparation of biotinylated and FITC-labelled water-soluble poly(acrylamide) derivatives of phosphorylcholine and their application as ligands in the study of binding properties of boar seminal proteins and CRP from *Limulus polyphemus* hemolymph.

2. Results and Discussion

2.1. *Preparation of Biotinylated Water-Soluble Poly(acrylamide) Derivatives of Phosphorylcholine*

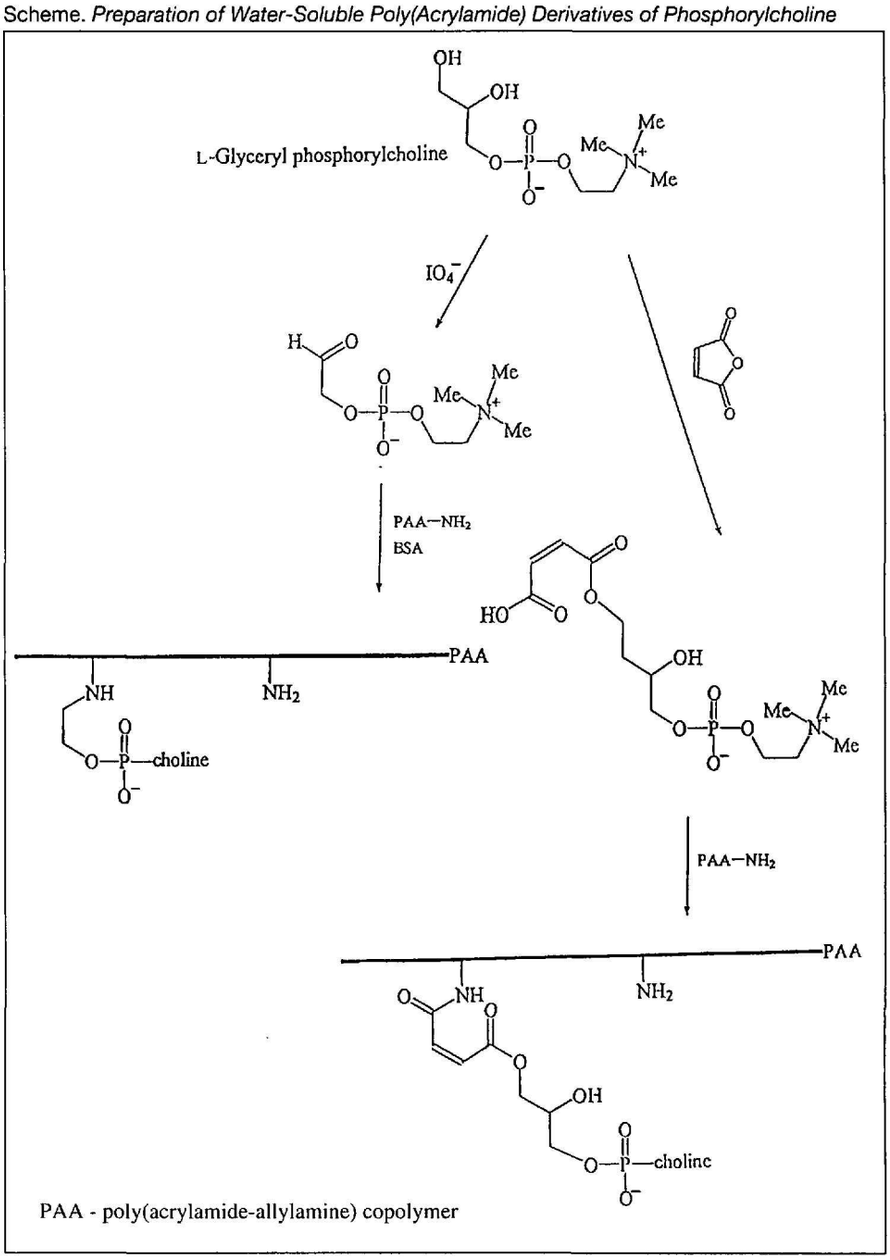
The starting poly(acrylamide-allylamine) copolymer was prepared by copolymerization of acrylamide and allylamine without cross-linking agent, as described previously [9]. Three different ratios of the reaction components were used, as given in the *Table*. The amount of allylamine incorporated was evaluated from NMR spectra (*Table*). The preparation A was used for the phosphorylcholine conjugation.

The first step of coupling was the preparation of the L-glyceryl phosphorylcholine derivative; two different reactions were carried out. In the first case, L-glyceryl phosphorylcholine was oxidized with periodate and the aldehyde groups being formed reacted with the amino group of the poly(acrylamide-allylamine) copolymer; after reduction with $NaBH_3CN$, a stable secondary amine was formed. In the second case, L-glyceryl phosphorylcholine was maleinylated with maleic anhydride, and the carbodiimide reaction was used to couple this derivative to the amino groups of the poly(acrylamide-allylamine) copolymer; a stable amide bond was formed (*Scheme*). Unsubstituted amino groups of the poly(acrylamide) derivative of phosphorylcholine were used for label-

Table. *The Ratio* (Based on Integral Intensities in NMR Spectra) *of Acrylamide to Allylamine Units in the Poly(Acrylamide-Allylamine) Copolymer*

Copolymer	Content of units in the reaction mixture		Content of units in the resulting copolymer	
	Acrylamide (mg)	Allylamine (µl)	Molar ratio	*w/w* ratio
A	800	1000	11:1	14:1
B	800	500	19:1	24:1
C	800	250	28:1	36:1

Scheme. *Preparation of Water-Soluble Poly(Acrylamide) Derivatives of Phosphorylcholine*



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ling with biotin or FITC. The content of biotin in the prepared derivatives did not differ significantly (0.3–0.4 mmol/100 g).

2.2. *NMR Spectra*

The ^{1}H-NMR spectrum of the starting copolymer exhibits broad signals for backbone CH_2 protons at δ 1.4–1.9, $CHCONH_2$ protons at δ 2.2–2.5, for $CHCH_2NH_2$ protons at δ 1.9–2.2, and signals of the diastereotopic protons CH_2NH_2 at δ 2.8–3.1 and 3.2–3.4. In addition, weak signals of an unexchanged portion of NH protons appear at δ 6.9, 7.4, 7.6, and 7.7. The signal at $\delta \sim 1.25$ (with intensity varying with the amount of allylamine used) shows that allylamine units are at least partly incorporated to the copolymer in a head-to-head manner rather than head-to-tail. All following calculations are based on the presumption that all signals of CH protons in acrylamide units are located in the region of δ 1.4–2.5 together with backbone protons of allylamine units, and that signals at 2.8–3.4 belong to CH_2 NH_2 protons only.

^{13}C-NMR Spectra of copolymers exhibit groups of signals of acrylamide units at δ ~37 (CH_2), 44 (CH), and 181 (CO). Groups of signals of low intensity could be observed at $\delta \sim$ 33 and 49 (allylamine units) and at $\delta \sim$ 179 and 182 (carbonyl close to the allylamine unit).

Moreover, ^{1}H-NMR spectra may be used to determine the residual amount of unreacted monomeric units, which exhibit multiplets of sharp lines at δ 3.65 and 3.75, J_{gem} = 11 Hz, J_{vic} = 4 Hz and 7 Hz ($-CH_2NH_2$ of allylamine).

The ^{1}H-NMR spectrum of a sample with coupled phosphorylcholine, obtained by oxidation of L-glyceryl phosphorylcholine, contains, besides the signals of the copolymer, one sharp signal at δ 3.24. The chemical shift is in good agreement with the reference values for the $-\overset{+}{N}Me_3$ group of choline. Other signals of choline and oxidized glycerol partly overlap with signals of the copolymer and are partly visible as multiplets in the region of δ 3.5–4.8 ppm. No additional signals were found in ^{13}C-NMR spectra in comparison to the starting copolymer. The comparison of integral intensities of the singlet (9 H) at δ 3.24 with intensities of signals of $-CH_2NH_2$ gives the amount of phosphorylcholine coupled to poly(acrylamide) copolymer: *ca.* 0.9×10^{-3} mol/100 g of copolymer.

The ^{1}H-NMR spectrum of a sample with coupled phosphorylcholine, obtained *via* binding maleinylated L-glyceryl phosphorylcholine, contains a sharp signal for the $-\overset{+}{N}Me_3$ group of choline at δ 3.24. Broad signals of olefinic protons of unsymmetrically substituted maleinate at δ 5.9 and 6.3 are present. Again, the comparison of intensities gives the amount of coupled phosphorylcholine: *ca.* 3.7×10^{-3} mol/100 g of copolymer.

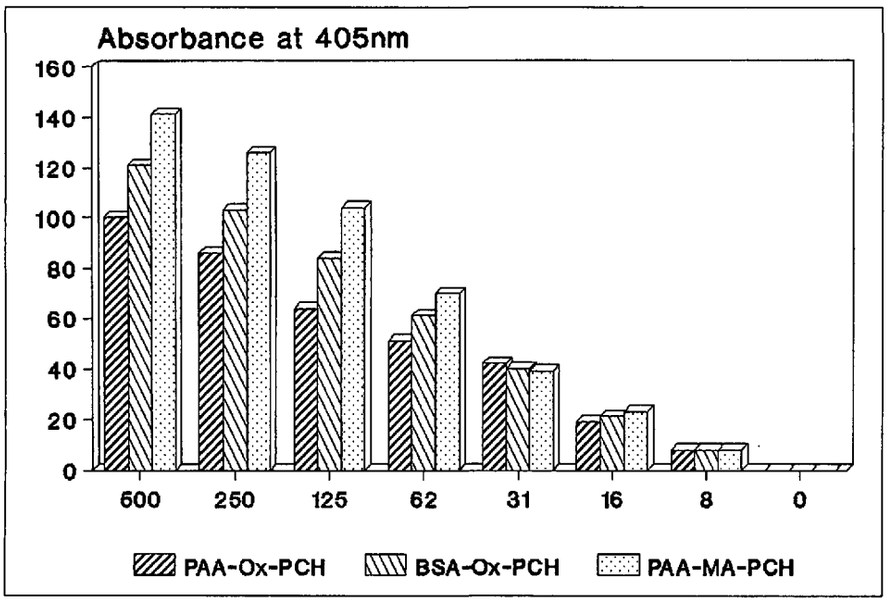

Fig. 1. *Comparison of the interaction of CRP from* Limulus polyphemus *hemolymph with biotinylated derivatives of phosphorylcholine coupled either to poly(acrylamide-allylamine) copolymer (PAA) or bovine serum albumin (BSA).* Ox-PCH = oxidized L-glyceryl phosphorylcholine and MA-PCH = maleinylated L-glyceryl phosphorylcholine were used for coupling; concentration of the used solutions of poly(acrylamide) or BSA derivative of phosphorylcholine: 100 µg/ml; concentration of CRP: 0–500 µg/ml).

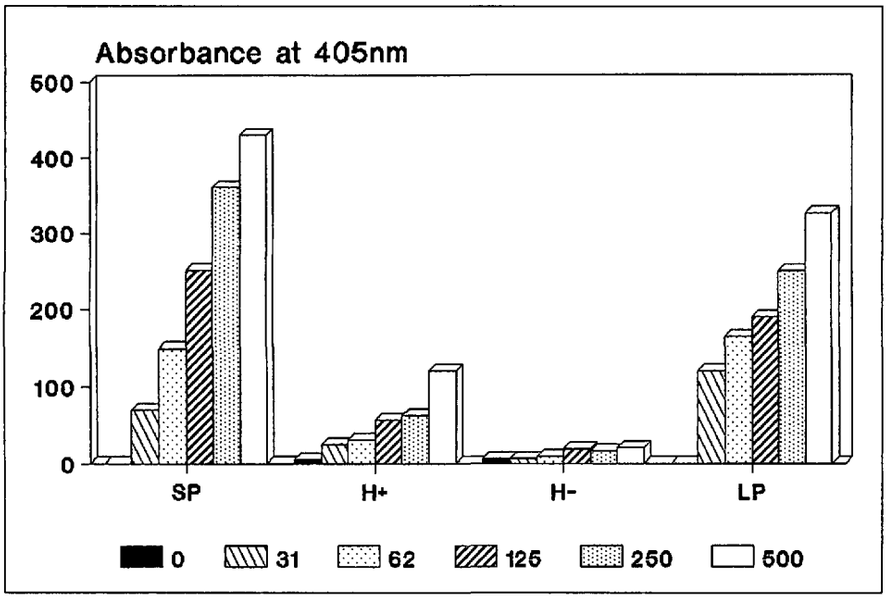

Fig. 2. *Interaction of boar seminal plasma proteins and CRP from* Limulus polyphemus *hemolymph with biotinylated poly(acrylamide) derivative of phosphorylcholine.* (SP: boar seminal plasma proteins, H+: heparin-binding and H–: non-heparin-binding proteins of boar seminal plasma, CRP: C-reactive protein from *Limulus polyphemus* hemolymph; concentration of the used solutions of phosphorylcholine derivative: 100 µg/ml; concentration of proteins: 0–500 µg/ml).

2.3. *Application of the Prepared Biotinylated and FITC-Labelled Phosphorylcholine Derivatives*

A known phosphorylcholine-binding protein was chosen to show that the prepared derivatives can be used for binding studies. ELBA proved that both types of biotinylated derivatives of phosphorylcholine (containing either periodate-oxidized or maleinylated L-glyceryl phosphorylcholine) interacted with CRP from *Limulus polyphemus* (*Fig. 1*). Similar results

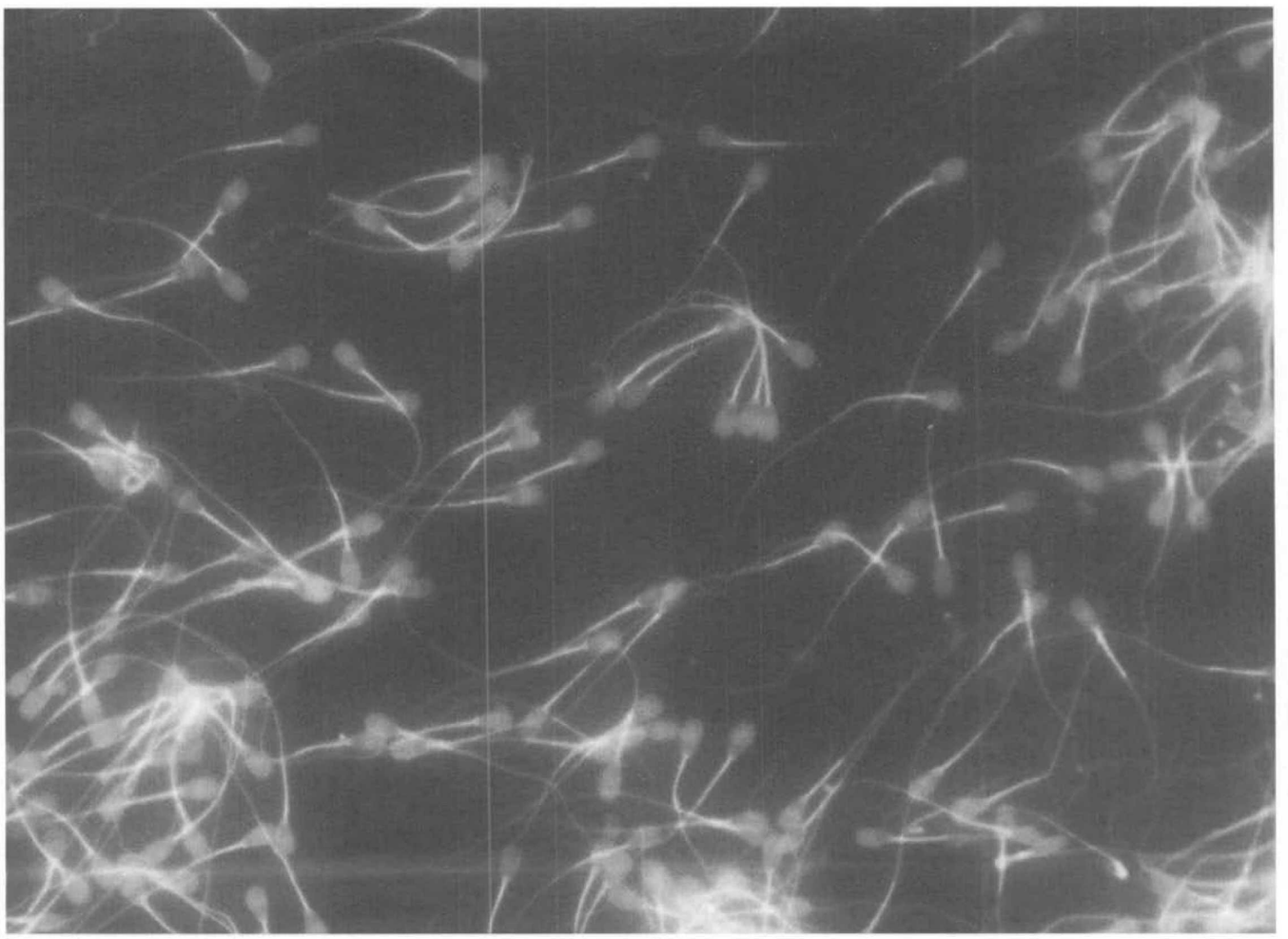

Fig. 3. *Phosphorylcholine-binding sites on ejaculated bull sperm visualized using fluorescence of FITC-labelled derivatives of the ligand*

were obtained with periodate-oxidized L-glyceryl phosphorylcholine coupled to bovine serum albumin, prepared according to *Stults et al.* (*Fig. 1*) [6]. The presence of free phosphorylcholine inhibited the interaction of CRP with the biotinylated poly(acrylamide) derivative of the ligand (not shown).

The prepared derivatives were used to show the phosphorylcholine-binding properties of proteins from boar seminal plasma (*Fig. 2*). Complete boar seminal plasma proteins were characterized by a high phosphorylcholine-binding activity. After the separation of boar seminal plasma by affinity chromatography on immobilized heparin, phosphorylcholine-binding activity was detected in the fraction of heparin-binding proteins (H+) even though the binding activity of these proteins was lower than that of complete seminal plasma proteins. This finding is in an agreement with results described by *Calvete et al.* [14]. The presence of Ca^{2+} ions did not affect the phosphorylcholine-binding activity of the H+ fraction.

FITC-labelled poly(acrylamide) derivatives of phosphorylcholine and fluorescence microscopy were used to show binding sites for this ligand on the surface of bull sperm (*Fig. 3*).

Our results have shown that the prepared biotinylated poly(acrylamide) derivatives of phosphorylcholine in combination with the ELBA method represent a useful tool to study phosphorylcholine-binding properties of proteins. The ELBA method was originally developed for saccharide-binding studies [8–11]. An advantage of this method, in comparison to precipitation experiments, is the fact that only small amounts are needed. FITC-labelled derivatives of the studied ligand were useful in direct binding studies using fluorescence microscopy or flow cytometry.

3. Experimental

3.1. *Reagents*

1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide, was purchased from *Fluka* (Buchs, Switzerland), and *N*-hydroxysuccinimidobiotin, L-glyceryl phosphorylcholine, $CdCl_2$ complex, phosphorylcholine, fluorescein isothiocyanate, Avidin peroxidase, ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), and CRP from *Limulus polyphemus* hemolymph from *Sigma*. Boar seminal plasma proteins (SP) were obtained by centrifugation (600 g, 20 min, 5°) of ejaculate to separate spermatozoa and seminal plasma. Heparin-binding proteins (H+) and non-heparin-binding proteins (H–) were isolated by affinity chromatography of boar seminal plasma on heparin-poly(acrylamide) gel [12].

3.2. *Preparation of Phosphorylcholine Derivatives*

3.2.1. *Preparation of Water-Soluble Poly(acrylamide-allylamine) Copolymer*

Water-soluble poly(acrylamide-allylamine) copolymer was prepared by copolymerisation of acrylamide and allylamine, as described previously [9]. The following ratios of acrylamide to allylamine in the polymerisation mixture were used: 800 mg/1000 µl, 800 mg/500 µl, 800 mg/250 µl.

3.2.2. *Preparation of* L-*Glyceryl Phosphorylcholine Derivatives and Their Coupling to Poly(acrylamide-allylamine) Copolymer*

Free L-glyceryl phosphorylcholine was prepared from its $CdCl_2$ complex (500 mg) using *AG 501 X-8* (D) mixed-bed ion-exchange resin as described by *Stults et al.* [6]. The combined filtrate was evaporated to a clear syrup (260 mg).

i) For maleinylation, the syrup of free L-glyceryl phosphorylcholine (260 mg) was dissolved in anhydrous DMF (15 ml). Malein anhydride (110 mg) and 4-(dimethylamino)pyridine (50 mg) were added, and the mixture was shaken overnight at room temperature. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide (250 mg) was added and shaking continued for 3 h. The solution of poly(acrylamide-allylamine) copolymer (400 mg in 15 ml 0.1M Tris-HCl buffer, pH 8.5) was added. After stirring for 24 h, the reaction mixture was dialyzed against distilled water and lyophilized.

ii) Periodate oxidation of L-glyceryl phosphorylcholine was carried out using the modified procedure described by *Stults et al.* [6]. For coupling to poly(acrylamide-allylamine) copolymer, reductive amination [9] was performed. Free L-glyceryl phosphorylcholine dissolved in distilled water (5 ml) was mixed with 5% sodium periodate (1 ml) and with poly(acrylamide-allylamine) copolymer (400 mg). The reaction mixture was shaken at room temperature for 2 h, and ethylene glycol (3 ml) was added. After 30 min of stirring, the reaction was stopped by adding $NaBH_3CN$ (80 mg). After further shaking for 30 min, the reaction mixture was dialyzed against distilled water and lyophilized. Analogous conditions were used for the coupling of periodate-oxidized phosphorylcholine derivative to BSA. Biotinylation of poly(acrylamide) and BSA derivatives of phosphorylcholine was carried out as described previously [8].

3.3. *Enzyme-Linked Binding Assay (ELBA)*

The procedure used was described by *Novotná et al.* [10]. The solution (100 µl) of proteins (10 µg/ml in 0.05M $NaHCO_3$, pH 9.6) was applied to wells and incubated overnight at 4°. After rinsing with washing buffer (0.01M Tris·HCl, pH 7.4, containing 0.85% NaCl, 3 mM $CaCl_2$, 0.2% *Tween*), the wells were deactivated with 200 µl of 3% BSA in washing buffer (2 h at 4°). After washing, a solution of either biotinylated poly(acrylamide) derivatives of phosphorylcholine (100 µg/ml in 0.01M Tris·HCl, pH 7.4) was applied to each well (100 µl) and incubated for 2 h at 37°. After washing, 25 ng of avidin peroxidase in 100 µl of 0.1M Tris·HCl, pH 8.0, containing 1% BSA, was added to each well and incubated for 1 h at 37°. After washing, the enzyme reaction was initiated by addition of 100 µl of 0.01% ABTS in 0.1M phosphate-citrate buffer at pH 4.6, containing 0.09% H_2O_2, into each well. After 30 min incubation at 37°, the reaction was stopped by adding 50 µl of 1% sodium dodecyl sulfate. The biotinylated poly(acrylamide) copolymer containing no phosphorylcholine served as a control. Absorbance at 405 nm was measured using a microplate reader (*SLT-Spectra, SLT-Labinstruments*, Vienna, Austria).

3.4. *Localization of Polysaccharide-Binding Sites on the Sperm Surface*

For a topological localization of the polysaccharide-binding sites, a fluorescein-labelled polysaccharide derivative and fluorescency-microscopic techniques were used.

Slide preparation: the drop of the sperm washed with PBS (20 µl) and a drop of fluorescein-labelled phosphorylcholine derivative (20 µl, 1 mg/ml) were mixed, applied on the slide, and dried. Fixation was done in 5% formaldehyde for 30 min. Then the slides were washed three times with distilled water, dried, and observed in the fluorescence microscope using excitation with UV light at 360 nm.

3.5. *NMR Spectra*

^{1}H-NMR Spectra were recorded on a *Varian INOVA* 400 MHz instrument at 40°, D_2O was used as solvent. Spectra were referenced to the singlet signal of the methyl groups of 2-methylpropan-2-ol (δ = 1.25 for ^{1}H and δ = 31.6 for ^{13}C, respectively), which was used as an internal standard. The assignment of proton signals in the copolymer is based on the measurement of three samples of copolymer prepared by copolymerization of acrylamide with varying portions of allylamine. This assignment was in addition validated by the homonuclear ^{1}H{^{1}H}COSY spectra. ^{13}C-NMR Spectra were recorded at 100 MHz using the same instrument and under the same conditions as mentioned for ^{1}H-NMR spectra.

3.6. *Analytical Methods*

The amount of coupled biotin was determined using HABA (2-hydroxy-azobenzene-2'-carboxylic acid)-avidin reagent [13]. The amount of phosphorylcholine in the prepared derivatives was evaluated by NMR.

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[1] S.P. Ballou, I. Kushner, *Adv. Intern. Med.* **1992**, *37*, 313.
[2] N.Z. Nguyen, A. Suzuki, S.M. Cheng, G. Zon, T.Y. Liu, *J. Biol. Chem.* **1986**, *261*, 10450.
[3] L. Desnoyers, P. Manjunath, *J. Biol. Chem.* **1992**, *267*, 10149.
[4] L. Leblond, L. Desnoyers, P. Manjunath, *Mol. Reprod. Dev.* **1993**, *34*, 443.
[5] E.B. Oliveira, E.C. Gotschlich, T.Y. Liu, *J. Immunol.* **1980**, *124*, 1396.
[6] N.L. Stults, Y.C. Lee, A.H. Craig, K. Kichitaro, K. Seichi, T. Isamu, K. Toshioki, T.-Y. Linn, *Anal. Biochem.* **1987**, *161*, 567.
[7] A. Rifai, S.S. Wong, *J. Immunol. Methods* **1986**, *94*, 25.
[8] J. Liberda, V. Jonáková, M. Tichá, *Biotechnol. Techn.* **1997**, *11*, 265.
[9] J. Klein, M. Kraus, M. Tichá, B. Železná, V. Jonáková, J. Kocourek, *Glycoconjugate J.* **1995**, *12*, 51.
[10] V. Novotná, L. Mikeš, P. Horák, V. Jonáková, M. Tichá, *Int. J. Biochromatogr.* **1996**, *2*, 37.
[11] A. Chadli, M. Caron, M. Tichá, R. Jourbet, D. Bladier, J. Kocourek, *Anal. Biochem.* **1992**, *204*, 198.
[12] M. Tichá, B. Železná, V. Jonáková, K. Filka, *J. Chromatogr.* **1994**, *656*, 423.
[13] M.M. Green, *Methods Enzymol.* **1970**, *18A*, 418.
[14] J. Calvete, M. Raida, M. Gentzel, C. Urbanke, L. Sanz, E. Töpfer-Petersen, *FEBS Letters* **1997**, *407*, 201.