then it is reasonable that the effect survives the hemolysis process to a large extent.

To investigate the reason for the previously observed[2] decrease in the effect of IPP incubation on reductase efficiency for aged cells, extracts were made of fresh and 30-day cells, according to the method of Hegesh et al.[8]. A comparison of the methemoglobin reductase activity of the 'crude enzyme extract' for fresh and aged erythrocytes is shown in the table. The similarity in reductase activity of the 2 extracts indicate that the activity of the reductases does not decrease during 30 days storage. Control experiments in which the reductase activity of 26-day-old red cells before and after incubation with IPP were compared and indicated no change in activity caused by the incubation. Further, disc gel electrophoresis of the 'crude enzyme extracts' were carried out according to the method of Kaplan[9] and showed no significant differences between the reductases from fresh and 26-day-old cells or between IPP incubated and nonincubated 26-day-old cells. Since the difference in the effect of IPP incubation between fresh and aged cells does not lie with the methemoglobin reductase then perhaps one of the enzymes in the pentose phosphate pathway is responsible.

The activity of glucose-6-phosphate dehydrogenase has been reported to decline upon aging of the red cell[10]. Further, methylene blue is known to have less effect on reductase activity in cells deficient in glucose-6-phosphate dehydrogenase activity[11].

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Production of antibodies against bradykinin

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Summary. High-titer antibodies against bradykinin were raised in rabbits. 2 different conjugates of bradykinin were used for immunization: bradykinin coupled to human serum albumin via 1,5-difluoro-2,4-dinitrobenzene and bradykinin coupled to edestin via 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide. The sensitivity of the radioimmunoassay method is in the range of 1–50 pg of bradykinin. Cross-reaction of anti-bradykinin antisera occurred with kallidin and met-lys-bradykinin.

The sensitivity and specificity of a radioimmunoassay depends mainly on the sensitivity and the specificity of the reaction between the antigenic determinant(s) of the antigen, and the specific binding sites of the antibody population in the antiserum. Consequently, for establishing a sensitive radioimmunoassay, the production of an antiserum with a high specific antibody titer is of primary importance. Various factors, including the immunogenicity of the antigen used[1], are of significance for the specificity of the antibodies. Methods of producing antibodies against bradykinin have been described by various investigators[2–9], but in most cases, very low concentrations of antibodies were obtained (titers up to 1:1000).

The purpose of the present study was to reinvestigate the coupling methods used so far, in order to obtain an antigen of high immunogenicity.

Conjugation methods for the preparation of bradykinin immunogens

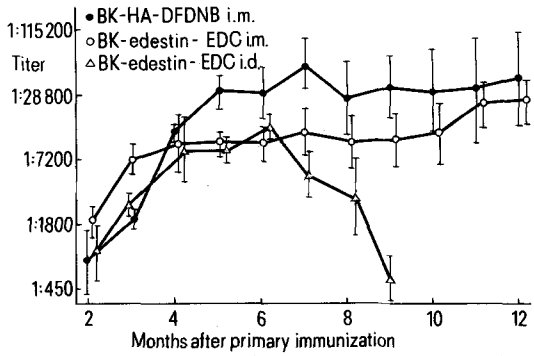
Method	Tager[10]	Goodfriend and Ball[2]
Bradykinin triacetate (BK)	10 mg	35 mg
Solvent for BK	1.0 ml 0.1 M potassiumphosphate + 7 M guanidine HCl pH 7.0	1.5 ml water
Coupling reagent	150 mg 1,5-difluoro-2,4-dinitrobenzene (DFDNB)	50 mg 1-ethyl-3(3-dimethyl-amino-propyl)carbodiimide HCl (EDC)
Solvent for coupling reagent	5.0 ml methanol	1.0 ml water
Protein carrier	20 mg human serum albumin	70 mg edestin
Solvent for protein carrier	1.0 ml 0.4 M sodiumboratebuffer pH 10.0	3.0 ml 0.1 M Tris-HCl pH 5.34
pH of reaction mixture	9.6	4.75
Reaction time	24 h at R.T.	1 h at 18 °C
Coupling rate	70–80%	10–25%
Number of BK-molecules/ molecule of protein carrier	10–11	6–15

Methods and material. 2 different bradykinin immunogens were prepared according to Goodfriend and Ball[2] and Talamo et al.[9]: bradykinin coupled to ovalbumin by means of carbodiimide and bradykinin coupled to human serum albumin by means of diisocyanate (referred to as BK-OA-EDC, and BK-HA-Isocyanate). Ovalbumin was purchased from Serva, Heidelberg; toluene-2,4-diisocyanate was purchased from ICN Life Sciences Group, Cleveland, Ohio. Furthermore, 2 new conjugates were prepared.

The 1st according to a method described by Tager[10]: Bradykinin-triacetate (Bachem, Bubendorf, Switzerland) was coupled to human serum albumin by means of 1,5-difluoro-2,4-dinitrobenzene (Merck, Darmstadt). This conjugate is referred to as BK-HA-DFDNB. 2ndly, the carbodiimide method was used in a modification of the procedure described by Goodfriend and Ball[2]. Bradykinin-triacetate was coupled to edestin (Serva, Heidelberg) by means of 1-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide HCl (Serva, Heidelberg). The reaction mixture was kept at pH 4.75 throughout the reaction time (autotitrator, radiometer). This conjugate is referred to as BK-edestin-EDC. Preparative details on these 2 conjugation methods are given in the table.

In order to determine the extent of coupling of bradykinin to the protein carrier, [^{125}I]-tyrosine^8-bradykinin (approximately 0.1 µCi) was added to all coupling reactions in combination with bradykinin-triacetate. The conjugates were dialyzed against 0.05 M NH_4HCO_3, pH 7.8, for 24 h at 4 °C. Chromatography of the antigens (BK-HA-DFDNB and BK-edestin-EDC) on sephadex G-50 f did not alter the specific radioactivity of the protein fraction. This indicates that free bradykinin was completely removed by dialysis. The antigens were stored at −20 °C until use.

For the immunization, a solution/suspension containing 200 µg of bradykinin coupled to the respective protein carrier was mixed with 3 vol. of complete Freund's adjuvant (Difco, Detroit, Michigan). The mixture was injected i.m. as an initial dose into the hind limbs of each of 5 male white New Zealand rabbits. At 4 weeks' intervals, i.m. boosting with half of the priming dose was performed. Blood for assaying antibody titers was obtained from the central artery of the ear 10–14 days after each booster injection. Additionally, 5 animals were immunized by a one-time multiple site intradermal injection of BK-edestin-EDC-conjugate[11]. For this purpose, a suspension containing 200 µg of the immunogen was emulsified in 3 vol. of complete Freund's adjuvant and injected at 30–40 sites of the shaved back-skin. Pertussis vaccine (0.5 ml, Behring, Marburg) was injected s.c. at a different site in order to increase antibody response. Antiserum titers were monitored every 4 weeks. All antisera were heated to 56 °C for 30 min and stored at −20 °C until use.



Antisera titers against bradykinin-antigens in rabbits during a period of 12 months of immunization. I.m. immunization was performed with bradykinin coupled to human serum albumin via difluorodinitrobenzene and bradykinin coupled to edestin via carbodiimide (BK-HA-DFDNB; BK-edestin-EDC; 100 µg/booster injection). Booster injections were given every 4 weeks. Intradermal immunization was performed once with BK-edestin-EDC; 200 µg of the antigen were injected at 30–40 sites on the shaved skin of the back. 5 animals for each group. The results are expressed as mean ±SD.

The radioimmunoassay was performed by a slight modification of the method described by Carretero et al.[12]: As a buffer, 0.1 M Tris-HCl, pH 7.4, containing 0.1% neomycin-sulfate and 1% human serum albumin (RIA-buffer) was used. The incubation mixture contained 100 µl of the antiserum dilution (approximately 50% binding), 360 µl of [^{125}I]-tyrosine^8-bradykinin (about 8000 cpm) and 100 µl of bradykinin standards ranging from 1 to 1000 pg and 100 µl of the unknown samples, respectively. The final volume was 560 µl. All samples were equilibrated for at least 6 h at 4 °C. Plastic equipment was used at all steps of the procedure in order to prevent kinin adsorption to surfaces. The tracer ([^{125}I]-tyrosine^8-bradykinin) was prepared according to Greenwood et al.[13]. Na^{125}I was purchased from Amersham Buchler, Braunschweig. To separate free kinins from antibody-bound kinins, dextrane-coated charcoal prepared according to Carretero et al.[12] was used. Charcoal (150 µl) was added to each sample, the mixture was shaken vigorously for 1 min and centrifuged at 3000 rpm at 4 °C. Subsequently, the supernatant was decanted and the precipitate counted for radioactivity in an automatic gammaspectrometer (Berthold, Wildbad). The standard curve was plotted from triplicate values. Bradykinin concentration of the unknown samples (n:3) was determined from this plot.

Results and discussion. Intermolecular coupling of bradykinin to the protein-carrier differs according to the coupling procedure. When EDC and isocyanate were used, 10–25% of bradykinin were bound to the protein-carrier, whereas 75–80% of bradykinin were bound with the DFDNB-method.

6 months after the 1st immunization, high titers of antibodies were raised only in rabbits treated with BK-edestin-EDC and BK-HA-DFDNB-conjugates. The titers ranged between 1:10,000 and 1:40,000. 1 animal, which had received bradykinin conjugated to HSA via DFDNB, produced an antiserum with a titer of 1:120,000. In all animals immunized with BK-HA-isocyanate and BK-OA-EDC, antibody titers were below 1:100.

During the initial phase of immunization (2–6 months), antibody titers were similar in intradermally (i.d.) and i.m. immunized animals. Following that period, antibody titers declined in rabbits immunized by the one-time i.d. method, whereas animals, in which the antigen had been i.m. injected, still produced antibodies of increasing titers, even 12 months after the beginning of immunization.

Cross-reaction of anti-bradykinin antisera occurred with kallidin and met-lys-bradykinin. The sensitivity of all antibodies was between 20 and 50 pg bradykinin per sample. For 1 antiserum against BK-edestin-EDC, sensitivity was up to 1 pg bradykinin per sample. These values correspond to the highest antibody titers and to the most sensitive antibodies against bradykinin so far[2–9].

In our experience, the use of a protein-carrier of high molecular weight, such as edestin (mol.wt 300,000), favours the antibody induction, probably due to its depot effect. Similarly a high coupling rate as obtained by the DFDNB-method is of significance. Furthermore, it is important to use the same strain of animals, which in this case corresponded to a strain that had been successfully immunized against various proteins, small peptides and steroids.

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Isolation and electrophoretic identification of exogenous histone from rat blood plasma

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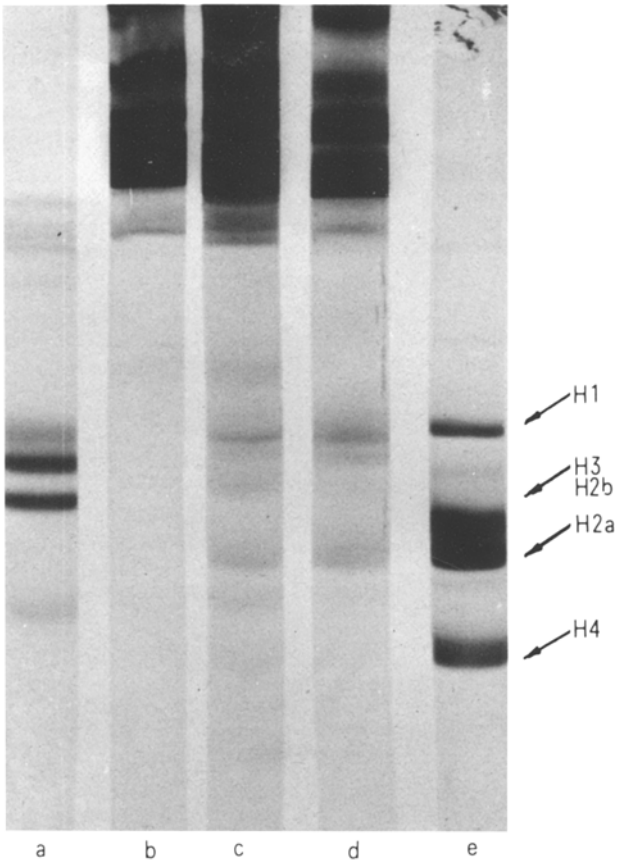
Summary. Histone fractions H1, (H2b+H2a) and H4 were isolated from rat blood plasma and electrophoretically identified 5 min after i.v. exogenous histone application.

The action of various chemical, mechanical, termic and toxic agents might result in cell destruction. Furthermore in case of structural and metabolic nuclear disturbance, some of the nuclear proteins-histones might leave chromatin and pass to other nuclear or cytoplasmic structures, or leave the cell[1-3]. Our previous studies have shown that histone proteins can be isolated from both normal and tumour rat blood plasma[4]. The electrophoretic pattern of isolated exogenous histones from rat blood plasma and the influence of globin fractions with the same electrophoretic mobility are the subject of the present study, carried out by the method previously employed[4].



Polyacrylamid gel electrophoresis of: *a* Globin; *b* blood plasma before i.v. application of exogenous histones; *c* untreated plasma after application; *d* treated plasma after application; *e* whole histone.

Material and methods. Wistar rats were i.v. injected 5 mg/100 g b. wt whole histone dissolved in 0.5 ml of 0.9% NaCl. 5 min later a blood sample was taken and centrifuged at 4 °C. The plasma so obtained was precipitated with trichloracetic acid to a final concentration of 18%. The precipitate was homogenized with 10 vol. of 0.25 M sucrose, 0.1 M Tris-HCl pH 7.4 and 0.003 M $CaCl_2$. Histones were extracted twice by homogenization of the sediment with 0.25 M H_2SO_4. The combined supernatants were clarified by filtration and histones were precipitated with 6 vol. of ethanol for a night at − 10 °C. The sediment thus obtained was washed and dissolved in 0.9 N CH_3COOH and 15% sucrose. Blood plasma samples obtained from each rat before and after histone injecting which were not treated in the aforesaid manner, served as controls. Whole histone was extracted according to Spelsberg and Hnilica[5] from liver chromatin obtained according to Tsanev and Russev[6]. Globin was obtained according to Teale[7]. Acrylamide gel electrophoresis was carried out according to Panyim and Chalkley[8].

Results and discussion. 5 min after an exogenous whole histone application histone fractions H1, (H2b+H2a), H4 are to be found in plasma corresponding to the standard histones (figure, c and e). Arginine-rich histones H3 only are missing, being probably adsorbed on erythrocyte membranes; passing over to subcellular structures is also probable[1,9]. Globin bands can be seen among histone fractions as a result of haemolysis due to partial erythrocyte destruction under the exogenous histone influence and dissociation of globin from haem because of acid buffer of gel electrophoresis (figure, a-c). Globin contaminations are eliminated after the above-mentioned plasma treatment and fractions H1, (H2b+H2a) become sharply outlined on the electrophoretic pattern (figure, a, c and d). However fraction H4 is less apparent in this case, probably due to partial loss at some stages of treatment (figure, c and d). Longer exposition was not carried out, since exogenous histones are eliminated up to 60–70% in 30 min and remain only 6–25% in plasma by the 6th h after application[10]. Our results demonstrate that histones present in rat blood