white (not shown), which suggests that avidin is essentially biotin-free. On the other hand, heating decreased biotin-binding values in some avian species, suggesting a smaller stability of their avidin to heat than that in the chicken.

The quail egg white showed an incomplete cross-reaction in the radioimmunoassay for chicken avidin (fig.), while the egg whites of other avian species could not prevent ^{125}I-labelled avidin from binding to antiserum. This result indicates differences in the antigenic determinants of avidin molecules as compared to chicken avidin.

The biotin-binding protein found in the chicken egg yolk[7,11] was demonstrated here to be a common constituent in the egg yolk of various avian species (table). This protein is distinct from avidin, since it is denaturated at 100 °C[7,10]. The biotin-binding activities in the egg yolks also varied considerably from species to species. No clear correlation was found between the biotin-binding activities in the egg white and yolk in different species. The lipid-free yolk material in all avian species studied did not show any cross-reaction in the avidin radioimmunoassay.

The egg white and yolk of the turtle also showed biotin-binding activity (table), but no cross-reaction in the avidin radioimmunoassay. No biotin-binding activity was found in the hard roe of the fishes, bull sperm or human seminal plasma. Jones and Briggs[5] discovered a low biotin-binding activity in fresh bull sperm. This discrepancy in results might originate in the microbiological avidin assay they used, since the growth of microbes could be inhibited by any growth inhibitor present in the bull sperm.

It has been proposed that the biotin-binding proteins might be widely distributed in the animal kingdom and play some fundamental role in the physiology of reproduction[3-5]. An antimicrobial[6,12] effect for avidin, and a biotin-transporting role[11] for yolk biotin-binding protein, have been suggested as their functions. Fishes and mammals[13-16] so far studied did not contain any biotin-binding protein similar to that found in egg whites and yolks. It seems possible that special biotin-binding proteins have evolved for reproductive purposes in amphibian, reptilian and avian eggs.

1 We thank Mr Jukka Peltonen and Mr Antti Karlin for the collection of the avian eggs with permission obtained from the Ministry of Agriculture, and Mr Reino Saarinen for the turtle eggs. The authors are indebted to Mrs Outi Kurronen, Miss Riitta Mero and Miss Tiina-Maija Mattila for technical assistance. This work was supported by the Ford Foundation Grants No. 760-0526 and No. 790-0665.

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A low molecular weight tracer molecule for immunocytochemistry. Identification of cytoplasmic actin

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Summary. Anti actin Fab-fragments were tagged to a small electron dense tracer molecule; ferrocene monocarboxylic acid (230 daltons). The conjugate stains actin filaments, which were found mainly in the core of microvilli.

Many technical efforts have been made to visualize antigenic structures immunocytochemically. The main obstacle has been that the methods depend upon very large tracer molecules such as ferritin[1]. The immunoperoxidase method[2] does not eliminate this problem either, as the enzyme-antibody complex is still too large in diameter to pass through the cell membrane. Attempts were also made to allow large molecules to penetrate the plasma membrane with membrane disrupting agents[3,4], or with enzymatic digestion of certain membrane components[5]; however, these manipulations resulted in the destruction of the cell shape. Thus most techniques are still far from being established for immunocytochemistry. We here present a more suitable staining procedure, which helps to avoid most of the difficulties mentioned above. The very small Fab-fragment - ferrocene carboxylic acid (FMCA) complex identifies intracellular antigens without background effects. The procedure is easy to handle, direct and does not result in the destruction of cellular ultrastructure.

Actin was isolated from Ehrlich mouse ascites tumour (MAT) cells according to Lazarides and Weber[6], purified by polymerization- depolymerization cycles[7] and by DNase-I affinity chromatography[8]. Actin was injected s.c. into male New Zealand rabbits in the presence of complete Freund's adjuvant (protein concentration: 1.5 mg/ml). This was repeated on days 8 and 40 after the 1st inoculation. The IgG fraction was isolated from the serum and purified by DEAE-52 ion exchange chromatography[9]. Fab-fragments were prepared according to Porter[10] and labeled with the iron-containing sandwich molecule FMCA, using a water soluble carbodiimide[11]. Fab-fragments (5 mg protein), FMCA (5 mg) and 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide (10 mg) were dissolved in 2.5 ml 10 mM sodium phosphate and gently stirred at 4 °C over night. The

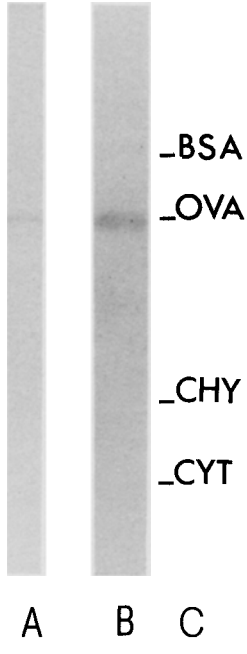

Figure 1. *A* Detection of MAT cell actin by the FMCA-Fab conjugate after SDS-polyacrylamide gel electrophoresis of purified actin. Actin was blotted from 15% gels on nitrocellulose filter (0.45 µm pore size, Millipore), incubated with the conjugate and stained with PEARL's solution. *B* A section of the gel was stained with Coomassie blue. *C* Marker proteins used are bovine serum albumin (BSA), ovalbumin (OVA), chymotrypsinogen A (CHY) and cytochrome C (CYT).

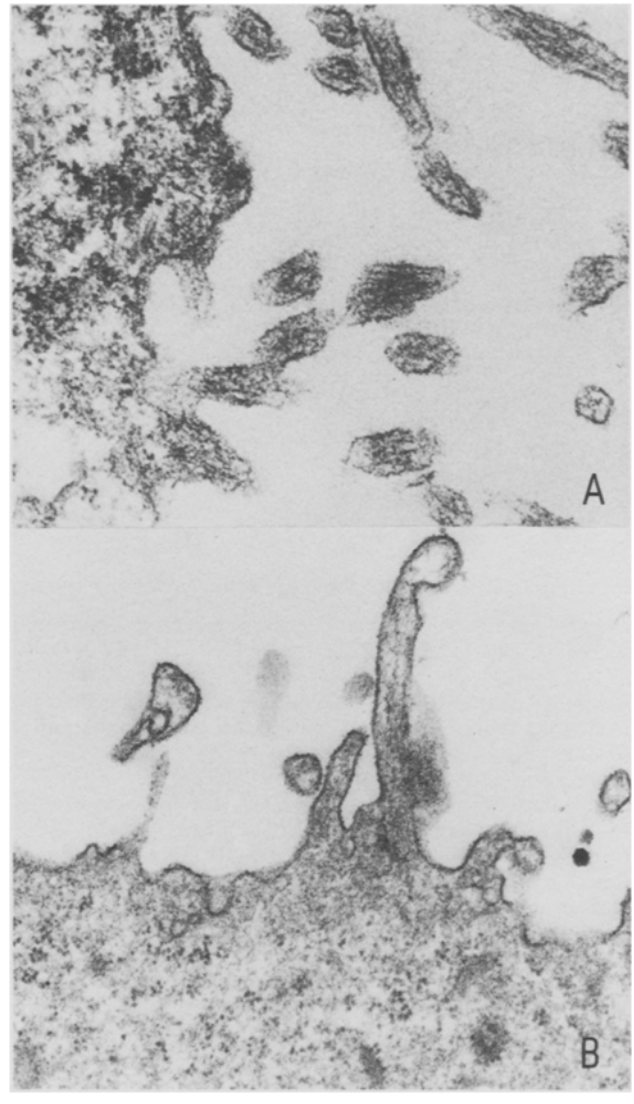

Figure 2. *A* MAT cells, incubated with FMCA-Fab-fragments to actin. Note the electron dense material within microvilli and beneath the plasma membrane. *B* Cells were processed as in A, but preincubated with specific, but untagged Fab-fragments. Microfilaments are not stained. × 32,000.

pH of the solution was repeatedly checked and held constant at 6.5. The precipitate formed at this stage was removed by low speed centrifugation (3500 × g, 1 h). The supernatant was applied to a Sephadex G 25 affinity column (2 × 60 cm), previously equilibrated with the same phosphate buffer as above. The conjugate was stored at −20 °C until use. The molar ratio of FMCA bound to 1 Fab molecule was estimated spectroscopically, reading the extinction at 280 nm and 435 nm using 0.65 mg/ml untagged Fab fragments and 1 mg/ml FMCA, respectively, as standards. The antibody activity of the conjugate was tested by blotting experiments as described[12]. For electron microscopy MAT cells were stabilized for 1 min at 37 °C in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid)-sodium salt, pH 6.8, 1 mM ethylene glycol-bis(β-aminoethylether)-N,N′-tetraacetic acid, 4% polyethylene glycol 6000[13], then incubated in the same buffer with 0.25% Triton X-100, washed with stabilization buffer, briefly fixed with a 3.75% sodium phosphate buffered (see above) formaldehyde solution, and washed again. Thereafter, cells were incubated with the conjugate at 37 °C for 1 h in the presence of 1% bovine serum albumin. As a control, cells were preincubated with specific, but unlabeled Fab-fragments to actin, and then with the conjugate. Specimens were extensively washed again, postfixed with 1% osmium tetroxide, and processed for electron microscopy.

The cyclopentadiene ring molecule FMCA (230 daltons) has 1 reactive carboxyl group, which can be gently coupled to the Fab molecule with the help of the carbodiimide[11]. Under these conditions, the Fab-fragments preserve their ability to precipitate the antigen: the conjugates cross-react with MAT actin in blotting tests (fig. 1, lane A). This can be visualized by Pearl's reaction[14]. Hence, the coupling procedure does not influence the antibody binding capacity of the conjugate. As far as we know from spectroscopic data, the conjugation of FMCA with Fab-fragments results in a 65:1 molar dye-to-protein ratio. The total mol.wt is about 65,000 daltons. It is therefore considered that the accumulation of Fe^{++}-ions around 1 Fab-fragment results in its contrast and its ultrastructural visualization (fig. 2). In figure 2A, MAT cell actin has been stained with the conjugate. However, actin cannot be demonstrated, if cells are preincubated with unlabeled, but specific Fab-fragments and then with the conjugate.

Most other immunocytochemical techniques have so far failed to give practically useful results[4,15–19]. The cell membrane must be damaged drastically so that large marker molecules are able to penetrate this barrier. The unavoidable consequence is the disorganization of the whole cytoplasm. Many serious problems of this kind are largely overcome by using the FMCA-Fab conjugate.

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Genetic variation in esteroproteases in the mouse submandibular gland

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Summary. 9 isozymes of esteroproteases were detected by column isoelectric focusing of submandibular gland extracts from four inbred strains of male mice. A marked strain variance in the esteroprotease isozymes was found among the strains.

The submandibular gland of mice is a rich source of biologically active proteins, such as nerve growth factor (NGF) and epidermal growth factor (EGF). In addition, this organ contains large quantities of trypsin-like enzymes with arginine-specific esteroprotease activity[1,2]. These enzymes and growth factors are androgen-dependent and are localized in serous-like granules in the cells of the secretory tubules[3-5]. Induction of the esteroproteases by androgen is good marker to use in studies on the mechanism of hormone action[6,7]. Several esteroproteases in the mouse submandibular gland have been isolated and their biochemical and immunological properties have been reported[8-10]. Some of the enzymes are thought to be involved in the processing of precursors of NGF and EGF[11-13]. Recently it has been shown that one of the esteroproteases in the mouse submandibular gland is under the control of genetic factors[14]. Here we report variations of several esteroproteases in different inbred strains of mice.

Materials and methods. Inbred mouse strains, BALB/cA, C57BL/10N, C3H/HeN and DBA/2N were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan) and were given standard laboratory chow and water ad libitum. Mice were killed by cervical dislocation between weeks 10 and 12 after birth and their submandibular glands were removed and homogenized with 9 volumes of 20 mM phosphate buffer (pH 7.0) in a glass-teflon homogenizer. The homogenate was centrifuged at $5000 \times g$ for 30 min and the supernatant was used for enzyme assay and isoelectric focusing.

Esteroprotease activity was measured by the method of Trautschold and Werle[15] with α-N-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The reaction mixture contained 0.5 mM BAEE, 1 mM NAD, 130 mM semicarbazide, 130 mM pyrophosphate-380 mM glycine buffer (pH 8.7), 100 units of alcohol dehydrogenase and the enzyme sample in a total volume of 1 ml. The reaction was followed by measuring the change in absorbance at 340 nm and 1 unit of enzyme activity was defined as the amount which hydrolyzed 1 µmole of substrate per min. Protein was determined by the method of Lowry et al.[16]. Column isoelectric focusing was carried out in a column with 50 ml capacity. A density gradient of 0-50% sucrose containing 1% ampholine (pH3.5-10) was prepared and the sample was layered on the middle of the gradient. After focusing for 24 h at 700 V at 0-2 °C, 1-ml fractions were collected.

Results and discussion. The table shows the esteroprotease activities in the submandibular glands of 4 inbred strains of mice. The activity was much higher in males than in females. In males, the specific and total activities of BALB/cA and C57 BL/10N strains were about twice those in C3H/HeN and DBA/2N strains.

Figure 1 shows the isoelectric focusing pattern of the esteroproteases in males. 9 isozymes with different isoelectric points were detected in the 4 strains examined, and these were named esteroproteases I-IX on the basis of isoelectric points (I, 4.7; II, 5.2; III, 5.6; IV, 5.9; V, 6.8; VI, 7.6; VII, 8.1; VIII, 8.8; IX, 9.8). Esteroproteases I, II, VII and VIII were found only in strain DBA/2N; III and V in strains BALB/cA, C57 BL/10N and C3H/HeN; IV in strains BALB/cA and C57 BL/10N; VI in strains BALB/cA and DBA/2N, and IX only in strain BALB/cA. Similar

Esteroprotease activity in the submandibular gland of mice

Strain	Sex	BAEE hydrolytic activity Units/mg tissue	Units/mg protein
BALB/cA	♂	19.5±0.56	169±3.9
	♀	0.8±0.09	7±0.4
C57BL/10N	♂	21.6±1.12	180±5.9
	♀	1.1±0.09	15±0.6
C3H/HeN	♂	10.5±0.94	91±3.7
	♀	1.8±0.08	17±0.8
DBA/2N	♂	12.4±1.01	104±3.1
	♀	1.4±0.06	13±0.8

Values are mean±SE for 6 mice.