Those in the upper cell neck region often contained large Ca^{++}-antimonate granules (figure 3), suggesting that they accumulated Ca^{++} during their migration through the cell neck region where numerous mitochondria were located[15]. Since mitochondria are known to sequester intracellular Ca^{++} in various non-muscle cells[22], they may serve as a source of Ca^{++} for coated vesicles during their transit through the cell neck region. The fact that coated vesicles found in the vicinity of apical microfilament bundles contained less Ca^{++}-antimonate precipitate (figure 5) than those in the cell neck region suggests that they release most of their stored Ca^{++} in the apical region. Although precise mechanisms of Ca^{++} accumulation and release are largely unknown, coated vesicles isolated from brain and other tissues have been shown to both biochemically and functionally resemble the sarcoplasmic reticulum[23-25]. Thus, the results of the present study are interpreted to indicate that coated vesicles in neuroepithelial cells are capable of sequestering Ca^{++} and, by functioning as a mobile source of intracellular Ca^{++}, may play a role in regulating contraction of apical microfilament bundles during uplifting of neural folds in the chick.

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Use of peroxidase-avidin conjugate for the demonstration of intracellular antigen[1]

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Summary. Horseradish peroxidase was coupled to avidin by periodate oxidation. Using the peroxidase-avidin conjugate together with biotin-labeled anti-chymotrypsinogen antibody, chymotrypsinogen was clearly demonstrated in the zymogen granules of the pancreas. The reaction between avidin and biotin is so rapid and stable that the avidin-biotin system can be effectively applied to immunohistochemical studies.

The strong affinity of avidin for biotin[3] has served as an effective tool for research in the immunohistochemical field. The avidin-biotin (A-B) system has been utilized mostly for the demonstration of antigens and syalyl residues on the cell surface[4-8]. Only the report of Heggeness and Ash[9] on the localization of actin and myosin in cultured muscle cells has delt with the demonstration of intracellular antigens. On the other hand, Guesdon, Ternynck and Avrameas[10] recently introduced a method for coupling enzymes to avidin with the aid of glutaraldehyde, and suggested the possibility of applying the A-B system to immunohistochemical staining. The present study was undertaken to develop a new method of preparing peroxidase-avidin conjugate, and to localize the chymotrypsinogen (CHT) in the pancreas with this conjugate in combination with biotin-labeled (biotinyl) anti-CHT antibody.

Materials and methods. 1 mg of bovine CHT (Sigma Chemical Co.) dissolved in 1 ml of phosphate buffered saline (PBS) was mixed with an equal volume of complete Freund's adjuvant, and injected s.c. into 2 rabbits. 4 weeks after the 1st injection, 0.1 mg of the antigen was administered as a booster injection. 1 week after the booster injection, antisera were harvested from the ear vein. Biotinyl-N-hydroxysuccinimide ester (BOSu) was prepared according to the method of Becker, Wilchek and Katchalski[11] and Bayer and Wilchek[12]. BOSu was coupled to goat IgG directed against rabbit IgG by the method described by Heggeness and Ash[9]. Horseradish peroxidase (HRP) (Sigma, Type VI) was coupled to avidin by periodate oxidation, which was followed by reductive alkylation. That is to say, HRP was oxidized with sodium periodate by the method of Wilson and Nakane[13]. The reactive aldehyde groups so formed react readily with amino groups of avidin.

In practice, the coupling procedure was as follows. 6 mg of HRP was dissolved in 1 ml of distilled water. To this was added 0.3 ml of freshly prepared $NaIO_4$ (21 mg/ml), and the solution was stirred for 20 min at room temperature. The oxidized HRP was dialyzed overnight against 0.001 M sodium acetate buffer, pH 4.0. Then, 20 µl of 0.2 M carbonate-bicarbonate buffer, pH 9.5, was added. Immediately after, 4 mg of avidin dissolved in 1 ml of 0.01 M carbonate-bicarbonate buffer, pH 9.5, was added. The HRP-avidin mixture was stirred gently for 2 h at room temperature in the dark. To this mixture was added 0.15 ml of 4% sodium borohydride ($NaBH_4$) dissolved in distilled water. The mixture was left for 2 h at 4 °C. Finally, the HRP-avidin conjugate was dialyzed against PBS for 2 days.

Application of the A-B system to immunohistochemical studies was made as follows. Mouse pancreas was taken and cut into small blocks in a drop of a mixture of equal volumes of 4% paraformaldehyde and 0.1% glutaraldehyde prepared in 0.1 M phosphate buffer, pH 7.4. The tissue

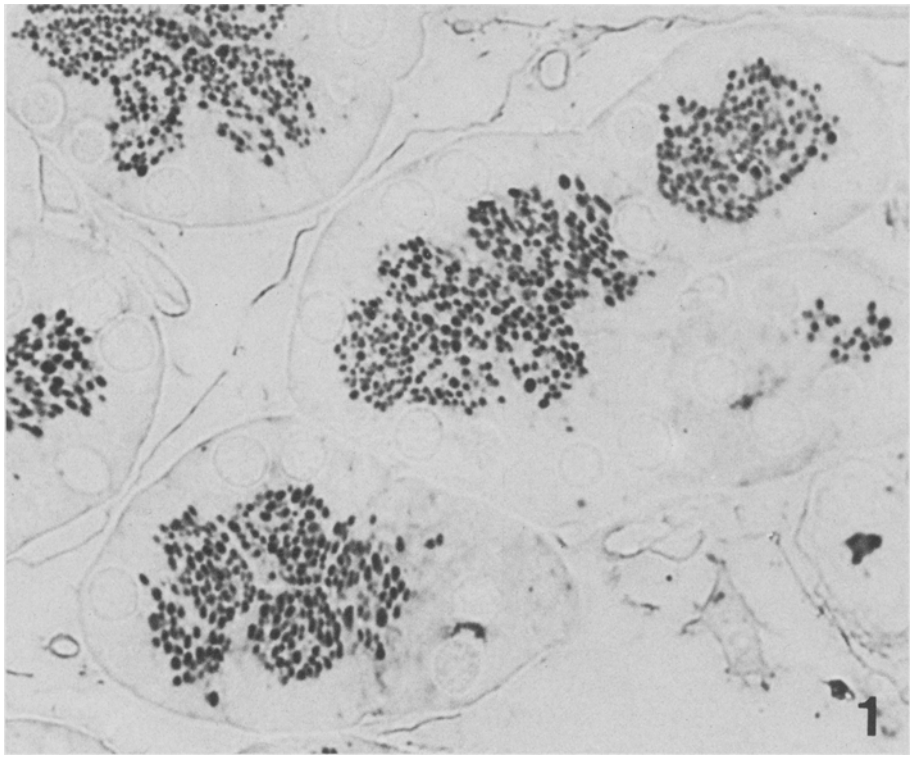

Fig. 1. Localization of chymotrypsinogen in mouse pancreas. The positive reaction is concentrated in the zymogen granules of the acinar cells. The mouse pancreas was fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde, and embedded in acrylamide[12]. Sections of 1 μm in thickness were prepared with a Porter-Blum ultramicrotome type MT-2 equipped with glass knives. The section was placed on a non-albuminated clean slide glass, air dried, and etched with 1% 2-mercaptoethanol for 15 min. The section was washed with several changes of PBS, and then with PBS containing 1% bovine serum albumin. The section was treated successively with anti-chymotrypsinogen rabbit IgG, biotin-labeled goat anti-rabbit IgG and with HRP-avidin conjugate. Finally, the section was colored by the diaminobenzidine reaction. × 400.

blocks were further fixed in the same mixture for 4 h, washed overnight with PBS and embedded in paraffin or in acrylamide[14]. Sections of 1–5 μm in thickness were first treated with anti-CHT rabbit IgG (0.1 mg/ml in PBS) for 30 min and washed with PBS for 30 min. The sections were then allowed to react with biotinyl goat IgG directed against rabbit IgG for 30 min and washed with PBS. Finally, HRP-avidin conjugate was applied to the sections for 5 min. The sections were washed with PBS and then reacted for 2–3 min with diaminobenzidine solution (10 mg of diaminobenzidine in 100 ml of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.001% H_2O_2). As controls, the following experiments were carried out; a) anti-CHT rabbit IgG was replaced by normal rabbit IgG; b) anti-CHT rabbit IgG was absorbed with excess bovine CHT and was used for the first step of staining; c) the sections were treated with non-labeled goat IgG directed against rabbit IgG and then with HRP-avidin; d) the specimens were reacted with biotinyl goat IgG directed against rabbit IgG and then with non-labeled avidin; and e) the specimens were stained directly with HRP-avidin conjugate to check the possible binding of HRP-avidin with naturally occurring biotin enzymes.

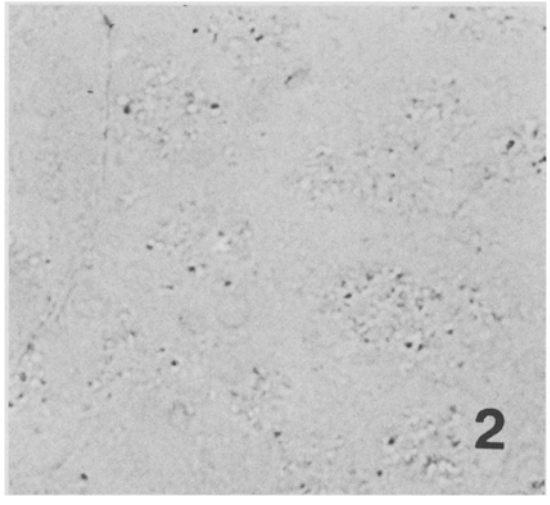

Fig. 2. Control specimen, treated with normal rabbit IgG in place of the antibody against chymotrypsinogen. No specific reaction is observed. × 300.

Results and discussion. By applying HRP-avidin conjugate to the sections, which had been previously treated with anti-CHT rabbit IgG and subsequently with biotinyl goat IgG directed against rabbit IgG, CHT was clearly localized in the zymogen granules in the acinar cells (figure 1). On the other hand, no specific reaction was recognized in the control specimens (figure 2). This suggests that the use of the A–B system is quite specific and effective not only for the demonstration of membrane associated antigens[4–8] but also for studies on the distribution of intracellular antigens, such as CHT. Although the distribution pattern of CHT in the acinar cells of the pancreas is the same as that obtained by the fluorescent antibody method[15] or by the HRP-labeled antibody method, the reaction between biotinyl antibody and HRP-avidin conjugate is far quicker and more stable than that between anti-CHT rabbit IgG and goat anti-rabbit IgG. Moreover, HRP-avidin conjugate can react with biotinyl antibody, even after the sections have been treated with biotinyl antibody and fixed with fixatives such as paraformaldehyde. The fixation does not disturb the A–B reaction[8]. These facts help to minimize the non-specific staining and prevent unexpected diffusion of the labeled antibody in the tissue. Thus, the use of the A–B system in immunohistochemical staining will contribute much to the enhancement of its specificity.

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Studies on muscle fibre splitting in skeletal muscle

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Summary. Autoradiographic, stereological and histological studies have been carried out to determine the origin of muscle fibre splitting which supposedly occurs during muscle hypertrophy. The results obtained clearly indicate that the supposedly 'split fibres' are a transient response probably derived from satellite cells and are not derived from pre-existing fibres by true splitting. Similarly, increases in muscle fibre size are not achieved by recruitment of satellite structures as indicated by lack of myonuclear recruitment.

Muscle fibre splitting has traditionally been regarded as a frequent non-specific feature of many chronic muscle diseases, notably in the muscular dystrophies. Its implications have attracted little attention though its biological importance has recently been emphasized[2,3]. Numerous reports have also indicated that severe exercise or muscular hypertrophy is associated with the development of similar but transient histological changes that are usually interpreted, though without evidence, as fibre splitting[4–12]. The present electron microscopical, stereological and autoradiographic study was undertaken to establish whether such a view is tenable.

Adult male mice weighing 20–25 g were randomly selected and subjected under sterile conditions and ether anaesthesia to a unilateral myectomy of the left tibialis anterior muscle of the hindlimb. It is known that removal of this muscle induces hypertrophy and fibre splitting in the ipsilateral extensor digitorum muscle[10,13,14]. During the operation great care was taken not to disturb any of the EDL muscles or their neurovascular supply. A sham operation, in which the tibialis anterior muscle was undisturbed, was carried out on the contralateral limbs of approximately one half of each experimental group of animals. The mice were allowed to recover for varying periods post-operatively prior to examination of their EDL muscles. It is well established that, even where deliberate trauma has been inflicted on rodent muscle sufficient to induce necrosis of a substantial proportion of fibres, satellite cell repair activities cease within 9 days[15].

At 7, 18, 35, 68, 98 and 128 days post-operatively specimens of 36 ipsilateral EDL muscles were obtained for electron microscopy[16,17] and enzyme histochemistry using routine procedures previously described[11,14]. Thick fresh frozen cryostat sections were also obtained which were stained to demonstrate succinate dehydrogenase activity (E.C.1.3.99.1). Thin sections of normal and hypertrophic EDL muscles were examined with a Philips 200 electron microscope during the early phases when the first occurrence of fibre splitting was expected to be most readily visible. The satellite cells clearly became most active at 7 and also at 18 days post-operatively as indicated by their increased electron lucency, by the formation of a distinct Golgi apparatus, by the occurrence of more numerous mitochondria and a marked reduction in the amount of peripheral nuclear heterochromatin (figure 1). The formation of satellite structures which were defined as those larger structures possessing clearly visible myofilaments and which lay in the same topographical relationship to

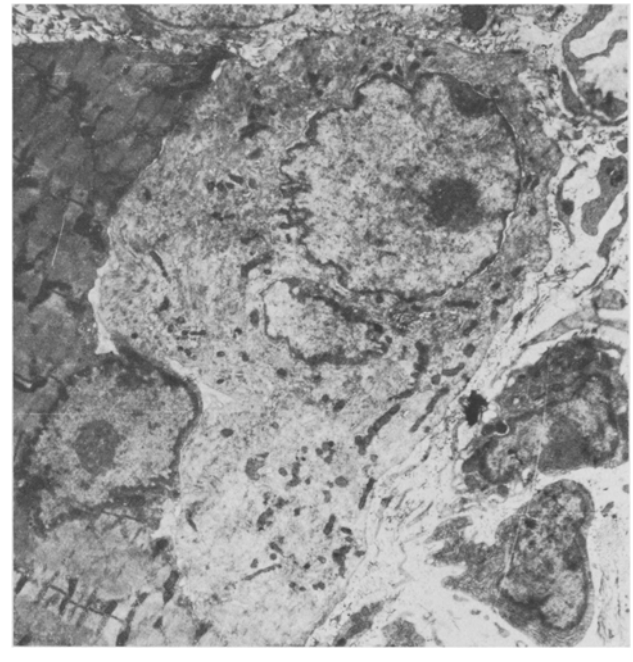

Fig. 1. Electron micrograph of satellite cell in 18-day hypertrophic EDL muscle. The cytoplasm is markedly electron lucent and is clearly increased in amount. The nucleus possesses a prominent nucleolus but little electron dense heterochromatin is present. Identification of satellite cells is facilitated by their close apposition to muscle fibres and lack of intervening basement membrane. ×4400.