(figure 3). However, when the number of EBs injected was more than 350 per mouse, the number of colonies became saturated at about 130 per lung. In the range that the number of EBs injected was less than 350 per mouse, the efficiency of colony formation was 35%. It may fluctuate by host and the populations of EBs from mouse. It is most likely that each colony in the lung is derived from single EBs so long as the number of EBs injected does not exceed 350 per mouse.

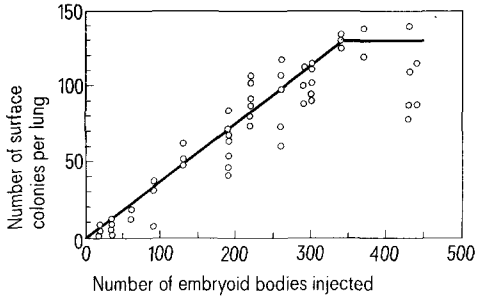

Fig. 3. Relationship between the number of embryoid bodies injected and the number of colonies formed on the lung surface.

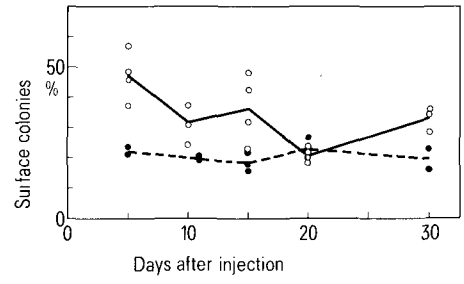

Fig. 4. Number of colonies on the lung surfaces of nonirradiated mice killed at various times after the injection. 2 experimental data are shown.

Figure 4 shows that the efficiency of the surface colony formation was nearly constant through the days after injection. This implies that a population of EBs capable of forming colonies is initially restricted and that the retarded colony formation rarely happens. By this phenomenon, a population of the lung colonies is expected to have the same age of injection, and we are able to compare the developmental stages and the differentiation potency among the colonies in the mice. Figure 4 shows that the teratomatous colonies in the lung were not rejected by host for about a month after injection.

EBs employed in the present experiment were 40–150 μm in diameter. When these large aggregates are injected i.v., they will certainly be first trapped in the capillary vessels of the lung and it will hardly be possible for them to go through the lung into the other organs. This would explain why EBs form colonies exclusively in the lung. We here stress that each colony may be derived from single EBs and may have almost identical environmental conditions. With these advantages, we can extend the utility of the system previously reported by Williams and Till[3]. All of colonies formed in the lung have one or more types of tissues[5]. Therefore, the lung colonies can also be investigated from the standpoint of developmental biology.

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Histochemistry of some trout respiratory muscles

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Summary. A histochemical study has been made of the main cranial muscles which produce ventilation movements of the rainbow trout. It is shown that a greater proportion of red(aerobic) fibres is present in those muscles known to be active during shallow ventilation than those which become active at greater ventilation volumes. An ordered recruitment of red, pink and white fibres within these muscles is also likely.

The cranial muscles of teleosts function in a co-ordinated manner to produce movements of the jaws, operculi and their associated skeletons during feeding and ventilation. The anatomy of the muscular and skeletal systems of a number of species has been described in some detail[2–4]. Recent electromyographical studies of lightly anaesthetised and free swimming fish has led to some understanding of the co-ordination of these muscles especially during respiration[5–11]. However, little is known about the fibre composition and metabolism of these muscles. In the present histochemical study the types and distribution of muscle fibres has been determined in 5 cranial muscles of the rainbow trout and the results correlated with previous investigations on their function[5,6].

Materials and methods. Rainbow trout (*Salmo gairdneri* Richardson) about 35 cm in length, were obtained from Midland Trout Hatchery, Nailsworth, Gloucestershire, and maintained in tanks at 15 °C. The muscles investigated (figure 1) were chosen because of their accessibility and varied function. A total of 6 fish were used in these investigations and the cranial muscles were dissected within 15 min of death which was by an overdose of MS 222 (Sandoz) anaesthetic. Blocks of muscle were mounted in 'Tyro-M-Bed' (Aerosol Marketing Chemical Co. Ltd, London) on cryostat chucks and rapidly frozen by plunging into liquid freon (Arcton 12, ICI) cooled to its melting point in liquid nitrogen (−159 °C). About 12 serial transverse sections 10 μm thick were cut from each block at −23 °C and mounted directly on coverslips.

Sections were stained for myofibrillar ATPase by a modified method of Guth and Samaha[12,13]. Fibre types were distinguished by preincubation for 1–10 min in a solution of 18 mM $CaCl_2$, 100 mM 2-amino-2-methyl-1-propanol pH 10.3 prior to staining for ATPase activity[13]. In contrast, fast white fibres which have a high myofibrillar ATPase activity biochemically[15] are more alkaline stable and are

unaffected by such periods of incubation. Another type of fibre can be distinguished in fish muscle on the basis of pH stability[13]. These so-called pink fibres usually have a pH stability at pH 10.3 several orders of magnitude greater than the white fibres together with other distinguishing features[13,14]. Sections were also stained for succinic dehydrogenase (SDHase), phosphorylase and lipid as described previously[15]. Fibre diameters were determined from photomicrographs by measuring the widest distance between the edges of the fibre in one plane.

Results and discussion. 4 basic fibre types could be distinguished on the basis of these stains. The staining characteristics, distribution and diameters of these fibre types are shown in figures 1 and 2. All muscles consist predominantly of fibres with high myofibrillar ATPase, intermediate phosphorylase and low succinic dehydrogenase activity. These fibres are analogous to the white fibres of the fast motor system of the trunk musculature[13-19]. Energy supply to fish white muscle is largely by anaerobic glycogenolysis from local glycogen stores[18,20]. Some areas (see figure 1) of all muscles studied, with the exception of the adductor mandibulae, contained fibres of high myofibrillar ATPase and an intermediate activity of SDHase. These regions show a wide distribution of fibre size (figures 2 and 3) with fibres with a diameter less than 50 µm showing the greatest staining for lipid and SDHase giving the muscle a mosaic appearance (mosaic white muscle) (figure 3). Pink fibres also with a high myofibrillar ATPase activity can be distinguished histochemically by their stability to alkaline preincubation at pH 10.3[13]. This type of fibre is present as a discrete band of muscle in the levator hyomandibulae et arcus palatini, adductor mandibulae and sternohyoideus and as isolated fibres scattered between the red and white fibre zones in the protractor hyodei muscle (figure 1). In the carp, pink fibres have been shown to have an intermediate myofibrillar ATPase activity to red and white fibres, abundant mitochondria and a high glycolytic capacity[17]. They are analogous to the fast oxidative glycolytic fibres of mammalian twitch fibres[20]. Red fibres which have high SDHase, low phosphorylase and low myofibrillar ATPase activities are always localised peripherally (figure 1). The proportion of slow red fibres in these muscles decreased in the following order: Levator hyomandibulae et arcus palatini > adductor mandibulae > protractor hyoidei > sternohyoideus > dilator operculi and covered a range from 30% to about 4% (figure 1).

The function of the jaw muscles of fish most clearly understood is that of respiration and this has been extensively studied in the trout[5,7,8]. The co-ordinated contraction of a fairly large number of jaw muscles causes volume changes in the buccal and opercular cavities and a concomittant pumping of water over the gills. Electromyographical evidence has shown that during shallow ventilation only 3 muscles are active namely the adductor mandibulae, adductor arcus palatini et operculi and levator hyomandibulae et arcus palatini[7,8]. These 3 muscles have the highest proportion of tonic red fibres. These types of fibres in vertebrates predominate in muscles which are adapted for continuous, sustained, low intensity effort of which respiratory movements under resting conditions provides a good example[20]. During deeper ventilation the hyohyoideus, sternohyoideus and protractor hyoideus come into operation[7,8]. The 2 of these 3 muscles studied had a lower

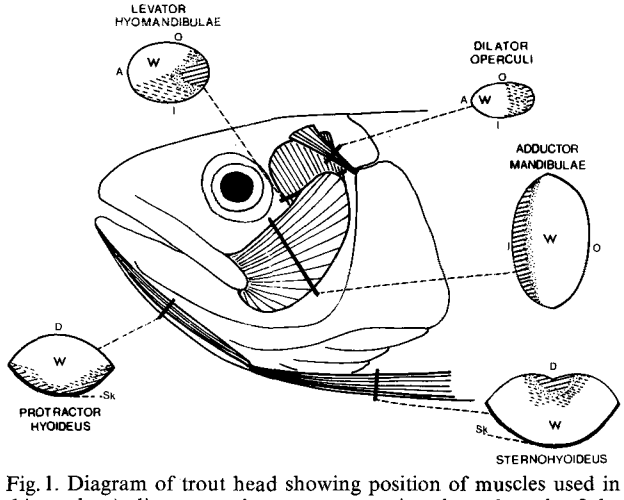

Fig. 1. Diagram of trout head showing position of muscles used in this study. A diagrammatic transverse section through each of the muscles is also shown and indicates distribution of different fibre types: ≡, Red, high aerobic, intermediate phosphorylase, low myofibrillar ATPase fibres. ▒, Pink, intermediate aerobic, high phosphorylase high myofibrillar ATPase fibres. ⁝White (M) low-intermediate aerobic, high anaerobic high myofibrillar ATPase fibres. □, White, very low aerobic, high anaerobic, high myofibrillar ATPase fibres. (Boundaries between these different regions are not well defined). Orientation of the sections is shown by D, dorsal; O, outside; I, inside; A, anterior; Sk, skin; W, white muscle.

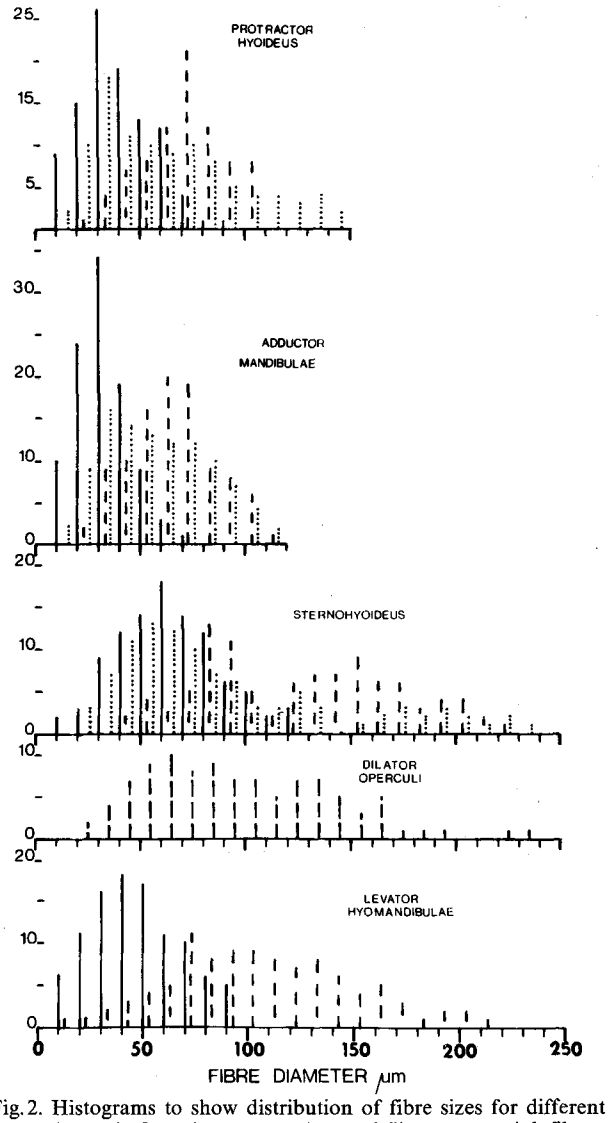

Fig. 2. Histograms to show distribution of fibre sizes for different types of fibre in 5 respiratory muscles. Red fibres, ——; pink fibres, ·····, white (M) fibres, — —; white fibres, – – –.

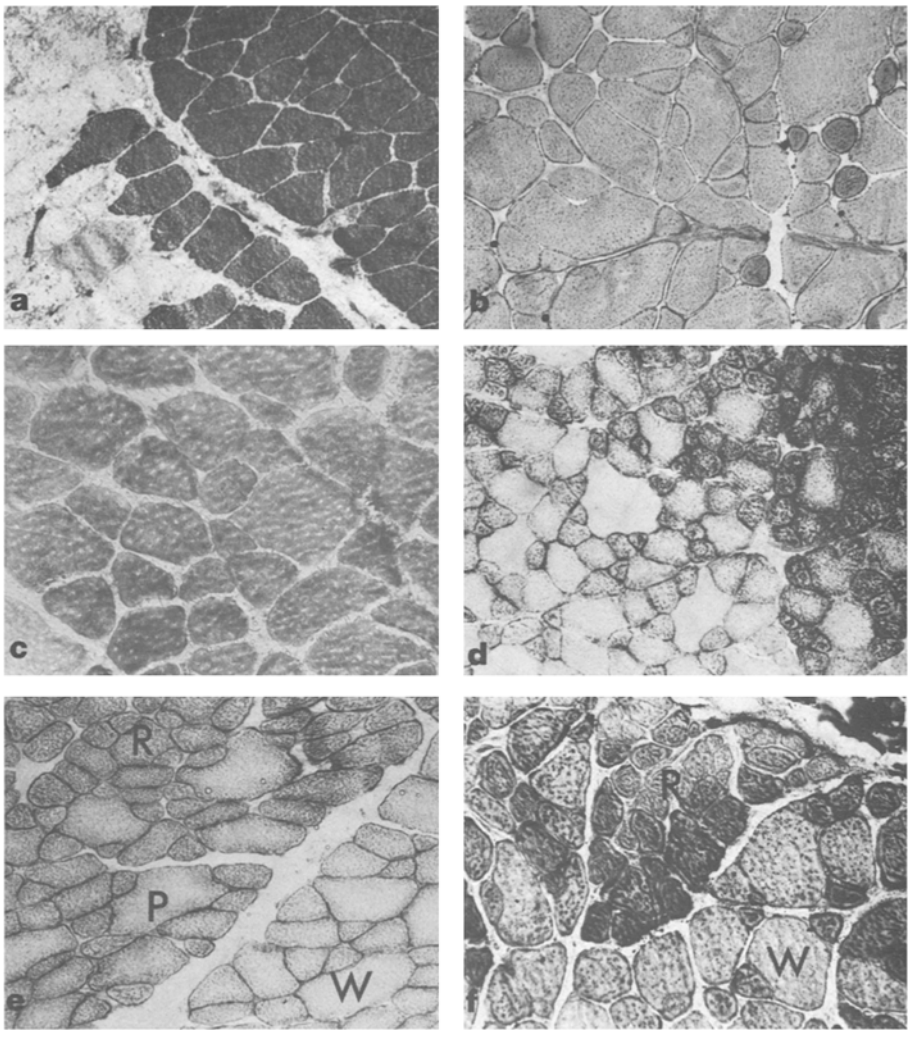

Fig. 3. *a* Sternohyoideus stained for myofibrillar ATPase activity following 1-min preincubation at pH 10.3 in 18 mM $CaCl_2$, 100 mM $CaCl_2$-amino-2-methyl-1 propanol at pH 10.3 showing small diameter low activity (red) and larger diameter high activity white fibres. × 68. *b* Section of sternohyoideus white fibres stained for lipid. Note low lipid content. Some isolated small fibres have a somewhat higher lipid staining in this muscle. × 68. *c* White fibres of the dilator operculi showing high phosphorylase activity. × 137. *d* Adductor mandibulae stained for lipid showing red (high lipid content) and mosaic pattern of pink fibres (see text). × 68. *e* Section of adductor mandibulae stained for succinic dehydrogenase showing red (R) pink (P) and white (W) fibres with high, intermediate and low activities respectively. Note pink fibres with diameters less than 50 μm have higher aerobic capacity than those larger diameter pink fibres. × 68. *f* A section of protractor hyoideus stained for lipid. This muscle contains only red (R) and white (W) fibres. × 137.

proportion of red fibres than those used for continuous ventilatory movements and probably rely partly on the anaerobic metabolism of the fast fibres during vigorous ventilation. Consistent with this pattern the lowest proportion of red fibres is found in the dilator operculi which only contracts in opercular abduction during strong ventilation and during coughing[7,8]. The sporadic nature of the activity of this muscle probably allows for a more anaerobic type of metabolism and presumably a fast muscle is required for the rapid abduction of the opercula, particularly during coughing. The role of cranial muscles in feeding in the trout has been much less extensively studied than their respiratory functions. Contraction of the adductor mandibulae to adduct the lower jaw plays a central role in catching prey which is usually taken in mid-water or at the surface[21]. The sternohyoideus functions in abduction of the hyoid and lower jaw during both feeding and swallowing[21]. Both of these muscles, particularly the sternohyoideus which only has an accessory role in respiration consist of predominantly fast white fibres (figure 1).

It therefore seems probable that there is a division of labour between different parts and between different jaw muscles which is somewhat analogous to that of the myotomal swimming muscles. The aerobic red slow fibres are probably more concerned with the continuous maintenance of respiratory activity while the anaerobic fast twitch fibres are predominantly employed for the vigorous jaw movements associated with feeding. However, there is probably an orderly recruitment of fibres, red, pink, white, from different regions of the cranial muscles in a way analogous to that seen in the lateral muscles during swimming[17]. This has already been noted, for example, in the adductor mandibulae of the dogfish where electrical activity has been recorded from different regions of this muscle during different types of activity e.g. shallow ventilation versus hyperventilation and coughing[6].

This functional interpretation contrasts with the different homologies of the cranial muscles investigated[22]. The sternohyoideus and protractor hyoideus being myotomic in origin, innervated by ventral root nerves (XII). Whereas the remainder are modifications of lateral plate muscles innervated by the special viscero-motor component in dorsal root nerves (V & VII).

1 This work was carried out when I. A. Johnston was holding an NERC Post-Doctoral Research Fellowship (1973–75).
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Differentiating abilities of avian somatopleural mesoderm[1]

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Summary. Quail-to-chick grafting experiments were performed on 2-day embryos in order to test the differentiating abilities of the somatopleure. After orthotopic and heterotopic transplantations of different parts of quail somatopleural mesoderm into chick embryos it is demonstrated that avian somatopleural cells differentiate into skeletal elements, smooth muscles, tendons and connective tissues. However, skeletal muscle fibres do not originate from somatopleural cells.

In previous studies using the quail-chick marker technique according to Le Douarin and Barq[2] it has been shown that the limb, thoracic and abdominal muscle cells are of somitic origin whereas the connective tissues and the tendons originate from the somatopleural mesoderm[3-11].

In contrast to our findings that the muscle cells solely differentiate from somitic cells, Chevallier et al.[9,10] have the idea that the somatopleure can also give rise to muscle fibres. These authors believe that under special conditions, the somatopleural mesoderm seems able to compensate for somitic deficiency. In view of this concept a new series of experiments was undertaken in order to test the differentiating abilities of the somatopleural mesoderm.

Material and methods. The experiments were carried out on 2-day chick and quail embryos (White Leghorn, *Coturnix coturnix japonica*). Parts of somatopleural mesoderm and adjacent ectoderm previously isolated from different levels of quail donors were orthotopically or heterotopically grafted on chick embryos. The affected somatopleural fragments had at that time not been invaded by myogenic cells from the somitic mesoderm.

The orthotopically implanted somatopleural fragments participate in the normal development and differentiation of limb and ventral body wall structures. Analysing their cellular composition, it can be stated that with the exception of the ribs the remaining cartilage elements and the complete connective tissue are made up of quail cells. The blood vessels can be identified as composite structures in which the endothelia mainly consist of chick cells whereas the surrounding connective tissue cells of the tunica media exhibit nuclei of the quail type (figure 1). From this it may be concluded that somatopleural cells can differentiate into smooth muscle fibres.

The skeletal muscular bulks within the operated regions are generally found to be of bispecific composition. While the tendons as well as the intra- and peri-muscular connective tissue are of quail (somatopleural) origin, the muscle fibres are of chick (somitic) origin. After histological examination of 50 embryos operated in this way no indication was found that somatopleural cells can form muscle fibres, since the myotubes exclusively contain nuclei of the chick type (figure 2). Moreover, hybrid myotubes cannot be observed.

From these results regarding the normal differentiating abilities of somatopleural cells, the question arises whether the somatopleural mesoderm in the absence of somites can undergo muscular differentiation. In order to test this possibility, in the 2nd experimental series, parts of somatopleural mesoderm and adjacent ectoderm of quail embryos were grafted into the coelomic cavity of chick embryos.

If the grafts consist of prospective limb mesoderm, well developed wings and legs with quite reasonable distal elements can be found. Examination of the pattern in such

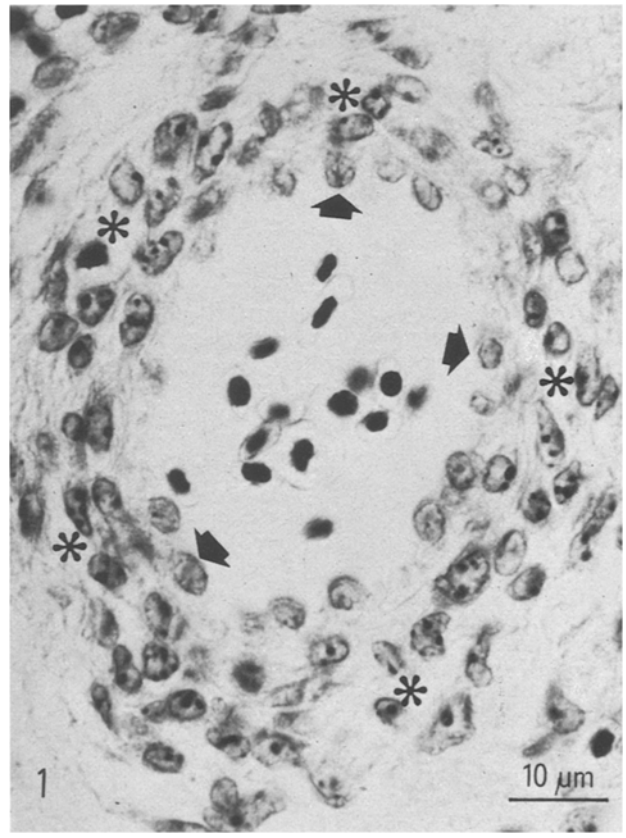

Fig. 1. Rudimentary blood vessel within a leg of a chick embryo 9 days after orthotopical replacement of somatopleural mesoderm by quail somatopleure. Endothelial cells (arrows) exhibit chick nuclei. The tunica media (asterisks) made up by quail cells. Feulgen-Rossenbeck reaction, post-stained with light green.