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Development before and after hatching of non-cholinergic excitatory innervation to the rectum via Remak's nerve in the fowl

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1 Development of the excitatory innervation to the rectal region of the intestine via Remak's nerve has been investigated in the rectum with Remak's nerve supply isolated from chicken embryos and young chicks aged less than two weeks.

2 Electrical stimulation of Remak's nerve produced a small contraction of the rectum isolated from chicken embryos on the 14th day of incubation (the earliest time examined). The contractile response was inhibited partially or totally by atropine (0.1 $\mu g\,ml^{-1}$) but enhanced by physostigmine (0.01 to 0.05 $\mu g\,ml^{-1}$), indicating its cholinergic nature.

3 During the embryonic stage, the proportion of the atropine-resistant component in the contractile response increased, and the contractile response became almost entirely atropine-resistant within the first week after hatching.

4 Later after hatching, the contractile response was increased in magnitude by atropine and reduced by physostigmine.

5 It is concluded that the excitatory innervation to the chicken rectum via Remak's nerve is cholinergic at the 14–16th day of incubation and is gradually replaced by a non-cholinergic innervation during embryonic development.

Introduction

The peripheral autonomic nerves develop from cells present in the neural crest or neural tube. The presence of Remak's nerve, which is ganglionated and ascends from the cloaca to the duodenum along the digestive canal, is characteristic of fowl. The anal end of Remak's nerve is connected with nerves originating from the pelvic plexus (Watanabe, 1972; Akester, 1979). At its oral end, it receives nerve fibres from the coeliac and mesenteric plexuses (Nolf, 1934; Yntema & Hammond, 1954; 1955; Bennett & Malmfors, 1970; Cantino, 1970). Pharmacological analysis of the mechanical responses of the rectum to electrical stimulation of Remak's nerve revealed that the nerve consists of cholinergic and non-cholinergic excitatory fibres, and adrenergic and non-adrenergic inhibitory fibres (Bartlet & Hasssan, 1971; Takewaki *et al.*, 1977; Komori & Ohashi, 1982; 1984; 1987). Cholinergic nerve fibres, due to their high content of cholinesterases, can be identified histochemically by cholinesterase-staining techniques in the myenteric plexuses of the intestine as early as on the 3rd day of incubation, and are as well developed as in adult chickens by the 13th or 14th day of incubation (Keller, 1976; Bunge *et al.*, 1978). Le Douarin *et al.* (1975) have observed cholinergic nerve-mediated mechanical responses in the isolated duodenum of the foetal chicken on the 15th day of incubation. Adrenergic nerve fibres can also be demonstrated with the fluorescent histochemical technique in the myenteric plexuses of the gastrointestinal tract of foetal chickens on the 16th to 18th day of incubation (Enemar *et al.*, 1965), and the content of noradrenaline and adrenaline reaches a level high enough to be detected by the extraction method (Konaka *et al.*, 1979). However, no paper dealing with development of non-adrenergic and non-cholinergic (NANC) neurones in the fowl has been published as yet.

The present study was carried out on the isolated rectum with Remak's nerve supply from foetal chickens at different embryonic ages and young

chickens aged less than two weeks in an attempt to determine how NANC excitatory innervation develops by recording mechanical responses to electrical stimulation of Remak's nerve.

Methods

Eggs, incubated for a different number of days, and chickens of either sex aged less than two weeks (Rhodehorn, Goto 360) were obtained from Goto chicken-raising company. Foetal and young chickens were decapitated and bled to death. The rectal region of the intestine was excised with Remak's nerve and the lumen was flushed clean with a physiological salt solution.

Measurement of mechanical responses

The isolated rectum with Remak's nerve supply was mounted horizontally in a 5 ml organ bath. The anal end was fixed with a pin on a black rubber board in the bottom of the organ bath, and the other was attached by thread to a transducer. The organ bath was filled and perfused (by use of a roller pump) at a flow rate of 2.5 ml min^{-1} with Krebs-Henseleit solution (composition (mM): NaCl 118.9, KCl 4.6, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgCl_2$ 2.4 and glucose 11.1), and kept at 30 ± 1°C while being bubbled with a mixture of 95% O_2 plus 5% CO_2. The anal cut end of Remak's nerve was placed in a fine bipolar suction electrode for stimulation of the nerve with trains of 20 square-wave pulses of 1 ms duration at 10 Hz and supramaximal intensity (80–90 V) delivered through an isolator (Nihon Kohden, SS-302J) from a stimulator (Nihon-Kohden, SEN-3013). Longitudinal changes in tension of the rectum were measured isometrically by a force-displacement transducer (Nihon Kohden, TB-612T) and recorded.

In some experiments, the isolated rectum was dissected, blotted on a filter paper and weighed for calculation of tension development per unit wet weight of the tissue.

The experimental values obtained were expressed as mean $\pm$ s.e.mean. The regression line was calculated by the least squares method. Statistical significance was tested by Student's t test.

Results

Embryos and chicks up to 3 days after hatching

Mechanical responses to electrical stimulation of Remak's nerve As shown in Figure 1, the wet weight of the rectal region of the intestine increased progressively with time after incubation until hatching on the 21st or 22nd day of incubation. On the 8th day of incubation, Remak's nerve trunk and its branches extending to the intestine could be observed under a binocular microscope. The isolated rectum showed spontaneous movements first between the 14th and 16th day of incubation. At this time in development, electrical stimulation of the anal cut end of Remak's nerve was effective in producing mechanical changes in the rectum. Of 14 rectums tested, contraction was elicited in 9 rectums, but in the remaining 5 only relaxation was obtained. The contractile response reached a peak tension within 4 s after the start of stimulation and faded to the base level within about 30 s. During the remaining embryonic period, the magnitude of tension development increased, and the time required to reach the peak tension of its onset was slightly shortened. The graph in Figure 2 presents a plot of change in tension development per unit wet weight tissue against the day of incubation.

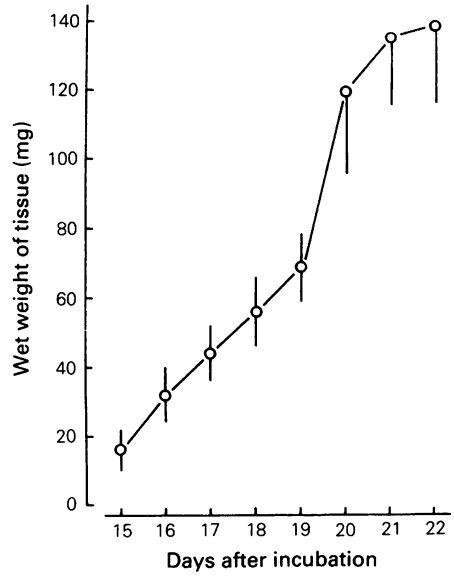

Figure 1 Increase in the wet weight of the rectal region of the intestine of chicken embryos with time after incubation. Ordinate scale: wet weight of tissue (mg). Abscissa scale: embryonic age (days after incubation). Hatching occurred 21 or 22 days after incubation. Each point represents the mean of three to six separate measurements. Vertical lines indicate s.e. means.

The results clearly indicate that Remak's nerve functions on the 14th day of incubation, and that it must have been in existence during an earlier period.

Effect of atropine The excitatory neurotransmitter responsible for the contractile response was exam-

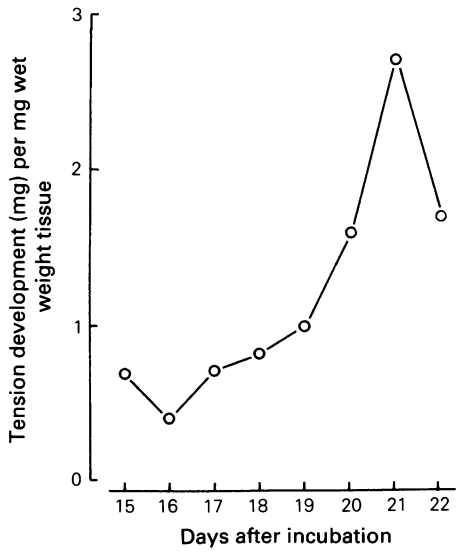

Figure 2 Development of contractile force of the rectum in the responses to Remak's nerve stimulation. Ordinate scale: tension development (mg) per mg wet weight tissue of the rectum. The mean tension development obtained from the three to six separate measurements was divided by the mean wet weight given in Figure 1. Abscissa scale: embryonic age (days after incubation). Trains of 20 square-wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for stimulation of Remak's nerve.

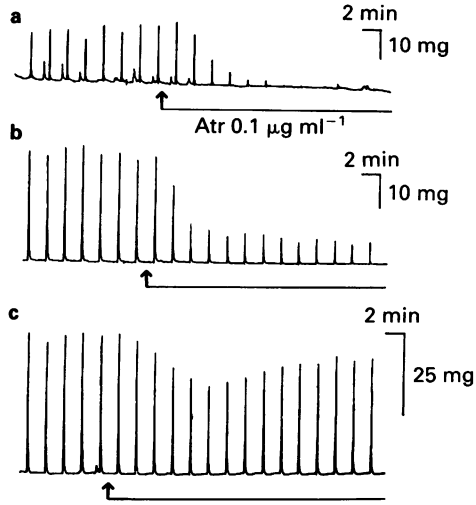

Figure 3 Effects of atropine (Atr 0.1 $\mu g\,ml^{-1}$) applied at arrows, on the contractile responses of the rectum to Remak's nerve stimulation at different ages. (a) At the 15th day of incubation; (b) at the 19th day of incubation; (c) 3 days after hatching. Trains of 20 square wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for Remak's nerve stimulation.

ined by use of atropine. In most rectums, atropine, 0.1 $\mu g\,ml^{-1}$, reduced the contractile responses (Figure 3). However, the contractile responses of some rectums were unchanged in some cases or were potentiated after application of atropine. The atropine-resistant contraction disappeared after application of tetrodotoxin (0.2 $\mu g\,ml^{-1}$), suggesting that the contractile response may be mediated by a neurotransmitter the action of which is atropine-resistant. Frequently the contractile responses of the rectum isolated from chicken embryos during the 14–16th day of incubation were converted to a relaxation in the presence of atropine. The proportion of rectums giving contractile responses resistant to atropine: to the total number of rectums increased with incubation time. Furthermore, the proportion of the atropine-resistant component relative to the total response in one rectum also increased with the day of incubation. This was also the case for the day after hatching. Figure 4 shows plots of the ratio between the magnitude of contractile responses before and after atropine against the embryonic age (days after incubation). It was found that there is a

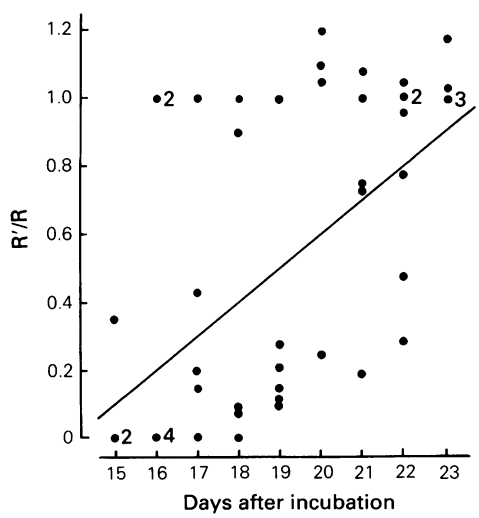

Figure 4 Ratio of magnitude of the atropine-resistant component (R') to the total contractile response (R) plotted against the embryonic age (days of incubation). Hatching occurred on the 21st or 22nd day of incubation. Figures attached to the dots show the number of the same observations. The calculated regression line for the dots is given as $Y = 0.1X - 1.4$ ($r = 0.58$, $n = 46$, $P < 0.01$), where $Y = R'/R$ and $X =$ the day of incubation.

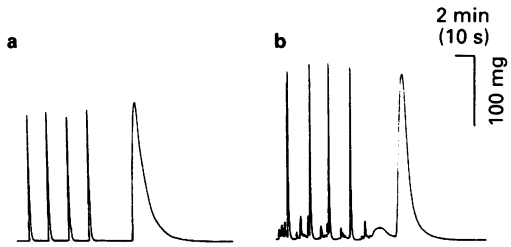

Figure 5 Effect of atropine on the contractile responses to Remak's nerve stimulation in the isolated rectum from a chick just after hatching. (a) Control; (b) 15 min after atropine, 0.1 $\mu g\,ml^{-1}$. The responses were recorded at two different speeds. Trains of 20 square-wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for stimulation of Remak's nerve.

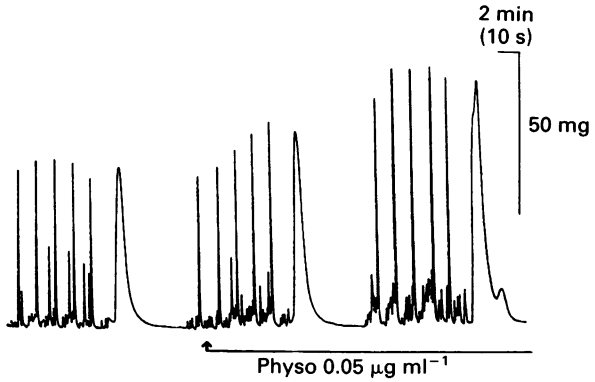

Figure 6 Effect of physostigmine (Physo 0.05 $\mu g\,ml^{-1}$) on the contractile responses to Remak's nerve stimulation in the isolated rectum from a chicken embryo on the 19th day of incubation. Trains of 20 square-wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for stimulation of Remak's nerve and the responses were recorded at two different speeds.

positive correlation between the magnitude of atropine-resistant components and the embryonic age ($r = 0.58$, $n = 46$, $P < 0.01$, the regression line, $Y = 0.1X - 1.4$).

Figure 4 also shows that the number of rectums in which the contractile responses were not inhibited by atropine (0.1 $\mu g\,ml^{-1}$) abruptly increased after hatching (12 of a total of 20 tested). Actually, in 4 of these 12 cases, the contractile responses were rather increased in magnitude by atropine (Figure 5). In the remainder (8 cases), the magnitude was first slightly reduced and then returned to its initial level even in the continued presence of atropine, and the restored magnitude remained unchanged after reapplication of freshly-prepared atropine. However, in eight other rectums the contractile responses were still reduced to different extents after application of atropine. The inhibitory effect of atropine usually faded to some extent before its removal, as shown in Figure 3c. In one of these 8 cases, the contractile response was obtained with two peaks. The main peak was markedly reduced, and the second peak was abolished by atropine. In general, the contractile response in the presence of atropine had the characteristic of faster decay than the control response (see also Figure 5).

Effect of physostigmine The effect of physostigmine, 0.01 to 0.05 $\mu g\,ml^{-1}$, on the contractile response to Remak's nerve stimulation was examined in the 20 rectums isolated from chicken embryos during the 16th–19th day of incubation. Physostigmine caused a significant increase of the contractile response in 18 of these, as shown by a typical result in Figure 6, and in the remaining two, this drug had no effect on the contractile response. As shown in Figure 7, the potentiating effects of physostigmine can be classified into 3 groups, In group (a) (6 cases), the response had only one peak before and after physostigmine treatment. In group (b) (8 cases) and group (c) (4 cases), the response displayed two components after physostigmine treatment; in group (b) the second component peaked, and in group (c) the first component peaked. No correlation was found between the response pattern and the embryonic age.

In addition, in all 18 cases, further application of atropine, 0.1 $\mu g\,ml^{-1}$, resulted in a marked reduction of the contractile response; the reduction varied from 39% to 100%.

The contractile responses in most rectums (about 75% of the total) isolated from chicks aged 1–3 days after hatching were reduced in magnitude by physostigmine (Figure 8), but in the remaining 25% physostigmine did not significantly change the responses.

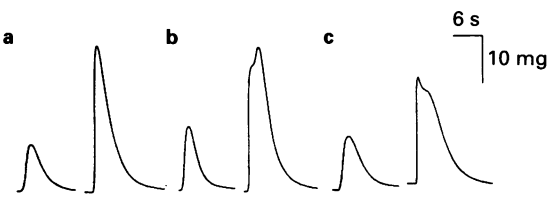

Figure 7 Different patterns of the contractile responses to Remak's nerve stimulation in the presence of physostigmine, 0.05 $\mu g\,ml^{-1}$, in isolated embryonic rectums. In each pair, left record is control, and the right record in the presence of physostigmine. (a) On the 17th day of incubation, right record, 20 min after physostigmine; (b) on the 18th day of incubation, right record, 18 min after physostigmine; (c) on the 17th day of incubation, right record, 28 min after physostigmine. Trains of 20 square-wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for stimulation of Remak's nerve.

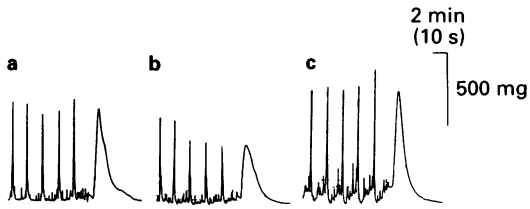

Figure 8 Effects of physostigmine, 0.02 $\mu g\,ml^{-1}$, and of atropine, 0.1 $\mu g\,ml^{-1}$, on the contractile responses to stimulation of Remak's nerve in the isolated rectum from a 3 day old chick. (a) Control; (b) 2 min after physostigmine; (c) 20 min after combined application of physostigmine and atropine. Trains of 20 square-wave pulses at 10 Hz were used for stimulation of Remak's nerve and the responses were recorded at two different speeds.

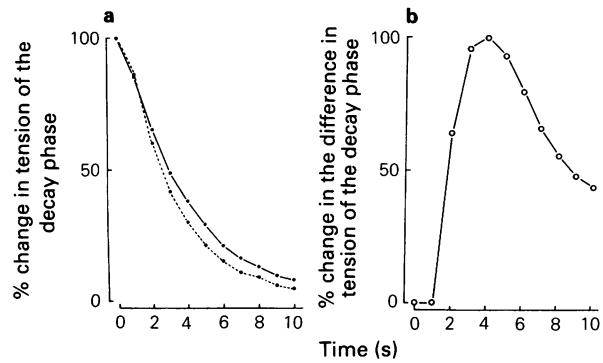

Figure 9 Difference in the time course of the decay phase of the contractile responses to Remak's nerve stimulation before and after application of atropine, 0.1 $\mu g\,ml^{-1}$, in the isolated rectums from chicks 1–3 days after hatching. (a) Comparison of the decay phases of the contractile responses, each point representing the mean of four separate measurements. Ordinate scale: relative tension in the decay phase of the contractile responses expressed as a percentage of maximal tension before and after application of atropine. Continuous line: before application of atropine. Dotted line: after application of atropine. Abscissa scale: time (s) after the peak. Trains of 20 square-wave pulses of 1 ms duration and supramaximal intensity at 10 Hz were used for stimulation of Remak's nerve. (b) Graph plotting the difference in the relative tension between before and after atropine in (a) against time (s) after the peak tension (the maximal difference at 4 s was taken as 100%). See text.

In all responses which were reduced in magnitude by physostigmine, the decay phase was slightly prolonged. The responses after additional application of atropine (0.1 $\mu g\,ml^{-1}$) (Figure 8c) were usually greater in magnitude than those before application of physostigmine.

Chicks 4–11 days after hatching

Effect of atropine Of the nine rectums isolated from 5–9 day old chicks which were examined, the contractile responses were reduced by atropine in only 3 cases, and the extent of the reductions was less than 20%. In the remainder (6 cases), the contractile responses were increased in magnitude. One of these 6 rectums responded with a biphasic contraction in normal solution, and the second component reached its peak about 20 s after the start of nerve stimulation. This late component was also cholinergic in origin since it was abolished after application of atropine (0.1 $\mu g\,ml^{-1}$).

The time course over which the contractile response decayed was invariably faster in the presence of atropine than in the control. Figure 9a shows plots of mean tension in the decay phase of four pairs of the contractile responses before and after atropine, in which the respective peak tensions were taken as 100%. Figure 9b presents the differences between the mean tensions in (a) as a percentage of the maximal difference (at 4 s) against time after peak tension. This graph revealed the time course of the atropine-sensitive component, probably mediated by cholinergic nerves, masked in the control response: it reaches the maximum 4 s after the peak. To confirm the presence of the masked atropine-sensitive component, another four pairs of responses showing the same peak magnitudes before and after application of atropine (0.1 $\mu g\,ml^{-1}$) were selected. The areas, defined by the contraction curve and the base line, were measured by a pattern analyzer, and these were compared before and after atropine application. The area before atropine was invariably greater than that after atropine. Furthermore, each of the areas was divided into two parts by drawing a vertical line from the point of peak to the base line, and the areas corresponding to the first and second halves of the contractile response were measured. Comparison between the mean areas before and after atropine was made for each part. The difference was statistically significant only in the area corresponding to the second half of the contractile responses.

Effect of physostigmine The effects of physostigmine (0.01 to 0.05 $\mu g\,ml^{-1}$) on the contractile responses of rectums from 4–11 day old chicks were essentially similar to those observed in the rectums isolated from younger chicks. In most rectums isolated from 8 to 11 day-old chicks, a contractile response consisting of two components was obtained, as previously reported by Komori & Ohashi (1984). The first component was larger and reached a peak tension in 2 to 3 s ($n = 7$) after the start of nerve stimulation. The second, smaller component began

10 to 15 s after the beginning of nerve stimulation, 10 to 15 s later reached a peak tension and faded completely within 30 s after the peak time. Physostigmine, 0.01 $\mu g\,ml^{-1}$, reduced the first component, but enhanced the second component. Atropine, 0.3 $\mu g\,ml^{-1}$, abolished the second component but enhanced the first component beyond its control magnitude in most cases.

Discussion

In chick embryos the excitatory innervation to the rectal region of the intestine via Remak's nerve can be demonstrated during the 14th to 16th day of incubation. This was the earliest time in development that we were able to examine tension changes in the rectum in response to stimulation of Remak's nerve. In almost all rectums examined, the pharmacological properties of the contractile response to stimulation of Remak's nerve indicated its cholinergic nature. Only a few rectums gave contractions resistant to atropine, possibly because they received innervation of non-cholinergic excitatory neurones at an extremely early stage. The contractile response required 4 s to reach the peak tension after the start of nerve stimulation. During the remainder of the embryonic stage, the time to reach peak tension was decreased to 2–3 s. This shortening of peak time may have resulted from maturation of the neuro-effector synapses and the smooth muscle.

An interesting finding is that there is a significant increase in the proportion of the atropine-resistant component in the contractile response to Remak's nerve stimulation during the embryonic stage, and the contractile response becomes virtually atropine-resistant within two weeks after hatching. The atropine-resistant contraction decayed with a faster time course than the control contraction. It was found that the contractile response that can be inhibited by physostigmine is enhanced by atropine. Thus, a chemical transmitter mediating the atropine-resistant contraction is unlikely to be acetylcholine, as previously described for older chicks (Bartlet, 1974; Takewaki *et al.*, 1977). Recently, adenosine 5'-triphosphate (Meldrum & Burnstock, 1985) and chicken neurotensin (Komori *et al.*, 1986) were proposed as candidates for this chemical transmitter.

The alteration of the nature of the excitatory innervation of Remak's nerve to the rectum during development could be explained in two different ways. One possible explanation is that newly developed nerve fibres are responsible for the atropine-resistant response, which may be derived from cells in the ganglia of Remak's nerve trunk in which cell bodies of non-adrenergic, non-cholinergic (NANC) neurones are located in adult chickens (Kanazawa *et al.*, 1980). There has been no pertinent information on the embryonic nerve cells in Remak's ganglia. The present results suggest that this neuromuscular transmission is regulated by an inhibitory cholinergic mechanism. The electrophysiological studies carried out in the rectum of adult chickens have provided evidence for this notion (Takewaki & Ohashi, 1977; Komori & Ohashi, 1964): the e.j.p. evoked by stimulating Remak's nerve in the rectum has been shown to be increased in amplitude by atropine with no change in the membrane properties of the smooth muscle. The present results, combined with these electrophysiological findings, lead us to suspect that the cholinergic fibres undergo changes in their function with growth; they are used exclusively to control smooth muscle cells during the early stage of development, and later to inhibit release of an unknown transmitter from the newly developed nerves (non-cholinergic excitatory nerves) through muscarinic receptors. Two or more weeks after hatching the cholinergic fibres are used exclusively to control non-cholinergic neuromuscular transmission.

Another possibility is that the well-developed cholinergic fibres acquire an ability to release another transmitter substance (in addition to acetylcholine), whose action is not antagonized by atropine. Recent studies suggest that single nerve fibres contain more than one transmitter substance which may be co-released by nerve impulses (Burnstock, 1981). According to this concept, the present results can be explained only by assuming that acetylcholine, released from the cholinergic nerve fibres, exhibits its action not only at the smooth muscle cells but also at the nerve terminals to inhibit release of these two substances through muscarinic receptors and that the former action is weakened with growth. However, in older or adult chickens, no membrane response favourable to co-release of these two transmitter substances was recorded from the rectal smooth muscle following stimulation of Remak's nerve (Komori, unpublished observations): there was no apparent membrane depolarization that was reduced by atropine, although exogenously applied acetylcholine produced membrane depolarization of the smooth muscle (Komori & Ohashi, 1984). It is very difficult to explain how acetylcholine co-released with an unknown transmitter substance can be used for the presynaptic inhibition with no influence on the smooth muscle after hatching. Thus, the second possibility seems to be less acceptable. If this unknown transmitter substance is identified, a number of reliable methods to localize and characterize neurones on the basis of their transmitter substance would give more information on the developmental change of innervation to the rectum via Remak's nerve in the chicken.

The cholinergic response, which reached peak

tension 15 to 25 s after the beginning of nerve stimulation, was also demonstrated and seen frequently in the rectum isolated from chicks 8 to 11 days after hatching. This cholinergic component is probably similar to that which was previously observed in older chickens (Komori & Ohashi, 1984). Because of its slow onset, the cholinergic contraction would be mediated by acetylcholine overflowing from the myenteric plexuses in which cholinergic interneurones may be driven by stimulation of Remak's nerve. Studies with the isolated, perfused rectum of the chicken have demonstrated the increase in acetylcholine concentration in the venous effluent following stimulation of Remak's nerve (Komori & Ohashi, 1984).

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