Cyclic AMP Assay: Litters of male Wistar rats aged 3, 7, 14, 28 days and 63 days as adults were used. The time of birth was determined within 12 hr. Rats were decapitated at 10 a.m. and the rectums removed. These tissues were scraped with a spatula to remove the mucosal layer. After preincubation in Locke’s solution maintained at 37°C, preparations
were incubated in the medium containing various concentrations of NE for various times. These tissues were immediately frozen and homogenized in 6% trichloroacetic acid. The tissue homogenate was extracted with ether and the residual ether expelled with a stream of air. The tissue extracts were neutralized to pH 7.5 with 1 M Tris buffer and purified according to the method of Walton and Garren (9). Measurement of cyclic AMP concentrations was carried out according to the protein binding method of Gilman (10).

Immunohistochemistry for cyclic AMP: Histochemical localization of cyclic AMP was determined by an indirect immunofluorescence procedure. Adult rats were decapitated, the rectums removed and frozen in isopentane chilled by acetone and a dry ice mixture. The frozen tissue was sectioned in a thickness of 4–6 \( \mu \)m in a Bright cryostat. Rabbit antiserum containing antibody against cyclic AMP which was prepared by the method of Steiner et al. (11), was obtained from the Research Laboratories of Yamasa Shoyu Co. Ltd., Choshi, Japan. The specificity of the antibodies was tested using radioimmunoassay (11). Relative binding affinity of AMP, ADP, ATP, GMP, GDP, GTP, adenosine, guanosine and cyclic GMP to antiserum against cyclic AMP was minimal. Values were less than 0.005%, when the ability of cyclic AMP to inhibit bindings of \(^3\)H-cyclic AMP was arbitrarily set at 100%. Fluorescein isothiocyanate (FITC)-labeled goat antibodies to rabbit IgG were used after shaking twice for 5 min with washed rat spleen acetone powder.

Drugs and chemicals: Drugs used were 1-norepinephrine bitartate (Sigma Chemical Co.), propranolol hydrochloride (Sumitomo Chemical Co.) and FITC-labeled goat antibodies to rabbit IgG (Behringwerke Institute). \([H] \) cyclic adenosine 3',5'-monophosphate purchased from New England Nuclear Corporation (Boston, Mass.), had a specific activity of 27.5 Ci/m mole. Other chemicals used were of reagent grade and were not purified further.

RESULTS

I. Basal levels and NE-induced elevation of cyclic AMP in the rectum of postnatal rats.

Cyclic AMP levels (approximately 2 pmole/mg wet wt.) in the tissues of rectums from adult rats were gradually lowered with incubation in Locke’s solution at 37°C and reached a steady state level 20 min after incubation (Fig. 1). Thus, preparations were incubated in the medium containing NE after 30 min preincubation and cyclic AMP levels after 30 min preincubation were used as basal levels. Elevation of cyclic AMP levels was dose-dependent and was maximal in a concentration of 10\(^{-4}\) M NE in the rectum from 28-day-old and adult rats and of 10\(^{-5}\) M NE in that from 3-, 7- and 14-day-old rats. The time courses of NE (10\(^{-4}\) M)-induced elevation of cyclic AMP levels in rectum from adult rats and of NE (10\(^{-5}\) M)-induced elevation of that from 7-day-old rats were examined. At 10 sec, elevation of cyclic AMP levels in the rectum from adult rats was maximal (170%) and was followed by a fall and subsequent stabilization of levels between 20 and 120 sec of incubation (Fig. 2). When rectal tissues were incubated with NE at various concentrations, the results were qualitatively similar to that of 10\(^{-4}\) M NE experiment. In rectal tissue from a 7-day-old rat, 10\(^{-5}\) M NE produced a maximal increase (120%) at 10 sec (Fig. 2). When propranolol, a \(\beta\)-adrenergic blocking agent was added to the medium 10 min before incubation with NE,
10^{-4} \text{ M} \text{ NE}-induced elevation of cyclic AMP levels was completely inhibited by pretreatment with 5 \times 10^{-5} \text{ M} \text{ propranolol} in the rectum from adult rats (Table 1). 10^{-5} \text{ M} \text{ NE}-induced elevation of cyclic AMP levels was also inhibited by pretreatment with 5 \times 10^{-5} \text{ M} \text{ propranolol in the rectal tissues from 7-day-old rats.}

As shown in Table 2, basal levels of cyclic AMP and maximal increases of cyclic AMP content induced by NE were measured in the rectum at different stages of development. During the first 2 weeks after birth, when the high basal levels of cyclic AMP were observed, we found no significant increase in NE responses to cyclic AMP contents, and thereafter,
the basal cyclic AMP levels rapidly declined, while NE responses significantly increased to those seen in tissues from adult rats.

**TABLE 1. Effect of β-adrenergic receptor blockade on NE-induced elevation of cyclic AMP levels**

| Drugs                          | Cyclic AMP Levels (pmol/mg wet wt.) |
|-------------------------------|-------------------------------------|
| None                          | 0.68 ± 0.07 (6)                     |
| NE (10⁻⁴ M)                   | 1.39 ± 0.16 (5)*                    |
| Propranolol (5 × 10⁻⁵ M)      | 0.64 ± 0.10 (6)                     |
| Propranolol (5 × 10⁻⁵ M) plus NE (10⁻⁴ M) | 0.69 ± 0.04 (6) |

Each tissue from adult rats was incubated in Locke’s solution for 30 min and then propranolol was added to the medium 10 min before incubation with NE for 10 seconds. *, significantly different from control value, (p<0.01). Values are means ± S.E.M. Numbers in parentheses indicate the number of determinations.

**TABLE 2. Basal levels and NE-induced increase of cyclic AMP in the rectum from rats of different ages**

| Days after Birth | Basal Level of Cyclic AMP (pmole/mg wet wt.) | Cyclic AMP Level in the Presence of NE (pmole/mg wet wt.) | % of Basal Cyclic AMP Level |
|------------------|-----------------------------------------------|-----------------------------------------------------------|----------------------------|
| 3                | 1.19 ± 0.29 (10)                              | 1.40 ± 0.10 (10)                                          | 118                        |
| 7                | 1.30 ± 0.13 (7)                               | 1.60 ± 0.22 (7)                                          | 123                        |
| 14               | 1.23 ± 0.10 (10)                              | 1.48 ± 0.22 (10)                                          | 120                        |
| 28               | 0.95 ± 0.09 (9)                               | 1.31 ± 0.10 (9)**                                         | 138                        |
| 63               | 0.86 ± 0.09 (9)*                              | 1.41 ± 0.16 (9)**                                         | 164                        |

Each tissue from 3-, 7- and 14-day-old rats was incubated in Locke’s solution containing 10⁻³ M NE, and that from 28-day-old and adult rats in solution containing 10⁻¹ M NE, for 10 seconds after 30 min preincubation. Values are means ± S.E.M. Numbers in parentheses indicate the number of determinations. *, significantly different from the value in basal level of 3-day-old rats (p<0.05). **, significantly different from the values in basal levels of 28-day-old and adult rats, respectively (p< 0.01).

II. Immunofluorescence study in the rectum from adult rats.

Immunofluorescence histochemistry was also performed in order to demonstrate cellular localization of cyclic AMP. To establish the specificity of the procedure, we showed that the serum from unimmunized rabbits and phosphate buffered saline (PBS) failed to produce significant staining and that when the antiserum to cyclic AMP was incubated overnight at 4°C with 5 × 10⁻³ M cyclic AMP, the staining pattern was no longer present. Furthermore, when the antiserum to cyclic AMP was incubated with 5 × 10⁻³ M concentrations of ATP, ADP, AMP or cyclic GMP, there was no evident effect on the cell staining. In control rat rectum, cyclic AMP immunofluorescence was predominantly localized in the lamina propria and submucosal layer, while minimal staining was found in the brush border area. Cyclic AMP immunofluorescence was also found in smooth muscle, crypts area, large postganglionic neurons and the margin of the myenteric plexus, in particular, in the cytoplasm, nuclear membranes and nucleoli in postganglionic neurons (Fig. 3). In the
rectum from a rat 3 min after intravenous administration of NE (5 μg/kg), cyclic AMP immunofluorescence was moderately increased in the lamina propria, brush border area and submucosal layer and was slightly increased in smooth muscle. On the other hand, cyclic AMP immunofluorescence was not seen in goblet cells in the control tissues or in those from the NE treated rats.

**DISCUSSION**

We have demonstrated herein for the first time that the adrenergic cyclic AMP generating system is present in the rectal smooth muscle of postnatal developing rats. Changes in cyclic AMP levels, adenylate cyclase and phosphodiesterase activities have been demonstrated in various organs of developing rats, skeletal muscle (12, 13), heart (13), liver (14) and brain (15). In all these tissues, the basal cyclic AMP levels gradually increased from prenatal to early postnatal stage. In rat and rabbit brains (5-7), the responsiveness of cyclic AMP generating systems to β-agonist was nil or very low in these stages. Our observations in rat rectum also revealed that shortly after birth, the basal level of cyclic AMP was highest and gradually decreased with aging. The high basal levels of cyclic AMP in young rats are supported by the findings that a high activity of adenylate cyclase and a low activity of phosphodiesterase was evident during 1 to 2 weeks after birth, in brain (5, 15) and liver slices (14). Elevation of the responsiveness of cyclic AMP generating system to NE may be largely due to the lowering of basal level of cyclic AMP. Adrenergic function was found to be present before the first appearance of adrenergic innervation in smooth muscle of human foetal small intestine (16). During the early stage of postnatal development, adrenergic relaxing function increased with age up to 14–28 days in rat rectum as described previously (17). These findings indicate a positive correlation between the development of the responsiveness of the cyclic AMP generating system to NE and that of adrenoceptive
relaxation.

Histochemical studies on alimentary tracts revealed two patterns of adrenergic innervation in the myenteric ganglia and in the smooth muscle (18, 19). In the rat stomach, adrenergic innervation to the myenteric plexus was evident even at birth, however, innervation to the smooth muscle was only apparent 7 days after birth (20). Age-related elevation in NE-stimulated cyclic AMP accumulation paralleled the development in adrenergic innervation to the smooth muscle. Thus, we attempted an immunohistochemical study to determine whether β-adrenoceptors are present in smooth muscle or in the myenteric plexus. In general, the localization of cyclic AMP immunofluorescence in the rectal tissue was similar to that seen in the jejunum of the rat as reported by Ong et al. (3), and Wegmann and Antakli (4), though there was no evidence of this immunofluorescence in myenteric ganglia. Furthermore, in our histochemical data, cyclic AMP immunofluorescence was found both in the smooth muscle and in the myenteric ganglia. NE application enhanced cyclic AMP immunofluorescence in smooth muscle. Our results suggest that the majority of β-adrenoceptors associated with the cyclic AMP generating system are localized in the smooth muscle of rat rectum. Cyclic AMP may also be involved in the mediation of synaptic transmission in myenteric ganglia as well as in bovine superior cervical ganglia (21), but further experiments are needed to determine whether or not NE is related in this transmission.

Acknowledgements: We thank Prof. M. Ui of Hokkaido University for pertinent discussion and Mr. A. Sato, Research Laboratories of Yamasa Shoyu Co. Ltd., Choshi, for the generous gift of antiserum to cyclic AMP.

REFERENCES
1) Bueding, E., Butcher, R.W., Hawkins, J., Timms, A.R. and Sutherland, Jr, E.W.: Effect of epinephrine on cyclic 3',5'-phosphate and hexose phosphates in intestinal smooth muscle. Biochim. Biophys. Acta 115, 173–178 (1966)
2) Robison, G.A., Butcher, R.W. and Sutherland, E.W.: Cyclic AMP. A. Rev. Biochem. 37, 149–174 (1968)
3) Ong, S.H., Whitley, T.H., Stowe, N.W. and Steiner, A.L.: Immunohistochemical localization of 3',5'-cyclic AMP and 3',5'-cyclic GMP in rat liver, intestine, and testis. Proc. natn. Acad. Sci. U.S.A. 72, 2022–2026 (1975)
4) Wegmann, R. and Antakli T.W.: Immunofluorescent localization of cyclic AMP in human jejunum and rat liver and jejunum. Cell. Mol. Biol. 22, 27–36 (1977)
5) Perkins, J.P. and Moore, M.M.: Regulation of the adenosine cyclic 3',5'-monophosphate content of rat cerebral cortex: Ontogenetic development of the responsiveness to catecholamines and adenosine. Mol. Pharmacol. 9, 774–782 (1973)
6) Harden, T.K., Wolfe, B.B., Sporn, J.R., Perkins, J.P. and Molinoff, P.B.: Ontogeny of β-adrenergic receptors in rat cerebral cortex. Brain Res. 125, 99–108 (1977)
7) Schmidt, M.J. and Robison, G.A.: The effect of norepinephrine on cyclic AMP levels in discrete regions of the developing rabbit brain. Life Sci. 10, 459–464 (1971)
8) Bitensky, M.W., Russell, V. and Blanco, M.: Independent variation of glucagon and epinephrine responsive components of hepatic adenyl cyclase as a function of age, sex and steroid hormones. Endocrinology 86, 154–159 (1970)
9) Walton, G.M. and Garren, L.D.: An assay for adenosine 3',5'-cyclic monophosphate based on the association of the nucleotide with a partially purified binding protein. Biochemistry 9, 4223–4229 (1970)
10) Gilman, A.G.: A protein binding assay for adenosine 3',5'-cyclic monophosphate. Proc.
11) Steiner, A.L., Kipnis, D.M., Utiger, R. and Parker, C.: Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proc. natl. Acad. Sci. U.S.A. 64, 367–373 (1969)

12) Hommes, F.A. and Beere, A.: The development of adenyl cyclase in rat liver, kidney, brain and skeletal muscle. Biochim. Biophys. Acta 237, 296–300 (1971)

13) Novák, E., Drummond, G.I., Shala, J. and Hahn, P.: Developmental changes in cyclic AMP, protein kinase, phosphorylase kinase, and phosphorylase in liver, heart, and skeletal muscle of the rat. Arch. Biochem. Biophys. 150, 511–518 (1972)

14) Christoffersen, T., Morland, J., Osnes, J.B. and Øyet, I.: Development of cyclic AMP metabolism in rat liver. A correlative study of tissue levels of cyclic AMP, accumulation of cyclic AMP in slices, adenylate cyclase activity and cyclic nucleotide phosphodiesterase activity. Biochim. Biophys. Acta 313, 338–349 (1973)

15) Schmidt, M.J., Palmer, E.C., Dettbarn, W.D. and Robison, G.A.: Cyclic AMP and adenyl cyclase in the developing rat brain. Develop. Psychobiol. 3, 53–67 (1970)

16) Hart, S.L. and Mir, M.S.: Adrenoceptors in the human foetal small intestine. Brit. J. Pharmacol. 41, 567–569 (1971)

17) Taniyama, K., Yoshida, N. and Tanaka, C.: Adrenergic function and cyclic AMP levels in the postnatal developing rat stomach and rectum. Proceedings 18th Int. Cong. Neurovegetative Research, p. 244–246, Tokyo (1977)

18) Costa, M. and Gabella, G.: Adrenergic innervation of the alimentary canal. Z. Zellforsch. 122, 357–377 (1971)

19) Jacobowitz, D.: Histochemical studies of the autonomic innervation of the gut. J. Pharmacol. exp. Ther. 149, 358–364 (1965)

20) Yoshida, N., Taniyama, K. and Tanaka, C.: Adrenergic innervation and cyclic adenosine 3',5'-monophosphate levels in response to norepinephrine in stomach of postnatal rats. J. Pharmacol. exp. Ther. (in press)

21) Kebabian, J.W., Bloom, F.E., Steiner, A.L. and Greengard, P.: Neurotransmitters increase cyclic nucleotides in postganglionic neurons: Immunocytochemical demonstration. Science 190, 157–159 (1975)