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STUDIES OF SEPARATION AND CHARACTERIZATION OF ACETYLCHOLINE RECEPTOR LABELED WITH TRITIATED DIBENAMINE

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Abstract—In order to label specifically ACh receptors with tritiated dibenamine, suitable pharmacological conditions of application of the drugs were examined on the isolated smooth muscle strip of the dog small intestine. The exposure to 4×10^{-5} M dibenamine for 90 min reduced irreversibly the sensitivity of the strip to ACh to less than 14 per cent of the original level. The pretreatment of this strip with 10^{-6} M atropine sulfate protected it from the inhibitory effect of dibenamine to ACh and after atropine was washed out, the exposure to tritiated dibenamine reduced the sensitivity of the strip to ACh to less than 9 per cent of the original level. In this way the labeling procedure was established as shown in Fig. 1. The radioactivity in the test preparation was found to be 34·8 per cent higher than the control. The homogenate was fractionated by differential centrifugation. The higher radioactivity difference between the test and control preparations was obtained in P_5 fraction (1000 *g* sup. and 45,000 *g* sed.). In order to characterize ACh receptor substances P_5 fractions of the test and control were treated with trichloroacetic acid, organic solvents and enzymes. The radioactivity difference between the test and control was higher in the trichloroacetic acid or organic solvent insoluble fractions than in the soluble fractions. But radioactive components of high molecular weight were transferred to the substances of low molecular weight, which were proved not to be dibenamine or dibenzylaminoethanol, after the treatment with proteolytic enzymes, such as Pronase or trypsin, and yet found in a fraction of high molecular weight after the treatment with hyaluronidase. These facts suggest that ACh receptor substances in the intestinal smooth muscle might be of proteinous nature.

IT HAS been proposed by Furchgott[1] that dibenamine exerts its blocking action by reacting irreversibly with various free receptors of smooth muscles and that each agonist or antagonist is able to protect the corresponding receptor from the action of dibenamine. After dibenamine has been applied to the smooth muscle preparation with atropine which has protected the acetylcholine (ACh) receptor from the attack of dibenamine and they have been washed out, ACh receptors may be specifically labeled with tritiated dibenamine by incubating the preparation with it.[2] According to the above program we tried to label ACh receptor of the smooth muscle with tritiated dibenamine and to obtain the subcellular fraction containing a large amount of receptor substances. The separation of the labeled receptor was then tried by differential centrifugation, trichloroacetic acid precipitation and organic solvents extraction. In order to study chemical nature of the receptor some enzymes were applied and transfer of the radioactivity to the fraction of small molecular weight was tested.

METHODS AND MATERIALS

Pharmacological tests

Adult dogs weighing 8–12 kg were anesthetized with pentobarbital sodium and exsanguinated. The small intestine was quickly excised and cut longitudinally and mesenteric, vascular and adipose tissues were snipped off from the smooth muscle layer.

The experiments were carried out with these intestinal smooth muscle strips of about 5 × 50 mm. The muscle preparations were suspended in a 30-ml organ bath with Tyrode solution maintained at 28°, and responses of the muscle to drugs were recorded on a kymograph with an ink-writing isotonic lever. Agonists except 5-hydroxytryptamine (5-HTP) were cumulatively added to the bath. All the data were the average of at least 10 experiments.

The tissues treated with drugs as shown in Fig. 1 and tested pharmacologically were then cut into small pieces, blended for 10 min with 9 vol. of Tyrode solution under ice cooling in a Waring–Blendor and lyophylized.

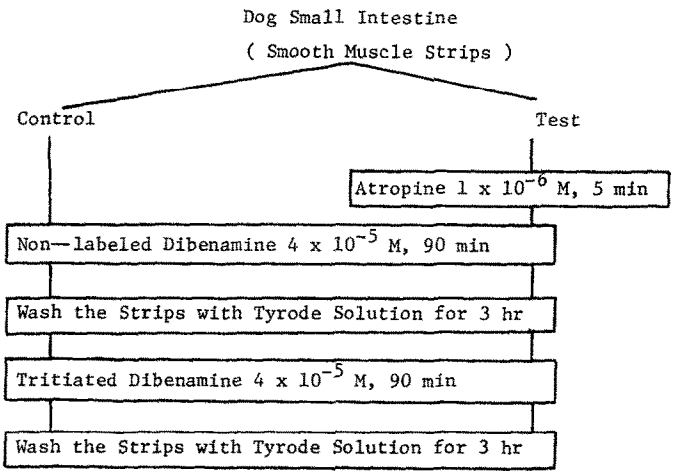

FIG. 1. Procedures of drug-treatment on the test and control preparations. Small pieces of the smooth muscle strips of the dog ileum were divided into the test and control groups, which were treated as follows: the test group was treated (i) with 10^{-6} M atropine and (ii) 5 min after with 4×10^{-5} M dibenamine for 90 min. It was then washed with Tyrode solution for 3 hr (iii). The preparation was then treated with ^{3}H-dibenamine 4×10^{-5} M for 90 min and washed with Tyrode solution for 3 hr. The control group was treated with dibenamine in the absence of atropine and quite similar to the test group thereafter. ACh receptor and the other receptors were equally blocked by this procedure with unlabeled dibenamine.

Tissue dispersion and fractionation

Initial dispersion. The smooth muscle tissue of the dog small intestine was cut into pieces of about 1 × 5 mm. The pieces were ground for 2 min in 9 vol. of 0·32 M sucrose solution, which was adjusted to pH 7·4 with 0·2 M Tris-(hydroxymethyl) aminomethane solution, in Waring–Blendor at middle speed, and homogenized in a glass tube with a Teflon pestle.

Centrifuging. The 10% (w/v) homogenate was centrifuged according to the procedure described in the previous paper[2] using Marusan Model 50V-1 and Hitachi Model 40p ultracentrifuges.

Determination of the radioactivity and total nitrogen. The radioactivity of a sample was measured by Packard Tri Carb liquid scintillation spectrometer as described previously[3] and total nitrogen by micro-Kjeldahl method.

Treatment with trichloroacetic acid, organic solvents and enzymes[4]

The highest radioactivity difference between the test and control was found in P_5 fraction ($P_2 + P_3$ fraction of Table 3). In order to study chemical natures of the receptor substances in P_5 fraction, it was fractionated in the manner as shown in Fig. 2.

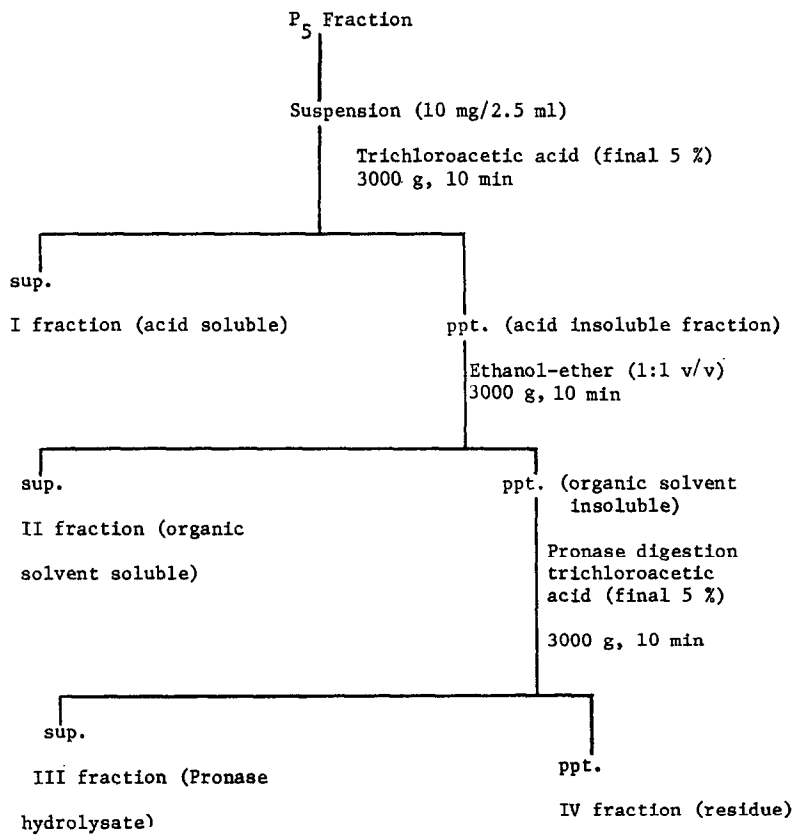

FIG. 2. Outline of fractionation procedure of P_5 fraction.

Treatment with trichloroacetic acid. P_5 fraction, about 10 mg (dry wt.), was placed in an ice-cold small glass homogenizer, and dispersed gently with a Teflon pestle with stainless steel stalk in 2·5 ml of distilled water and mixed with 2·5 ml of 10% trichloroacetic acid under ice cooling. After agitating for 30 min the suspension was centrifuged for 5 min at 3000 rpm. The sedimented pellet was washed twice with 5% trichloroacetic acid solution by repeated centrifugations.

Treatment with organic solvents. The trichloroacetic acid insoluble fraction was suspended in 5 ml of a mixture of (A) ethanol:ether (1:1, v/v) or (B) chloroform: methanol (2:1, v/v) as recommended by Folch *et al.*[5] These suspensions were mildly boiled for 5 min and agitated for 20 min at room temperature, and centrifuged for 5

min at 3000 rpm in (A), or filtered with a glass filter in (B). Precipitates were washed twice with each 5 ml of the same mixture.

Pronase digestion and separation of the digested components. Pronase digestion was performed on P_5 and the dried fraction insoluble to the organic solvents. The sample for digestion was suspended in 1 ml of 0·05 M Tris-acetate buffer solution of pH 7·2 and added to 1 ml of this medium containing 0·2 mg Pronase (Pronase P, a protease of Streptomyces griseus,[6] Kaken Co. Ltd. Tokyo, 45,000 P.U.K./g). The mixture was shaken for 6 hr in an incubator at 37° ± 1° after the addition of a drop of toluene, followed by the treatment with trichloroacetic acid as mentioned above. Thinlayer chromatography (TLC) was performed on the acid soluble materials after Pronase digestion. Trichloroacetic acid in the fraction was removed by ether. The plate was coated with Kiesel gel-G (Merck) in distilled water, and the solvent system used was a mixture of butanol-acetic acid-water, 3:1:1 by volume. After running the sample the plate was dried and stained by ninhydrin or Dragendorff reagent. Each spot scraped directly into the counting vials.[7] The radioactivity was determined in a Packard Tri Carb liquid scintillation spectrometer.

Trypsin and hyaluronidase digestions of P_5 fraction. The sample for trypsin digestion was suspended in 1 ml of medium containing 0·05 M Tris-acetate buffer (pH 8·0) and 0·1 mg trypsin (Difco, 1:250). The suspension (about 5 mg of P_5 fraction/ml) was shaken for 12 hr in the incubator at 37° ± 1° after the addition of a drop of toluene. The sample for hyaluronidase digestion was suspended in 1 ml of medium containing 0·1 M acetate buffer (pH 6·0) and 0·1 mg hyaluronidase[8] (Hyaluronoate glycanohydrolase, Tokyo-Kasei, 300 usp unit/mg) and incubated under the same condition as described in the trypsin digestion. After each enzyme digestion, the reaction mixture was passed through a 1 × 40 cm column of Sephadex G-75 (Pharmacia, Sweden) prepared as described by P. Andrews,[9] and was eluted with 0·05 M Tris HCl buffer (pH 7·2).

The tritiated dibenamine (60 C/M) was synthetized by Akao.[20]

RESULTS

Antagonistic effects of atropine and dibenamine to ACh on the dog small intestinal smooth muscle

The dose-response curve of ACh on the dog intestinal smooth muscle was located between concentrations of 10^{-5} and 10^{-8} M as given in Fig. 3 (curve 1). The sensitivity of these preparations to ACh was lower than that of guinea pig intestines, but sufficient for our purpose.

Dibenamine 4×10^{-5} M itself did not affect the muscle tone and the sensitivity of the strip to ACh was reduced to less than 14 per cent of the original by exposure to it for 90 min (curve 2) and did not return to a normal level within 10 hr. This irreversible inhibition of the response to ACh was not noticeable after 60 min incubation with 4×10^{-5} M dibenamine or after 90 min incubation with 2×10^{-5} M dibenamine.

Five min after the addition of 10^{-6} M atropine to the bath, 4×10^{-5} M dibenamine was applied for 90 min. Although the preparations so treated did not show considerable contractile response to ACh after washing with Tyrode solution for 2 hr, the sensitivity to ACh was restored completely to the original level after washing for 3 hr as given in Fig. 3 (curve 3). Lower concentrations of atropine could not protect sufficiently the preparation against the action of 4×10^{-5} M dibenamine.

In order to label the ACh receptors with tritiated dibenamine the smooth muscle strips, after the atropine and dibenamine added was eliminated, were treated with 4×10^{-5} M tritiated dibenamine for 90 min as described above and so the sensitivity of the strips to ACh was reduced to less than 9 per cent of the original after washing the strips with Tyrode solution for 3 hr as given in Fig. 3 (curve 4). The inhibitory effect of tritiated dibenamine was proved to continue for more than 10 hr.[2]

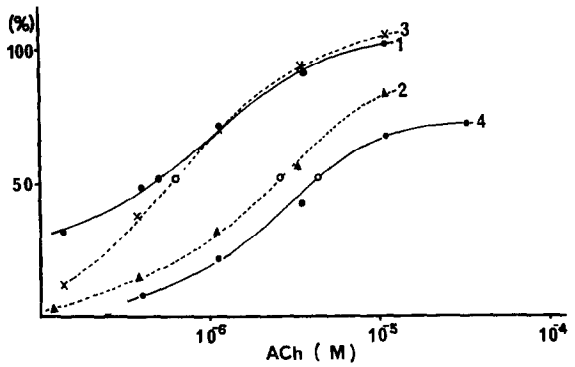

FIG. 3. Effects of atropine and dibenamine upon dose-response curves of ACh on Strips of the dog small intestine. Curve 1, ACh alone; curve 2, dibenamine 4×10^{-5} M; curve 3, atropine 1×10^{-6} M and dibenamine 4×10^{-5} M; curve 4, dibenamine 4×10^{-5} M after washing out atropine.

Influence of atropine on the responses to various stimulating drugs

The normal dose-response curves of the agonists were obtained on the isolated dog small intestines and it was recognized that the concentration range of histamine was situated between 1×10^{-4} M and 1×10^{-6} M, and that of 5-HTP between 1×10^{-6} M and 1×10^{-8} M. When the smooth muscle tissues had been treated with atropine and dibenamine as described above and washed with Tyrode solution for 3 hr, the sensitivities of the tissues to histamine and 5-hydroxytryptamine were not restored to the original level.

These findings suggest that there is no cross-protection of atropine between ACh and histamine or 5-HTP, and that the pretreatment of the smooth muscle tissues with atropine may protect only the receptor sites of ACh from the inhibitory effect of dibenamine.

The standard method for labeling the ACh receptor sites with tritiated dibenamine

From above results it is concluded that the sequences of the drug treatment on the test and control preparations as shown in Fig. 1 may be adequate for labeling of ACh receptors of the dog intestinal smooth muscle with tritiated dibenamine.

Comparison of radioactivity between the test and control homogenates

Aliquots of the homogenates of the test and control preparations were lyophylized, and the radioactivity of the test preparation was compared with that of the control one. The mean value of the radioactivity in the test preparation was 4600 dpm/0·1 mg nitrogen or 34 per cent higher than the control one as shown in Table 1.

TABLE 1. COMPARISON OF RADIOACTIVITY BETWEEN THE TEST AND CONTROL HOMOGENATES

Experiment	Test (T)*	Control (C)*
1	19,000	13,300
2	18,600	13,250
3	18,650	13,900
4	18,400	14,700
5	16,400	12,300
mean	18,210	13,500
S.E.	466	397

* radioactivity, dpm/0·1 mg nitrogen.

Subcellular distribution of the radioactivity in primary fractions

In order to identify the fraction having high radioactivity, the homogenate was fractionated by differential centrifugation and P_1 (1000 *g* sed.), P_6 (77,500 *g* sed.) and S (77,500 *g* sup.) fractions were preliminarily obtained. As given in Table 2, the radioactivity difference between the test and control in P_6 fraction was higher than that of P_1 or S fraction. P_6 fraction was then subfractionated in P_2 (10,000 *g* sed.), P_3 (45,000 *g* sed.) and P_4 (77,500 *g* sed.) as described previously,[2] and the distribution of radioactivity in each fraction was examined. The higher radioactivity difference between the test and control was obtained in P_2 and P_3 fractions, which were combined as P_5 fraction (Table 3). This suggests that P_5 fraction may contain a larger amount of ACh receptor substances than the others.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN P_1, P_6 AND S FRACTIONS OBTAINED FROM THE DOG INTESTINAL SMOOTH MUSCLE

Fraction	Test (T)*	Control (C)*	(T—C)*	(T—C)/C × 100
P_1	16,403 ± 704	13,979 ± 522	2423	17·4
P_6	23,767 ± 1,035	16,214 ± 919	7553	46·6
S	8152 ± 680	6847 ± 625	1305	19·1

* radioactivity, dpm/0·1 mg nitrogen.
Results are given as mean ± S.E. of four experiments.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN P_1, P_2, P_3 AND P_4 FRACTIONS OBTAINED FROM THE DOG INTESTINAL SMOOTH MUSCLE

Fraction	Test (T)*	Control (C)*	(T—C)*	(T—C)/C × 100
P_1	20,300 ± 1140	17,250 ± 832	3050	17·6
P_2	22,900 ± 1359	17,100 ± 723	5800	33·9
P_3	48,547 ± 2082	33,200 ± 1465	15,347	46·2
P_4	40,620 ± 1901	36,500 ± 1803	4120	11·3

*radioactivity, dpm/0·1 mg nitrogen.
Results are given as mean ± S.E. of six experiments.

Studies on chemical natures of ACh receptor

In order to study chemical natures of ACh receptor substances, P_5 fractions of the test and control were treated with trichloroacetic acid, organic solvents and enzymes.

Treatment with trichloroacetic acid. When P_5 fraction was treated with trichloroacetic acid, about 15 per cent of the total radioactivity was found in the trichloroacetic acid soluble fraction. However, the difference of this activity between the test and control was extremely small as shown in Table 4.

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN TRICHLOROACETIC ACID SOLUBLE AND INSOLUBLE FRACTIONS

Fraction	Test (T)*	Control (C)*	(T—C)*	(T—C)/total (T—C) × 100
Soluble	1697	1575	122	3·5
Insoluble	9179	5765	3414	96·5
Total	10,876	7340	3536	

* Radioactivity, dpm/mg P_5 fraction.
Results are given as the mean of three experiments.

Treatment with organic solvents. P_5 fraction was treated with a mixture of ethanol-ether (1:1) or chloroform-methanol (2:1). Table 5 shows the distribution of radioactivity in each fraction in the treatment with ethanol-ether mixture. The radioactivity of the fraction soluble in the mixture was about 10–15 per cent of the total one and the difference between the test and control was also comparatively small. In the treatment with chloroform-methanol mixture the result obtained was almost similar to that with ethanol-ether mixture.

TABLE 5. DISTRIBUTION OF RADIOACTIVITY IN ORGANIC SOLVENT SOLUBLE AND INSOLUBLE FRACTIONS

Fraction	Test (T)*	Control (C)*	(T—C)*	(T—C)/total (T—C) × 100
Soluble	960	798	162	5·5
Insoluble	6230	3450	2780	94·5
Total	7190	4248	2942	

* Radioactivity, dpm/mg P_5 fraction.
Results are given as mean of three experiments. Organic solvent was a mixture of ethanol-ether (1:1).

Distribution of radioactivity in the fractions obtained by the successive treatments with trichloroacetic acid, ethanol-ether mixture and Pronase. Ten mg of P_5 fraction was treated with trichloroacetic acid, ethanol-ether mixture and Pronase in the way described in Fig. 2, and four fractions (I, II, III and IV fractions) were obtained. The radioactivity was higher in the III fraction than the others, and the radioactivity difference between the test and control in the III fraction was also the highest among these fractions as given in Table 6. From these results it seems that the ACh receptor substances might be insoluble in trichloroacetic acid and the organic solvents and solubilized in trichloroacetic acid after Pronase digestion.

TABLE 6. DISTRIBUTION OF RADIOACTIVITY IN TRICHLOROACETIC ACID SOLUBLE, ORGANIC SOLVENT SOLUBLE, PRONASE DIGESTED AND THE RESIDUAL FRACTIONS

Fraction*	Test (T)	Control (C)	(T—C)	(T—C)/total (T—C) × 100
I	18,965	18,637	328	1·6
II	15,374	13,408	1974	9·7
III	45,527	29,052	16,475	82·6
IV	10,832	9428	1404	6·1
Total	90,698	70,525	20,181	

* I: trichloroacetic acid soluble fraction, II: organic solvent soluble fraction, organic solvent was a mixture of ethanol-ether (1:1). III: Pronase digested fraction, this was trichloroacetic acid soluble after Pronase treatment. IV: residual fraction.
Results are given as mean of four experiments.

Separation of the radioactive components in the III fraction with TLC. The III fraction was separated with TLC and five ninhydrin positive spots were obtained. Dibenamine and dibenzylaminoethanol which might be probably produced by the hydrolysis of bound-dibenamine were used as the authentic samples and the R_f's of these samples were compared with those of the components in the III fraction. The spots of the dibenamine and dibenzylaminoethanol appeared at higher R_f range than those of the components in the III fraction. The distribution of radioactivity in the seven fractions including dibenamine and dibenzylaminoethanol was qualitatively examined, and shown in Table 7. Low radioactivity in dibenamine or dibenzylaminoethanol fraction was recognized.

TABLE 7. DISTRIBUTION OF RADIOACTIVITY IN THE VARIOUS NINHYDRIN AND DRAGENDORFF POSITIVE SPOTS ON TLC OF THE III FRACTION OF TABLE 6

Fraction	R_f range (%)	Test (T) (dpm)	Control (C) (dpm)	T—C (dpm)
1	9·1–20·8	3630	2200	1430
2	20·8–30·8	3690	1535	2155
3	30·8–44·2	3570	1810	1760
4	44·2–54·2	3000	1600	1400
5	54·2–64·1	1160	782	378
Dib. (OH)	66·6–71·6	614	311	303
Dib.	83·3–87·5	600	295	305

Dib. (OH): Dibenzylaminoethanol, Dib.: Dibenamine.

Treatment of P_5 fraction with enzymes. It was shown by Sephadex-G 75 column chromatography that the radioactive components in P_5 fraction turned into substances of low molecular weight after the treatment with Pronase.[2] The radioactive peak was observed in a fraction of low molecular weight after the treatment with tryspin as well as Pronase, although the peak was found mainly in a fraction of higher molecular weight in the blind treatment without tryspin or Pronase. However, the radioactive peak was found mainly in a fraction of higher molecular weight after the treatment with hyaluronidase which has shown sufficiently enzymatic activity on the turbidimetric assay.

DISCUSSION

During the past decade the studies on drug receptors have been developed to their biochemical isolation and identification,[10–14] but these studies were scarce of biological evidence that the isolated material was the receptor itself. We decided the procedure to label specifically the ACh receptor with tritiated dibenamine, with the experiment examining the biological response of the isolated organ to the drugs. According to the procedure atropine exerts protective action against dibenamine on only ACh receptor of the isolated dog small intestinal tissue, and the response to ACh is restored to the original level after washing for 3 hr. However, the responses to histamine and 5-HTP were not restored to the original level by the same procedure after washing for 3 hr or even for 10 hr. The response to noradrenaline was not observed on this preparation of the dog.

From these results it was suggested that tritiated dibenamine might probably combine specifically with ACh receptor of the test preparation which had been treated as Fig. 1. When the radioactivities of the test and control preparations after the treatment of Fig. 1 were measured, considerable radioactivity was found in the test and also control preparations. However, radioactivity in the test preparation is 35–50 per cent higher than that in the control one. The difference seems to be derived from tritiated dibenamine combined with atropine-protected sites including ACh receptor.

Recently the results in our previous report[2] were criticized by Moran *et al.*,[15] but his opinion is not free from misunderstanding, for example, the difference of the radioactivity between the test and control homogenates was shown as 10–30 per cent in their paper in place of 34 per cent of our report.[2] After the publication numerous determinations of the radioactivity of the homogenates were done and the differences were found always between 35–50 per cent. In the study on the uptake of atropine by intestinal smooth muscle of the guinea pig Paton and Rang[16] found several binding sites of atropine, which are ACh receptor, binding site other than ACh receptor and nonspecific binding sites such as acceptors or silent receptors. Although the difference of radioactivity between the test and control in our experiments includes ACh receptor, it is not sure that the difference also contains the second atropine binding site of Paton, because atropine may not protect the binding of dibenamine to that site. The problem must be studied further.

In order to obtain the fraction containing a considerable amount of ACh receptors we tried to separate the fraction having the high radioactivity difference between the test and control by differential centrifugation as described previously.[2] As the higher radioactivity difference was obtained in P_5 fraction, it is very probable that P_5 fraction may contain a larger amount of ACh receptor substances than the others. On the other hand the radioactivity difference was very low in the soluble fraction of P_5 in the trichloroacetic acid or organic solvents. Moreover, when the components which were insoluble in trichloroacetic acid or organic solvents were treated with Pronase, a greater part of radioactive substances was solubilized in trichloroacetic acid solution. Furthermore it was found that the radioactive components in P_5 fraction were digestible with trypsin, but not with hyaluronidase.

From these results, it seems that the ACh receptor substances may not be lipids, polysaccharides and nucleic acids, and that protein may constitute an important portion of the ACh receptors of the intestinal smooth muscle.

Similar studies have been recently reported on labeling and characterizing the

α-adrenergic receptor of the rabbit aorta by Dikstein *et al.*[17] and Yong *et al.*[18] and that of the rat seminal vesicle by Lewis *et al.*[19] Interests of pharmacologists are seemed to center in adrenergic mechanisms and also in adrenergic receptors, but study of ACh receptor is easier, because the ileum which is the source of ACh receptor can be obtained in larger quantities than adrenergic organs such as aorta, seminal vesicle and vas deferens.

2-Dibenzylaminoethanol must be released when the dibenamine bound receptor is hydrolysed with protease. Trichloroacetic acid and ethanol-ether insoluble fraction of P_5 was hydrolysed with Pronase and the hydrolysate was treated with trichloroacetic acid. The acid soluble fraction (Fraction III of Fig. 2) was analysed by TLC. The difference of radioactivity was not located in the spot of dibenzylaminoethanol. Consequently the active site of the receptor was still bound with dibenamine in the fragment of small molecular weight. It could not be identified at this step.

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