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An Assay Method for Secretin in Crude Preparations containing Pancreozymin

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An assay method for secretin in crude preparations containing unknown amounts of pancreozymin has been developed, using rats.

For several hours after urethan injection there was a marked fall in the volume of pancreatic juice correlated with the decrease of protein output of rat pancreas stimulated with secretin-pancreozymin mixture, then both reached steady-state levels. In this condition, where the sensitivity to pancreozymin was reduced, the pancreatic response was linearly related to the logarithm of the dose of crude or partially purified secretin in the ranges of 0.05—0.4 and 0.5—4.0 Crick, Harper and Raper (CHR) units, respectively. Within this range, the activity of crude secretin could be determined by four- and three-point assay procedures with an error of 10 percent or less.

The validity of the assay values for the crude preparations using the three-point design was confirmed by the recovery of secretin activity as highly purified secretin (free from pancreozymin) from the crude materials.

Keywords——rat; secretin; pancreozymin; twin crossover assay; four-point assay; three-point assay

Several methods have been reported for the assay of secretin in various animals[2–5] under anesthesia, and at present the potency of secretin can be determined with an estimation error of 10—15 percent[6] for highly purified preparations.

None of the assay methods for secretin so far reported has been examined for applicability to crude secretin preparations containing pancreozymin, which has a marked hydrelatic action and enhances the response of animals to secretin.[7] However, in the early studies on secretin and also in some recent work, secretin preparations which probably contained pancreozymin have been used as reference standards. In addition, some discrepancies have been observed between the secretin values of various standards,[5,6,8] which might have been due to pancreozymin contamination. In order to clarify the relative potencies of the various standards and to determine the potencies of secretin preparations at various levels of purity, an assay method for secretin in the presence of pancreozymin is required.

In this communication, we describe a simple assay method for secretin in crude or partially purified preparations containing pancreozymin, using a three- or four-point assay procedure in rats.

Materials and Methods

Secretin——GIH standard secretin, batch No. 17491 (75 clinical units of secretin plus 1 mg of cysteine/ampule) was purchased from the Karolinska Institute, Stockholm, Sweden, and was used as a standard to

1) Location: *a) Koishikawa 4, Bunkyo-ku, Tokyo 112, Japan*; *b) Kawashima-cho, Hajima-gun, Gifu 483, Japan.*
2) A.C. Ivy, G. Kloster, G.E. Drewyer, and H.C. Leuth, *Amer. J. Physiol.*, **95**, 35 (1930).
3) V. Mutt and U. Soderberg, *Arkiv. Kemi*, **15**, 63 (1959).
4) J.W. Love, *Q. Jl. Exp. Physiol.*, **42**, 279 (1957).
5) N.G. Heatley, *J. Endocr.*, **42**, 535 (1968).
6) S. Tachibana, "Gastro-Entero-Pancreatic Endocrine System—A Cell-Biological Approach," Ed. by T. Fujita, Igaku Shoin Ltd., Tokyo, 1973, p. 174.
7) N.G. Heatley, *J. Endocr.*, **42**, 549 (1968).
8) G.F. Stening, M. Vagne, and M.I. Grossman, *Gastroenterology*, **55**, 687 (1968).

define the value of our own standard secretin, on the assumption that 1 clinical unit is equal to 4 Crick, Harper and Raper (CHR) units.[6]

Standard secretin (100 CHR units of secretin plus 5 mg of cysteine/ampule), batch No. 510603, prepared from a highly purified porcine secretin (5400 CHR units/mg) and free from pancreozymin,[6] was used as a standard throughout the present experiments.

Porcine duodenum extracts (0.3—3 CHR units/mg) and a partially purified secretin (100—200 CHR units/mg),[6] which are intermediate products in the commercial production of this hormone by Eisai Co., were used as test samples.

Both of the standard secretins were dissolved in sterile physiologic saline containing bovine serum albumin (1%) to give a concentration of 20 CHR units/ml, and the crude duodenum extracts and the partially purified secretin were dissolved in saline to give concentrations of 10 mg/ml and 0.5 mg/ml.

Pancreozymin——Partially purified pancreozymin (300 Ivy dog (ID) units/mg) free from secretin was prepared from porcine duodenum extract according to the method described by Tachibana.[6]

The Operation——Male Wister-Imamichi rats weighing 280—450 g were fasted for at least 18 hr before each experiment. After a single intraperitoneal injection of a 48% (w/v) solution of urethan (0.27 ml per 100 g body weight), they were operated on according to the method described by Love.[4] The pylorus was ligated to reduce the effect of gastric juice on the pancreatic secretin[6] in all of the animals.

Measurement of Pancreatic Responses to Secretin and Pancreozymin——The pancreatic response of rats to a single intravenous injection of secretin was measured in terms of the volume of pancreatic secretion using a graduated capillary glass tube (about 10 μl/cm) which was connected to the pancreatic duct by a polyethylene tube.[4,6] The pancreatic response to pancreozymin was measured in terms of the extra protein secreted into the pancreatic juice. Pancreatic juice drawn into the graduated capillary tube was diluted with 20 ml of distilled water after measurement of the volume, and the optical density at 280 nm was determined with a Hitachi 124 spectrophotometer.

Assay Procedure

Principle——For several hours after intraperitoneal injection of urethan, there is a marked fall in the amount of protein output in parallel with a reduction in the volume of pancreatic juice in a rat pancreas stimulated with pancreozymin-secretin mixture, then both reach steady-state levels. In the steady-state, the hydrelatic effect of pancreozymin is reduced, and so it should be possible to determine the potency of secretin in crude preparations by using low doses of test samples to minimize the interference by pancreozymin.

Four-point Assay——Operated rats were used for the assay of crude secretin 3.5—4 hr after urethan injection. The body temperature of rats was kept at 35—36°. In each assay, "high" and "low" (half of the high) doses of a test sample (TH, TL) and the reference standard (SH, SL) were given under a random schedule. The administration of secretin at doses below 0.5 CHR unit was carried out at intervals of 60 min, and doses above 0.5 CHR unit were given at intervals of 80 min. The pancreatic response was measured in μl for 25 or 35 min before and after the administration, and the mean pancreatic response was calculated as the volume "after" minus that "before."

The potency of the sample was calculated by comparison of the mean responses to the unknown sample and to a known reference standard, using an equation given in The Pharmacopoeia of Japan.[9]

Three-point Assay——A three-point assay method was used for the assay of large numbers of crude secretin preparations. Two different doses of the standard (SH 0.2; SL 0.1 CHR unit) and one dose of a test sample (at a dose level intermediate between SH and SL) were injected at intervals of 45 min. Three test samples were assayed at the same time in 3 rats, administering the doses in a different order for each animal as follows; Rat I: SH, T_1, SL, T_2, SH, T_3, SL, Rat II: SH, T_3, SL, T_1, SH, T_2, SL, and Rat III: SH, T_2, SL, T_3, SH, T_1, SL.

The pancreatic response was measured in μl for 30 min after administration (R) of each dose, and the basal pancreatic secretions were also determined for 15 min before administration (B_b) and during 30 to 45 min after administration (B_a). The mean pancreatic response was calculated as $R-(B_b+B_a)$. The potency of the test samples was calculated using the reported equation for the three-point assay of agonists.[10]

Results

Stimulating Effect of Pancreozymin on the Pancreatic Response to Secretin

It is well known that pancreozymin has a marked hydrelatic action and increases the secretion of pancreatic juice induced by secretin.[7] In an attempt to quantify the effect of this hormone, the pancreatic responses to secretin in the presence and absence of pancreozymin were measured using rats anesthetized 60 min before the determination.

9) The Pharmacopoeia of Japan, 8th Edition, D-230 (1971).
10) "Pharmacological Experiments on Isolated Preparations," Ed. by W.L.M. Perry, E. and S. Livingstone Ltd. Edinburgh and London, 1968, p. 14.

Repeated administration of a highly purified secretin (1 CHR unit) caused no appreciable change of the pancreatic response, as shown in Fig. 1a; a steady secretion of pancreatic juice and a steady protein output were obtained.

On the other hand, the pancreatic response to highly purified secretin (1 CHR unit) supplemented with pancreozymin (1 or 0.125 ID unit) or to crude secretin preparations (about 1 CHR unit of secretin containing an unknown amount of pancreozymin) was different from that to the purified secretin, as shown in Fig. 1b and 1c. An extremely large volume of pancreatic juice was secreted initially on administration of secretin containing pancreozymin, and a gradual fall in the volume was observed at each subsequent administration, correlated to the decrease of protein output.

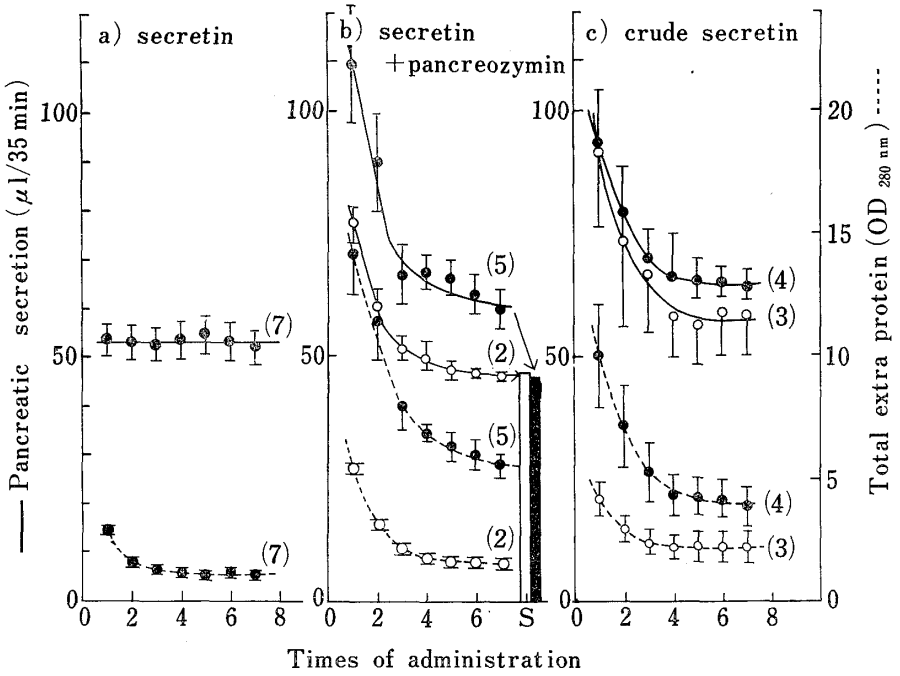

Fig. 1. Effect of Pancreozymin on the Pancreatic Response to Secretin in the Rat after Urethan Injection

Soon after the operation (within 1 hr after the pre-operative urethan anesthesia), various secretin preparations equivalent to about 1 CHR unit were administered repeatedly at intervals of 35 min through the cannula at the femoral vein, and the volume of pancreatic juice and the amount of protein output during each interval was determined as described in the text.

a) a highly purified secretin (1 CHR unit).

b) a highly purified secretin (1 CHR unit) plus pancreozymin
(●: 1ID unit, ○: 0.125 ID unit).
The open and closed bars indicate the mean pancreatic responses of each group to 1 CHR unit of standard secretin.

c) crude secretin preparations containing pancreozymin,
●: Batch No. 062, ○: Batch No. 902.

Numbers of rats are shown in parenthesis, and the results are given as means±S.D.

A steady-state secretion of pancreatic juice was obtained after 4-6 administrations, *i.e.*, at 3.5—4 hr after urethan injection. At this time the pancreatic response to secretin (1 CHR unit) supplemented with 0.125 ID units of pancreozymin was essentially the same as that to 1 CHR unit of secretin, as shown in Fig. 1b by an open bar; however, the administration of 1 ID unit of pancreozymin added to secretin still produced a response, and marked increase of pancreatic secretion was observed, as shown in Fig. 1b by a closed bar and closed circles.

It was concluded that the stimulating effect of pancreozymin on secretin activity is due to the extra output of protein and bicarbonate into the pancreatic juice, inducing extra water output due to their osmotic effect, especially in the early period after urethan injection. How-

ever, the presence of a small amount of pancreozymin in secretin had no significant effect on the secretin activity in rats under steady-state conditions more than 3.5 hr after urethan injection.

Relation between Pancreatic Response and the Logarithm of Secretin Dose in Rats 3.5 hr after Urethan Injection

No further fall in the volume of pancreatic juice was observed in the rat 3.5 hr or more after urethan injection upon repeated administration of a secretin-pancreozymin (0.2 CHR unit-0.025 ID unit) mixture or crude secretin preparations (0.1—0.4 CHR unit). Therefore, using rats in this state, the mean pancreatic responses to 4 different doses of secretin ranging from 0.05 to 0.4 CHR unit or from 0.5 to 4.0 CHR units were determined, using various secretin preparations (Fig. 2). One group of 3—4 rats was used in each assay and doses were given in random order at intervals of 60 or 80 min.

The rats were pretreated 3 times with 1 CHR unit of a purified secretin before the assay to obtain a steady basal secretion of pancreatic juice. The mean response to the administration of secretin was measured as "the total secretion during 25 or 35 min after the dose" minus "the basal secretion for 25 or 35 min before the dose".

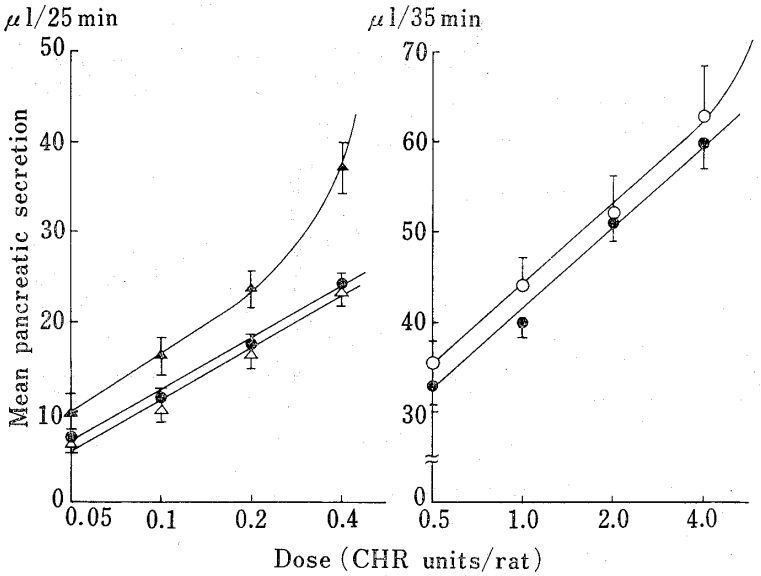

Fig. 2. Relationship between the Logarithm of the Dose of Various Secretin Preparations and the Flow of Pancreatic Juice in Rats 3.5 hr after Urethan Injection

Various secretin preparations with potencies covering the ranges of 0.05—0.4 and 0.5—4.0 CHR units (chosen on the basis of preliminary assay) were injected in random order at intervals of 60 or 80 min, and the mean pancreatic secretion in the 25 or 35 min after each injection was determined as described in the text.
●: a highly purified secretin (5400 CHR units/mg).
○: a partially purified secretin (180 CHR units/mg).
▲: an isopropanol extract from crude secretin (3 CHR units/mg), Batch No. 062.
△: a crude secretin (0.5 CHR unit/mg), Batch No. 902.
One group of 3—4 rats was used in each assay, and the results are given as means±S.D.

The pancreatic response was linearly related to the logarithm of the dose of highly purified secretin in the ranges of 0.05 to 0.4 and 0.5 to 4.0 CHR units, as shown in Fig. 2a and 2b. Similar responses were also observed with both partially purified secretin (0.5 to 4.0 CHR units) and crude secretins (0.05 to 0.4 CHR unit). However, administration of doses above these ranges caused unexpectedly large increases in pancreatic secretion, probably due to the contaminating pancreozymin. These results indicate that the assay of secretin in crude and partially purified

preparations using rats in the steady state is possible in the low dose range, where contaminating pancreozymin has no appreciable effect.

Reproducibility of the Four-point Assay of Secretin

It was reported by Tachibana[6] that it was difficult to determine the potency of secretin accurately by a four-point assay in rats, even if 10 or more rats were used, whereas using a twin crossover design, it could be determined with a estimation error of 10—15 percent in 2—4 rats.

We therefore reinvestigated the confidence limits and accuracy of secretin assay using the four-point design in comparison with the twin crossover design. The same solution of highly purified secretin was used as both the standard and test sample. 1 CHR unit was employed as the high dose of the standard and test sample, and the low dose was half of the high dose.

The four-point assay using 2—3 rats indicated the potency of the test sample to be 99, 100 or 104% of the standard, very close to the value of 95, 100 or 105% obtained by the twin crossover design in 2—3 rats, as shown in Table I. The errors of both assay methods appeared to be very similar at about ±5%. The confidence limits in the four-point assay were also similar to those in the twin crossover assay, as shown in Table I (L value).

Table I. Reproducibility of the Assay Values of Secretin obtained by the Twin Crossover and Four-point Methods in Rats

Experiment No.	No. of rats	Twin crossover design			Four-point design		
		Ratio of T/S (%)	95% confidence limits	L value	Ratio of T/S (%)	95% confidence limits	L value
1	3	95	82—110	0.125	99	85—116	0.134
2	2	100	90—120	0.115	100	90—110	0.079
3	3	105	98—110	0.061	104	89—112	0.135

Standard secretin dissolved in saline containing bovine serum albumin (20 CHR units/ml) was used as both standard and test samples, and the potency of the test sample was determined three times by the twin crossover and four-point methods.
SH=TH: 1.0 CHR unit and SL=TL: 0.5 CHR unit.

Assay of Crude Secretin by Four- and Three-point Assays

The contents of secretin in the crude preparations were determined by four- and three-point design methods using 3 rats in each assay. The rats were pretreated with 1 CHR unit of a purified secretin as described above, prior to the assay.

In the four-point assay, two different doses of the test sample corresponding as closely as possible to the doses of the standard (SH, 0.2; SL, 0.1 CHR unit) were injected in random order at intervals of 60 min.

In the three-point assay, the test sample (at a dose level intermediate between SH and SL) was administered between the injections of SH and SL. There was an interval of 45 min between successive injections.

The activity of secretin in nine crude preparations was determined twice by different operators to investigate the estimation errors in the four- and three-point assays. The results are shown in Table II. A similar value of secretin activity was obtained for each preparation by both operators in both methods. The estimation error in the four-point design appeared to be about 10 per cent or less, although the "L" value (95% confidence limit) was slightly larger than that for the assays of a purified secretin, as shown in Table I.

The potency of secretin determined by both operators using the three-point assay was well correlated with that obtained by the four-point assay (correlation coefficient, 0.976). Thus, the three-point assay is satisfactory for the determination of secretin in crude preparations.

Table II. Assay of Crude Secretin Preparations by the Four- and Three-point Methods

Sample No.	Four-point design				Three-point design			
	[I] operator A (units/mg)	[II] operator B (units/mg)	[III] average (units/mg)	Difference from the mean (%)	[I'] operator A (units/mg)	[II'] operator B (units/mg)	[III'] average (units/mg)	Difference from the mean (%)
1	0.54 (0.26) *	0.55 (0.24) *	0.55	1.8	—	—	—	—
2	1.39 (0.28)	1.27 (0.11)	1.33	4.5	—	—	—	—
3	1.36 (0.30)	1.30 (0.13)	1.33	2.3	—	—	—	—
4	0.90 (0.12)	0.94 (0.39)	0.92	2.2	1.05	1.00	1.03	1.9
5	0.62 (0.27)	0.59 (0.27)	0.61	1.6	0.58	0.60	0.59	1.7
6	1.16 (0.28)	1.35 (0.31)	1.26	7.1	1.46	—	—	—
7	0.51 (0.32)	0.62 (0.13)	0.57	8.8	0.66	—	0.66	—
8	0.72 (0.26)	0.73 (0.37)	0.73	1.4	0.74	0.64	0.69	7.2
9	0.78 (0.17)	0.90 (0.19)	0.84	7.1	0.90	0.80	0.85	5.9
10	1.00 (0.22)	—	1.00	—	1.02	—	1.02	—
11	—	—	—	—	0.76	0.90	0.83	8.4
12	—	—	—	—	0.76	0.73	0.75	1.3
13	—	—	—	—	0.79	0.79	0.79	0
Mean ± S.D.				4.1 ± 2.7				3.8 ± 3.07

SH=0.2 and SL=0.1 CHR unit.
Each assay was performed in three rats. The correlation coefficient r between [I] and [II] was 0.9586 ($p<0.01$); that between [I'] to [II'] was 0.8323 ($p<0.05$); and that between [III] to [III'] was 0.9763 ($p<0.01$).
* 95% confidence limits (L values) are shown in parentheses.

Validity of the Estimated Potency of Secretin in Crude Preparations

The three-point assay of secretin is convenient for estimating the potencies of large numbers of crude preparations, because it is possible to determine the potency of three samples a day using 3 rats, while only one sample can be tested in a day using the four-point method.

The validity of the values obtained by the three-point assay for crude secretin preparations was confirmed by a recovery experiment; a highly purified product free from pancreozymin was produced from the crude preparation by five steps of purification. The recovery of secretin in the purified final product thus obtained corresponded well to the potency of the starting material determined by this method, as shown in Table III. Allowing for a loss of secretin in each purification step of 10%, a recovery of 50% is reasonable.

Table III. Recovery of Purified Secretin from the Crude Preparations by a Five-step Procedure

	Starting material			Final product			Percent recovery of activity (%)
	Total weight (kg)	Specific activity (CHR units/mg)	Total activity (CHR units)	Total weight g	Specific activity (CHR units/mg)	Total activity (CHR units)	
Batch 1	13.5	0.4	5.4×10^6	2.0	1500	3.0×10^6	55
Batch 2	15.0	0.8	1.2×10^7	3.3	1800	5.9×10^6	49
Batch 3	16.0	0.6	9.6×10^6	2.9	1600	4.6×10^6	48

The potencies of the crude secretin preparations were determined by the 3-point procedure in the range of 0.1—0.2 CHR unit, and the potentcies of the purified products were determined by a twin crossover procedure in the range of 2—4 CHR units using 3 rats.

Discussion

It has been reported that various problems arise in the rat method for the assay of secretin, *e.g.* deviation from linearity in the dose-response relation and differences in sensitivity to secretin between animals.[5] However, similar problems have also been observed in the cat[4] and dog,[11] and they may simply be due to pancreozymine contamination of the secretin tested, as mentioned in the introduction.

We have shown here that the stimulating effect of pancreozymin on secretin activity is too large to permit accurate determination of the potency of secretin in the presence of an unknown amount of pancreozymin in the rat. However, the sensitivity to pancreozymin gradually falls with time, and beyond 3.5—4 hr after urethan injection, the pancreatic response to 1 CHR unit of secretin was not significantly affected by 0.125 ID unit of pancreozymin, as shown in Fig. 1, suggesting that assay of secretin in crude preparations containing a limited amount of pancreozymin would be possible.

In fact, we found that the pancreatic dose-response relation with crude or partially purified secretin preparations containing unknown amounts of pancreozymin closely paralleled that with highly purified secretin within a limited range of doses in rats in the steady state, although considerable deviation from linearity was observed, due to a significant increase of the pancreatic secretion, at higher dose levels. Within this range the activity of crude secretin could be determined with good accuracy and reproducibility by both four-point and three-point assay procedures.

The validity of the assay values for crude preparations was supported by the results of a recovery experiment in which highly purified secretin was isolated from the crude starting material; the recovery of secretin was consistent with the secretin content determined in the crude preparation by the three- and four-point assays.

For the assay of secretin in terms of the volume of pancreatic secretion, it is important to use rats that are as heavy as possible. The mean pancreatic response to 0.2 CHR unit of secretin apparently increased with increase of the body weight, while the ratio of the basal secretion to the total secretion decreased, as shown in Fig. 3. Therefore, we used rats weighing more than 330 g for the assay of crude secretins.

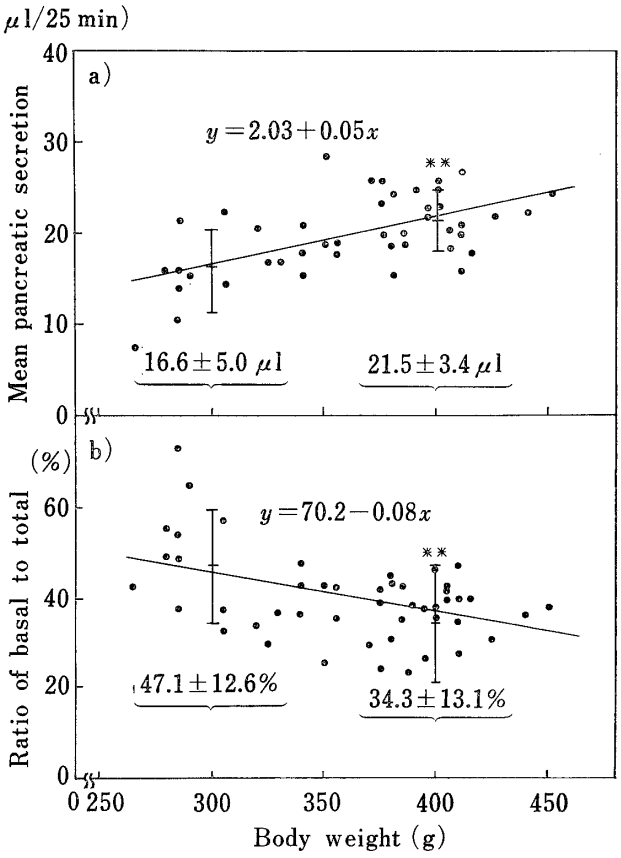

Fig. 3. Effect of the Body Weight of Rats on the Mean Pancreatic Response to 0.2 CHR Unit of Secretin

The mean pancreatic response (the total minus basal secretin of pancreatic juice, μl) to 0.2 CHR unit of standard secretin, chosen at random from secretin assay data in rats at our laboratory is shown. Significant differences (t test) were observed in the mean pancreatic secretion and also in the ratio of basal to total secretion between rats weighing 280—330 g and those weighing more than 330 g ($p<0.01$).

11) A.C. Ivy and H.M. Janacek, *Acta Physiol. Scand.*, **45**, 220 (1959).

Thus, the three-point assay of crude secretin in the rat is a simple and convenient method suitable for estimating large numbers of samples. The four-point assay is suitable for the accurate determination of secretin activity in partially or highly purified preparations, and can replace the twin crossover assay.

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