by the signal at δ 14.34 in the NMR spectrum, which disappeared on adding heavy water.[8]

From the evidences described above, I should be the red form of 1,5-di(*p*-sulfamoylphenyl)-3-phenylformazan, shown in Chart 1.

Experimental[9]

Isolation of I——To a solution of 1.7 g of sulfanilamide dissolved in a mixture of 5 ml of concentrated HCl and 40 ml of H_2O was added 1 g of $NaNO_2$ freshly dissolved in 5 ml of H_2O with stirring under ice-water cooling at 5°. To the resulting diazotized sulfanilamide solution were added successively 3.0 g of benzaldehyde freshly distilled and 6.0 g of $Na_2S_2O_4$ with shaking. To the mixture was added 50 ml of 3N NaOH and warmed at 60° for 20 min. After cooling, the reaction mixture was neutralized with dilute HCl, and separated precipitates were collected. The product was washed with H_2O, air-dried, and extracted with acetone.

The above procedure was repeated ten times, and the combined extract was dried over Na_2SO_4, concentrated *in vacuo*, poured onto a column packed with about 200 g of neutralized Al_2O_3,[10] and eluted with acetone to afford three fractions. The second and main fraction left I when its elutae was concentrated.

Dark red needles, mp 236.5—237.5° (from acetone). Yield 116 mg. *Anal.* Calcd. for $C_{19}H_{18}O_4N_6S_2$: C, 49.77; H, 3.96; N, 18.33. Found: C, 49.71; H, 3.97; N, 18.14. UV $\lambda_{max}^{0.2N\ NaOH}$ nm(log ε): 525 (4.77).

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9) UV spectrum was measured by a Shimadzu Double-40 Multiconvertible spectrophotometer in a cell of 10 mm optical length, IR spectrum by a Nihonbunko 701G infrared spectrophotometer in KBr tablet, NMR spectrum by a Nihondenshi PS-100 NMR spectrometer at 100 MHz with tetramethylsilane as an internal standard. The melting point is uncorrected.

10) Commercial activated alumina (Merck, 100 mesh) was dispersed in H_2O, neutralized with 10% HCl and filtered. After washing with H_2O, it was air-dried and activated at 120° for 10 hr.

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Studies on the Passage of α-Chymotrypsin across the Intestine. III.[1] Quantitation of α-Chymotrypsin in the Mesenteric Perfusate by Single Radial Immunodiffusion

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In our preceding experiment,[1] an immunoreactive substance against the anti-chymotrypsin serum was found in the mesenteric perfusate of rats after administrating α-chymotrypsin (CT) in the intestinal lumen. Although this fact suggests the transmittance of CT into the circulatory system through the intestinal wall, it would be prefered to determine

1) Part II: C. Moriwaki, K. Yamaguchi, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **22**, 1029 (1974).

2) Location: *Funakawara-cho, Ichigaya, Shinjuku-ku, Tokyo.*

the amount of the substance transmitted by various methods in order to confirm its absorption. Recently the single radial immunodiffusion technique was developed by Mancini, *et al.* and Fahey and McKelvey[3] as a relatively simple quantitative determination method for serum proteins. In this experiment the recovery amount of CT in the mesenteric perfusate after the intestinal administration of CT was determined by using both the single radial immunodiffusion technique and the esterolytic activity upon L-tyrosine ethyl ester (TEE).

Material and Method

CT and the Anti-CT Rabbit Serum——A purified CT and the rabbit antiserum against CT which were described in the preceding paper[1] were used in this experiment. A single precipitin band which hydrolyzed N-acetyl-DL-phenylalanine β-naphtyl ester (APNE) was observed in the immunoelectrophoresis of the CT and the anti-CT serum.

Determination of Esterolytic Activity——Esterolysis of TEE by CT was measured in accordance with the method of Takenaka and Schwert.[4] To 3 ml of 0.002M TEE in 0.05M Tris buffer (pH 7.0), 0.2 ml of a sample was added and the increase of the optical extinction at 234 nm was determined by Hitachi UV spectrophotometer UV-VIS type 139.

Single Radial Immunodiffusion——An antibody containing agar diffusion plate was prepared according to the method of Mancini, *et al.*[3a] The antiserum was diluted with 0.05M Veronal buffer, pH 8.6, heated to about 55°, and then mixed with an equal volume of 3% molten agar (60°) in the same buffer. Sodium azide was then added to this mixture as a preservative. The antibody-agar mixture was poured into the mold and 1.5 mm thick diffusion plates were prepared. Various concentrations of CT and the samples (concentrated mesenteric perfusates) in a volume of 7 μl were put separately into the wells of 2 mm diameter with a microsyringe (Jintan Termo Co., Osaka). The plates were kept in a humid chamber in the horizontal position at 4°. The diameters of the precipitin rings were measured with a vernier scale after 6 days of immunodiffusion.

Determination of Esterolytic Activity of CT-Anti-CT Complex——The immunodiffusion plates were washed with 0.15M NaCl and then with distilled water to eliminate the soluble proteins from the plates. They were then dried under moist filter paper. The dried plates were soaked for 60 min at 25° in the solution of APNE and fast blue RR of Uriel.[5a] After washing with 2% acetic acid, a precipitin ring possessing esterolytic activity was stained red-violet.

Mesenteric Perfusion——The perfusion of the mesenteric blood vascular system was executed with Krebs–Ringer solution as described previously.[1] Following a preliminary perfusion for 30 min, each rat was given 15 mg of CT in 0.3 ml of physiological saline in a ligated segment of the jejunum. An aliquot of the perfusate, which was collected from the mesenteric vein in 3 fractions every 30 min for 90 min, served for the assay of the esterolytic activity on TEE. A mixture of the rest of the perfusate and dry Sephadex G-50 (160 mg/ml) was put into a syringe with Millipore filter, and a concentrated perfusate was effluxed through the filter. The volume was adjusted to 1/5 of its initial one and it was submitted to a single radial immunodiffusion.

Result and Discussion

When various concentrations of CT solution (10—100 μg/ml) were applied to a plate containing the antiserum against CT, the precipition rings formed and expanded with time. The expansion of rings stopped after 6 days, and there was a linear relationship between the applied CT concentrations and the areas of the final rings. Furthermore, this CT-anti-CT complex maintained APNE splitting activity, and the measurement of the ring area was facilitated by the clarity of the red-violet ring rim. The size of ring did not change after the staining. Fig. 1 is the calibration curve for determination of CT concentration, which was obtained on the 50 times diluted antiserum agar plate after one week diffusion. This method enabled us not only to identify CT by its immunological specificity and by its enzymic

3) *a*) G. Mancini, A.O. Carbonara, and J.F. Heremans, *Immunochemistry*, **2**, 235 (1965); *b*) J.L. Fahey and E.M. McKelvey, *J. Immunol.*, **94**, 84 (1965).
4) G.W. Schwert and Y. Takenaka, "Methods of Enzymatic Analysis," H–U Bergmeyer de. Academic Press, New York, 1963, p. 802.
5) *a*) J. Uriel, *Ann. N. Y. Acad. Sci.*, **103**, 956 (1963); *b*) J. Uriel, "Antibodies to Biologically Active Molecules," Pergamon Press, Oxford and New York, 1966, p. 181.

activity, but also to do quantitative analysis of CT of 10 μg/ml upward by the direct measurement of the coloured area.

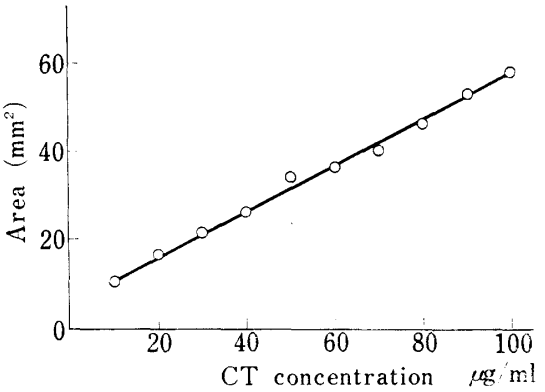

Fig. 1. Calibration Curve for Determination of CT Concentration by Single Radial Immunodiffusion

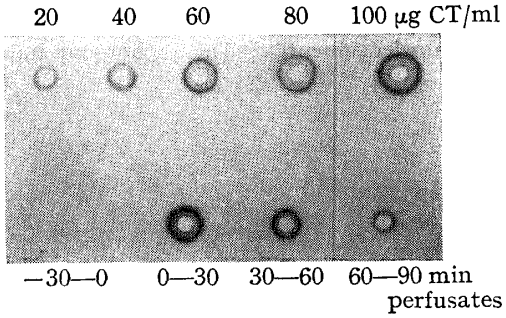

Fig. 2. Precipitin Rings formed by the Mesenteric Perfusates on an Immunodiffusion Plate

After treatment in APNE medium

Employing this method, the amount of CT transmitted across the intestinal wall into the mesenteric perfusate was estimated quantitatively. Though the perfusate collected before CT administration in the intestine did not give any immunochemical reaction even being concentrated, the perfusates which flowed out during 0 to 30, and 30 to 60 min after CT administration formed large precipitin rings, but a small ring was obtained with that during 60 to 90 min. Immersing these plates in the APNE medium, all rings changed their colour to red-violet by splitting the substrate (Fig. 2). These results indicate that the immunochemically and enzymatically active CT was transferred into the mesenteric vein across the intestinal wall. The amount of the CT in each perfusate was estimated from the calibration curve and shown in Table I. The mean recoveries of the enzyme in the first, second, and third 30 min perfusates from 10 rats were 0.31±0.05, 0.22±0.03, and 0.16±0.04%, respectively, and the total recovery in 90 min perfusate was about 0.7%.

This result was confirmed by the determination of TEE-esterolytic activity of the perfusate witout concentration process (Table I). The mean recoveries of the activity in the first, second, and third 30 min perfusates were 0.28±0.06, 0.18±0.05, and 0.12±0.04%, respectively, and the total recovery in 90 min was about 0.6%. Slightly higher recovery percentages were obtained in the immunodiffusion method than in the determination of esterolytic activity, but the recovery patterns of CT obtained in both methods were similar. The esterolytic activity upon N-acetyl-L-tyrosine ethyl ester in the mesenteric perfusate was also determined in our previous investigation.[1] The present data agreed well with the previous one in which the recovery in 90 min was 0.51%.

Table I. Immunoreactive Substance Possessing APNE Hydrolyzing Potency and TEE-esterolyticActivity in the Mesenteric Perfusate

	Amount in 30 min perfusate (μg CT equivalent)				
	—30—0	0—30	30—60	60—90	Total
Immunoreactivity	0	47.3± 7.9	34.1±5.7	24.3±6.1	105.7±15.4
TEE-esterolytic activity	0	38.0±10.0	24.6±7.4	18.1±6.1	80.7±21.5

Thus CT amount in the mesenteric perfusate was determined by three different methods including the tracer experiment with ^{131}I-CT,[1] and all experiments gave almost identical

results. The authors also reported the passage of CT through the sacs of everted rat intestine *in vitro* and the appearance of ^{131}I-bound protein in the systemic blood serum after the intestinal administration of ^{131}I-CT.[6] All of these experimental results are suggestive of the passage of CT from the intestine into the vascular system.

Acknowledgement The authors are indebted to Miss T. Ono for her assistance.

6) H. Moriya, C. Moriwaki, S. Akimoto, K. Yamaguchi, and M. Iwadare, *Chem. Pharm. Bull.* (Tokyo), **15**, 1662 (1967).