1,3-Dimethylpyrimido[4,5-*b*]quinoline-2,4(1*H*,3*H*)diones (1,3-Dimethyl-5-deazaalloxazines) (IIa—d)——A mixture of the adduct (I) (0.02 mol) and DAD (0.06 mol) in sulfolane (5 ml) was heated at 180° for 30 min. After cooling, the crystals which separated were collected by filtration and recrystallized from acetic acid to give pale yellow prisms (Table II).

5-(*p*-Tolyl)-1,3,7,9-tetramethylpyrido[2,3-*d*: 6,5-*d'*]dipyrimidine-2,4,6,8(1*H*,3*H*,7*H*,9*H*)tetrone) (III)——Compound Ib (0.4 g, 0.01 mol) was heated in sulfolane (4 ml) at 200° for 2 hr. The reaction mixture was diluted with water and allowed to stand overnight. The resulting crystals were collected by filtration and dried. Recrystallization from ethanol gave colorless plates (0.25 g, 63.6%), mp 335°, MS *m/e*: 393 (M^+). *Anal.* Calcd. for $C_{20}H_{19}N_5O_4$: C, 61.06; H, 4.87; N, 17.80. Found: C, 60.88; H, 4.71; N, 17.65.

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Guanylate Cyclase in Carrageenin Granuloma Tissue of Rat[1)]

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Guanylate cyclase activity was examined in inflammatory granuloma tissue induced by subcutaneous injection of carrageenin solution in the rat. Enzymological properties of guanylate cyclase in this tissue, such as pH optimum, metal ion requirement, *etc.*, were examined, with special reference to the intracellular distribution of the enzyme. Differences were found between guanylate cyclase in the soluble fraction and that in the particulate fraction in the response to Mn^{2+} and Triton X-100 in this tissue.

Keywords——guanylate cyclase; carrageenin granuloma; inflammation; Triton X-100; Mn^{2+}; subcellular fractionation

It has been reported that guanosine 3',5'-monophosphate (cyclic GMP) is involved in inflammatory processes, such as the release of lysosomal enzymes,[3)] chemotaxis,[4)] *etc.* Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) is responsible for the production of cyclic GMP, and the presence of two types of guanylate cyclase, soluble and particulate types, which differ from each other in molecular weight, metal ion requirement, and antigenicity, has been reported in several tissues.[5)] However, few studies have been carried out on guanylate cyclase in inflammatory tissues, and seldom on the two types of this enzyme.

In this paper, we describe the activity of guanylate cyclase in an experimental inflammatory granuloma tissue induced by subcutaneous injection of carrageenin solution in the rat. The presence and properties of soluble and particulate types of this enzyme in the granuloma tissue were also studied.

Experimental

Materials——GTP-8-^{3}H and cyclic GMP-8-^{14}C were purchased from the Radiochemical Centre; creatine kinase, phosphocreatine, and neutral alumina (type WN 3) were from Sigma Chemical Co.; cyclic GMP from

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1) A part of this work was presented at the 51st Annual Meeting of the Japanese Biochemical Society, Kyoto, November, 1978.
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Kohjin Co.; GTP from Yamasa Co.; bovine serum albumin from Armour Pharmaceutical Co.; and Dowex 1×8 (100—200 mesh) from Dow Chemical Co. Carrageenin was kindly supplied by Taisho Pharmaceutical Co.

Inflammatory granuloma pouch was induced in male rats of the Wistar strain, weighing 130—160 g, by subcutaneously injecting 4 ml of 2% carrageenin solution in saline into the dorsal region, where 5 ml of air had been subcutaneously injected 1 day earlier to form a space.[6] The granuloma tissue was collected 5—10 days after carrageenin injection, and freed from the exudate and adhering tissues. The tissue was minced and then homogenized with 10 volumes of 0.25 M sucrose solution containing 1 mM EDTA and 0.1% ethanol (pH 7.4). Where indicated, the homogenate was treated with 0.5% Triton X-100 for 30 min at 4°. Soluble and particulate fractions were obtained by centrifuging the homogenate at 105000 ***g*** for 60 min.

Guanylate Cyclase Activity——Guanylate cyclase activity was measured in terms of the formation of cyclic GMP-^{3}H from GTP-^{3}H during incubation with the homogenate or fraction. The assay system comprised 50 mM Tris-HCl, 2.7 mM cyclic GMP (added for protection of the product), 1 mM GTP and GTP-^{3}H (2×10^6 cpm), 4 mM $MnCl_2$, 40 μg/250 μl creatine kinase, 15 mM phosphocreatine, 10 mM theophylline, and the tissue preparation in a total volume of 250 μl. The reaction was initiated by addition of the tissue preparation, and the total mixture was incubated at 37° for 15 min, unless otherwise stated. Next, 50 μl of 0.5 N HCl was added to stop the reaction, and the mixture was boiled for 1 min. To the final mixture, 50 μl each of 0.1 M Tris-HCl (pH 8.0) and cyclic GMP-^{14}C (3.5×10^4 cpm) were added, the latter being added as a standard to correct for losses of cyclic GMP-^{3}H in the later procedure.

Separation of cyclic GMP from other guanine nucleotides and nucleosides was achieved by sequential column chromatographies on neutral alumina and Dowex 1×8.[7] The formation of cyclic GMP was calculated based on the radioactivity of cyclic GMP-^{3}H measured with a liquid scintillation spectrometer (Packard Tricarb, type 3320), and guanylate cyclase activity was expressed as pmol of cyclic GMP formed/min/mg protein. Protein concentration was determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Results

Guanylate Cyclase Activity in Carrageenin Granuloma Tissue

The enzymological properties of guanylate cyclase in carrageenin granuloma tissue were examined first, since they have not previously been studied in this tissue. As shown in

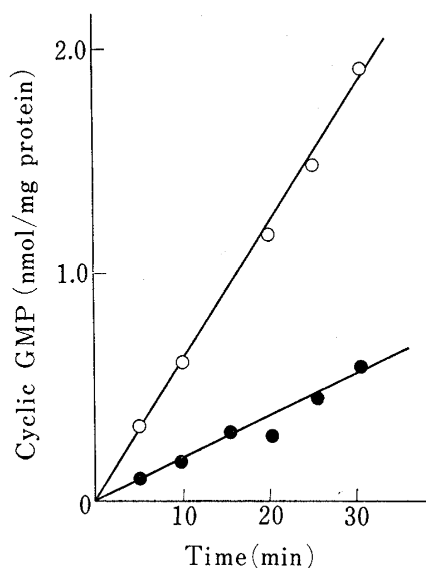

Fig. 1a. The Time Course of Cyclic GMP Formation

Standard reaction mixtures containing 4 mM $MnCl_2$ and 270 μg protein of 10 day granuloma homogenate treated with (○) or without (●) 0.5% Triton X-100 were incubated at 37° for the indicated times.

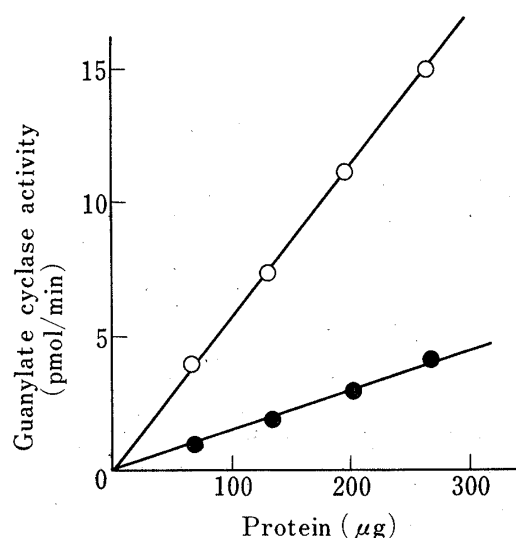

Fig. 1b. Guanylate Cyclase Activity as a Function of Enzyme Concentration

Standard reaction mixtures containing 4 mM $MnCl_2$ and various amounts of 10 day granuloma homogenate treated with (○) or without (●) 0.5% Triton X-100 were incubated at 37° for 15 min.

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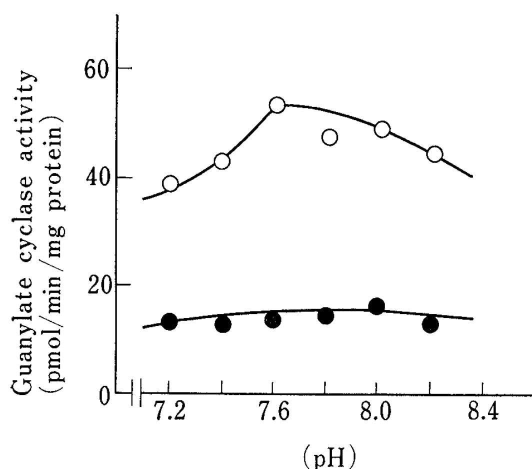

Fig. 1c. Effect of pH on Guanylate Cyclase Activity

Standard reaction mixtures containing 4 mM $MnCl_2$ and 250 μg protein of 10 day granuloma homogenate treated with (○) or without (●) 0.5% Triton X-100 were incubated at 37° for 10 min.

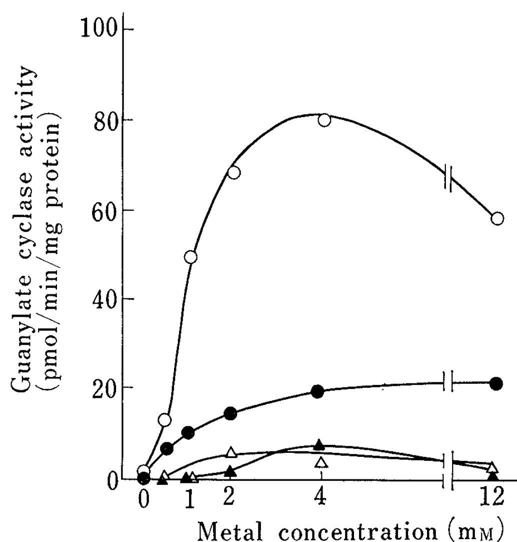

Fig. 1d. Effects of Metals on Guanylate Cyclase Activity

Standard reaction mixtures containing 210 μg protein of 10 day granuloma homogenate treated with (○, △) or without (●, ▲) 0.5% Triton X-100 were incubated at 37° for 15 min in the presence of various concentrations of $MnCl_2$ or $MgCl_2$.
—○— and —●—, $MnCl_2$; —△— and —▲— $MgCl_2$.

Table I. Changes in Guanylate Cyclase Activity in Granuloma Tissue after Injection of Carrageenin Solution into Rats

Days after injection	Guanylate cyclase activity[a]
5	10.3±1.7[b]
7	13.6±1.3
10	21.6±3.5

a) pmol of cyclic GMP formed/min/mg protein.
b) Mean±S.E.

Fig. 1a, the formation of cyclic GMP by the homogenate of the granuloma tissue obtained on the 10th day after carrageenin injection was linear, at least up to 30 min, during incubation at 37° in the presence or absence of Triton X-100. Fig. 1b shows that the formation of cyclic GMP was proportional to the amount of the homogenate added. Fig. 1c shows that the pH-activity curve of guanylate cyclase in this tissue has a broad optimum around pH 8.0 in the absence of Triton X-100, but shows a peak at 7.6 in the presence of Triton X-100. Irrespective of the presence of Triton X-100, the activity of guanylate cyclase was markedly stimulated by Mn^{2+}, and to a lesser extent by Mg^{2+}, as shown in Fig. 1d. Table I shows the change in the activity in the granuloma tissue after carrageenin injection. The activity per mg protein of the homogenate increased gradually from the 5th day to 10th day after the injection, but the above-mentioned properties remained essentially the same.

Localization of Guanylate Cyclase in Carrageenin Granuloma Tissue

To investigate the presence of soluble and particulate types of guanylate cyclase in carrageenin granuloma tissue, the homogenate of the tissue obtained on the 10th day after carrageenin injection was centrifuged at 105000 ***g*** for 60 min to separate the supernatant and precipitate (soluble and particulate fractions, respectively). As shown in Table II, about 60% of the guanylate cyclase activity in the homogenate of the granuloma tissue was distributed in the particulate fraction. Addition of Triton X-100 (0.5%) to each of the

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fractions after separation increased the activity in the particulate fraction 4.4-fold, which might be attributed to the solubilization of guanylate cyclase associated with the membranous structure in this fraction. However, the activity in the soluble fraction was also increased 1.7-fold by the addition of Triton X-100. Therefore, the increase in guanylate cyclase activity on Triton treatment may not be due simply to solubilization, but may also be result of activation of this enzyme by the surfactant. As a next step, the distribution of guanylate cyclase in each of the fractions was examined after treatment of the homogenate with 0.5% Triton X-100 at 4° for 30 min prior to fractionation. In this case, about 66% of the activity was recovered in the soluble fraction, as shown in the same table, suggesting release from the particulate fraction in addition to the activation, since the total activity was almost the same as that measured when Triton X-100 was added after fractionation. The activity remaining in the particulate fraction after Triton treatment of the homogenate before fractionation seemed to be due to the enzyme tightly bound to membranous structures in the granuloma tissue cells.

Table II. Guanylate Cyclase Activities in Soluble and Particulate Fractions of Carrageenin Granuloma Tissue in the Rat

Fraction	Protein (mg)	Guanylate cyclase activity			
		Specific activity[a]		Total activity[b] (%)	
		Measured without Triton[c]	Measured with Triton	Measured without Triton	Measured with Triton
Before fractionation	73.9	23.4	72.0	1.73(100)	5.32(100)
Fractionated without treatment					
Soluble	33.9	18.4	30.9	0.62(36)	1.05(20)
Particulate	34.1	30.8	136.7	1.05(61)	4.66(88)
Fractionated after treatment with Triton X-100[d]					
Soluble	44.5		79.0		3.51(66)
Particulate	28.4		66.5		1.89(35)

a) pmol of cyclic GMP formed/min/mg protein.
b) nmol of cyclic GMP formed/min.
c) Triton X-100, 0.2%.
d) 0.5%, 30 min, 4°.

Table III. Effects of Divalent Cations on Guanylate Cyclase Activity in Carrageenin Granuloma Tissue

Fraction	Cation	Concentration (mM)	Guanylate cyclase activity[a]	
			Measured without Triton X-100[b]	Measured with Triton X-100
Soluble	Mn^{2+}	4	18.0	26.9
		12	34.1	33.9
	Mg^{2+}	4	7.7	10.5
		12	14.9	8.2
	Ca^{2+}	4	11.7	7.2
		12	12.1	11.6
Particulate	Mn^{2+}	4	28.3	73.8
		12	22.8	69.7
	Mg^{2+}	4	13.5	8.1
		12	19.1	15.4
	Ca^{2+}	4	11.2	8.0
		12	12.5	10.0

a) pmol of cyclic GMP formed/min/mg protein.
b) 0.2%.

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Guanylate Cyclase in the Soluble and Particulate Fractions

As shown in Table III, guanylate cyclase activity in both the soluble and particulate fractions was markedly stimulated by Mn^{2+}, and to lesser extents by Mg^{2+} and Ca^{2+}; these results are similar to those observed with the homogenate. However, guanylate cyclase activity in the soluble fraction differed from that in the particulate fraction in its mechanism of stimulation by Mn^{2+} and Triton X-100, since the former was stimulated by higher concentrations of Mn^{2+} irrespective of the presence of Triton X-100, whereas the latter activity decreased at higher concentrations of Mn^{2+}, though it was stimulated by Triton X-100 to the same extent at both high and low concentrations of Mn^{2+}. The findings suggest that guanylate cyclase in the soluble fraction might be different in some way from that in the particulate fraction in carrageenin granuloma tissue.

Discussion

Inflammatory granuloma tissue experimentally induced by the subcutaneous injection of carrageenin in rats is known to proliferate rapidly. Studies on guanylate cyclase in this tissue may cast light on the involvement of cyclic GMP in cellular proliferation and on the role of cyclic GMP in inflammatory reactions. Though the presence of adenylate cyclase was reported in a granulation tissue induced by the subcutaneous implantation of viscose-cellulose sponge,[9] guanylate cyclase has not previously been studied, to our knowledge, in an experimental inflammatory tissue such as that reported here. However, guanylate cyclase in carrageenin granuloma tissue of the rat appears to have enzymological properties similar to those of the enzymes from other tissues. The specific activity of this enzyme in granuloma tissue was similar to that in other tissues.[10] Thus, it is difficult to correlate guanylate cyclase activity directly with the proliferation rate of this tissue. Direct measurement of cyclic GMP in this tissue may be subject to large errors, however, since the compound is easily decomposed during the experimental procedure for the preparation of the specimen.

The intracellular distribution of guanylate cyclase has been examined in various mammalian tissues[3a-c,11] and cultured cells,[12] with special emphasis on the relationship between the enzyme in the soluble fraction and that in the particulate fraction. In the carrageenin granuloma tissue, we also found guanylate cyclase activity in both fractions, the activity being higher in the particulate fraction after conventional fractionation by centrifuging the homogenate at 105000 ***g***. The actual state of guanylate cyclase in the particulate fraction of this tissue was not clarified, but the enzyme was probably associated with endoplasmic reticulum and/or plasma membrane, as reported in the heart,[13] since the activity was significantly transferred to the soluble fraction by treatment with Triton X-100.

As to the relationship between guanylate cyclase in the soluble fraction and that in the particulate fraction of the carrageenin granuloma tissue, some differences were found in the responses to Mn^{2+} and Triton X-100. A decrease in the activity in the particulate fraction and an increase in that in the soluble fraction in the presence of high concentration of Mn^{2+}, as observed in this tissue, were also noted in the renal inner medulla of the rat.[11] These findings suggest the existence of soluble and particulate types of guanylate cyclase in these tissues. The differences between the two types of this enzyme remain to be elucidated in detail.

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