Biochemical Pharmacology, Vol. 26, pp. 2049–2053. Pergamon Press, 1977. Printed in Great Britain.

THE EFFECTS OF PROSTAGLANDIN $F_{2\alpha}$ ON VASCULAR PERMEABILITY AND COLLAGEN AND NON-COLLAGEN PROTEIN SYNTHESIS OF PREFORMED CHRONIC CARRAGEENIN GRANULOMA IN RATS

KAZUO OHUCHI, HIROSHI SATO and SUSUMU TSURUFUJI

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aramaki, Aoba, Sendai 980, Japan

(*Received* 29 *June* 1976; *accepted* 25 *February* 1977)

Abstract—Prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) was administered into the pouch fluid of 7-day-old carrageenin granuloma in rats and the effects of PG $F_{2\alpha}$ on vascular permeability and collagen and non-collagen protein synthesis of the granuloma were investigated. At a dose of 10 μg of PG $F_{2\alpha}$, vascular permeability as measured by the leakage of radioiodinated human serum albumin into the pouch fluid was not inhibited, but at doses of 50, 100 and 250 μg of PG $F_{2\alpha}$, vascular permeability was inhibited significantly. The incorporation of [^{3}H]proline into collagen hydroxyproline and into non-collagen protein of carrageenin granuloma was also inhibited by local administration of PG $F_{2\alpha}$. The mechanism of the effects of PG $F_{2\alpha}$ is discussed.

A number of biologically active endogenous and exogenous substances, possibly the chemical mediators of inflammatory and anti-inflammatory processes, have been investigated in relation to their roles upon inflammatory processes [1–8].

Concerning the relation of prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) to inflammatory processes, some work has been done in relation to the acute, transient form of the inflammatory responses [9–15], but there are few reports which deal with preformed chronic granulomatous inflammation. We have been investigating drug action on chronic inflammation in rats designing to disclose chemical mediators, if any, which control the process of chronic granulomatous inflammation [1–5].

The present investigation was intended to clarify whether PG $F_{2\alpha}$ would interfere with any aspects of preformed granulomatous chronic inflammation provoked by carrageenin in rats. It has been shown that vascular permeability, collagen synthesis and non-collagen protein synthesis in chronic granulomatous inflammation tissue can be inhibited significantly by a single local administration of PG $F_{2\alpha}$.

MATERIALS AND METHODS

Carrageenin granuloma pouch. Carrageenin granuloma pouches were made according to the procedure of Fukuhara and Tsurufuji [1]. Male rats of Donryu strain, weighing 110–140 g, 42–44 days old, were injected with 6 ml of air in the dorsum subcutaneously to make an air sac and 24 hr later with 4 ml of a 2% solution of carrageenin (Seakem No. 202, Marine Colloid Inc., U.S.A.) in 0.9% NaCl into the preformed air sac. The carrageenin solution was sterilized by autoclaving at 120° for 15 min prior to the injection. Seven days after the carrageenin injection, a granuloma pouch involving inflammatory exudate with a volume of 15–20 ml had developed and was used in the following experiment.

Purification of radioiodinated human serum albumin. Commercially available radioiodinated human serum albumin were found to contain small amounts of free radioiodine or radioiodine bound small molecular weight substances in different degrees in each lot. The purification of radioiodinated human serum albumins was carried out by Sephadex G-100 column chromatography. ^{125}I-labeled human serum albumin ([^{125}I]HSA, Iodinated (^{125}I) Human Albumin Injection, 2.5 μCi/mg albumin, Kaken Kagaku, Co. Ltd., Japan) and ^{131}I-labeled human serum albumin ([^{131}I]HSA, Radioiodinated Serum Albumin (RISA), 345 μCi/μM, Dainabot Co., Ltd., Japan) were used. One ml of [^{125}I]HSA or [^{131}I]HSA solution was loaded on a Sephadex G-100 column (column size; 1.5 × 30 cm) and eluted with 0.9% NaCl (elution speed: 60 ml/hr; fraction volume: 1.2 ml/tube). The radioactivity of ^{125}I or ^{131}I was counted in each tube. The fractions containing human serum albumin (fraction numbers: 13 to 17) were gathered and diluted appropriately with 0.9% NaCl into about 1 μCi of [^{125}I]HSA or [^{131}I]HSA per 0.2 ml. These diluted solutions were used in the following vascular permeability experiments.

Vascular permeability measurements in the granuloma pouch. Vascular permeability in the granuloma was measured according to the procedure of Tsurufuji *et al.* [5]. In brief, a 1 μCi aliquot of the purified [^{125}I]HSA in 0.2 ml of 0.9% NaCl was injected into the right femoral vein of the rats bearing the granuloma pouches. After 30 min, 1.0 ml of the inflammatory fluid in the granuloma pouch was withdrawn, through a syringe attached with a 1/3 mm needle, to measure the leakage of [^{125}I]HSA into the pouch fluid through local vascular networks. Immediately after

the sampling of the pouch fluid, PG $F_{2\alpha}$ (Prostaglandin $F_{2\alpha}$, Fuji Yakuhin Kogyo Co. Ltd., Japan) solution or its vehicle in a volume of 0.2 ml was injected locally into the pouch fluid. Five min after the drug treatment, a 1 μCi aliquot of the purified [^{131}I]HSA in 0.2 ml of 0.9% NaCl was injected into the left femoral vein, and after 30 min, 1.0 ml of the pouch fluid was again taken out and served as a sample to measure the leakage of [^{131}I]HSA. The animals were sacrificed just after the second sampling of the pouch fluid and all the fluid in the pouch was collected to measure its volume.

The radioactivity of ^{125}I and ^{131}I was measured in an automatic well type scintillation counter Aloka JDC-751 (Nihon Musen Co. Ltd., Japan). The radioactivity of ^{125}I was counted in the operation mode adjusted for counting the pulses upon photoelectric effect of 35 KeV gamma ray of ^{125}I. The radioactivity of ^{131}I was counted by selective counting of pulses which correspond to 360 KeV gamma ray of ^{131}I, separated from the radiation of coexisting ^{125}I.

The radioactivity of [^{125}I]HSA and [^{131}I]HSA in all the pouch fluid of each rat was calculated and expressed in terms of the percentage of the particular radioactivity injected into the rat. The percentage of [^{125}I]HSA radioactivity, and the percentage of [^{131}I]HSA radioactivity which leaked into the pouch fluid were used as indices of vascular permeability in the granuloma pouch before and after PG $F_{2\alpha}$ treatment, respectively. The ratio of the control group was almost unity. Therefore, a ratio was determined in order to express the change in vascular permeability obtained under the influence of PG $F_{2\alpha}$ treatment.

Incorporation of [^{3}H]proline into collagen hydroxyproline. Rats were subcutaneously injected with [^{3}H]proline (generally labeled L-[^{3}H]proline, 63.0 Ci/m-mole, Daiichi Pure Chemicals Co. Ltd., Japan), 10 μCi per 0.1 ml per 100 g body weight, at 5 min after PG $F_{2\alpha}$ or its vehicle, into the pouch fluid. Thirty min later, the rats were sacrificed by cutting the carotid artery and the granuloma tissue was carefully dissected free from surrounding fat, muscle and non-granulomatous subcutaneous tissues. The granuloma tissue was washed with ice-cold 0.9% NaCl and minced with scissors. A portion of it was autoclaved twice with distilled water at 120° for 1 hr to obtain the collagen fraction as gelatin, which was hydrolyzed with 6 N HCl in a sealed glass tube at 105° for 16 hr. The hydrolysate was evaporated to dryness and [^{3}H]hydroxyproline was determined according to the procedure of Juva and Prockop [16]. The results were expressed as disintegrations per min per μg of collagen hydroxyproline.

Incorporation of [^{3}H]proline into non-collagen protein. A portion of minced granuloma tissue was homogenized by a Vir-Tis 45 homogenizer in ice-cold water for 5 min, mixed with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 1000 *g* for 5 min. The pellet was washed twice with ice-cold 5% TCA containing 1% L-proline and then boiled for 15 min at 90° to solubilize the collagen. The resultant insoluble fraction was washed twice with ice-cold 5% TCA and dissolved in 1 N NaOH. A portion of it was used for the determination of protein by the method of Lowry *et al.* [17]. Another portion of it was neutralized with AcOH and aliquot of it was mixed with 10 ml of triton-toluene scintillation cocktail (PPO 7 g, POPOP 0.1 g, in toluene 667 ml and triton X-100 333 ml) and the radioactivity was measured in a Packard Tri-Carb model 3380 liquid scintillation spectrometer correcting for quenching by external standardization. The results were expressed as disintegrations per min per μg of protein.

RESULTS

Vascular permeability inhibition by PG $F_{2\alpha}$. Table 1 shows the inhibition effect of PG $F_{2\alpha}$ treatment on vascular permeability of the granuloma pouch. No change in vascular permeability was observed at a

Table 1. Effect of PG $F_{2\alpha}$ on vascular permeability of carrageenin granuloma

Exp. No.	Treatment	No. of Rats	Vascular Permeability Index Ratio§	% of Control
I	Control	5	0.96 ± 0.08	100
	PG $F_{2\alpha}$ 10 μg	8	0.97 ± 0.08	101
	PG $F_{2\alpha}$ 100 μg	6	0.61 ± 0.04‡	63.5
II	Control	7	1.19 ± 0.11	100
	PG $F_{2\alpha}$ 50 μg	8	0.72 ± 0.66†	60.5
III	Control	3	1.10 ± 0.16	100
	PG $F_{2\alpha}$ 50 μg	4	0.67 ± 0.08*	60.9
	PG $F_{2\alpha}$ 250 μg	4	0.48 ± 0.04‡	43.6

Results are given as mean ± S.E. Results marked (*, †, ‡) show statistically significant difference between PG $F_{2\alpha}$ treated group and corresponding control group.

* $P < 0.025$, † $P < 0.01$, ‡ $P < 0.005$.

§ Vascular Permeability Index Ratio = $\frac{\%\text{ of [}^{131}\text{I]HSA radioactivity leaked into the pouch fluid (post-treatment)}}{\%\text{ of [}^{125}\text{I]HSA radioactivity leaked into the pouch fluid (pre-treatment)}}$

Experiment I and III: PG $F_{2\alpha}$ was dissolved in a mixture of 7.5% $NaHCO_3$–0.9% NaCl (1:4, v/v) by sonication. Rats received 0.2 ml of PG $F_{2\alpha}$ solution (10 μg, 100 μg per rat in Experiment I, 50 μg, 250 μg, 250 μg per rat in Experiment III) in the pouch fluid. Control rats received 0.2 ml of the vehicle.

Experiment II: PG $F_{2\alpha}$ was dissolved in 0.9% NaCl containing 1% EtOH. Rats received 0.2 ml of PG $F_{2\alpha}$ solution (50 μg per rat) in the pouch fluid. Control rats received 0.2 ml of the vehicle.

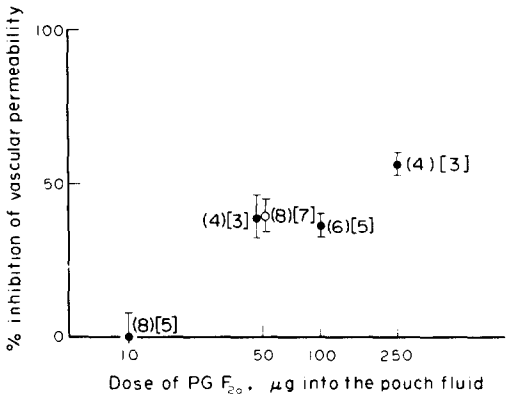

Fig. 1. Inhibition of vascular permeability of carrageenin granuloma by various doses of PG $F_{2\alpha}$. Each point represents the mean of percent inhibition of vascular permeability. Figures in parentheses and in brackets mean the number of PG $F_{2\alpha}$ treated rats and the number of corresponding control rats used. The vertical lines represent standard errors of the means. ●, PG $F_{2\alpha}$ was dissolved in 7.5% $NaHCO_3$-0.9% NaCl (1:4, v/v) by sonication; ○, PG $F_{2\alpha}$ was dissolved in 0.9% NaCl containing 1% EtOH.

dose of 10 μg/0.2 ml, but at doses of 50 μg, 100 μg and 250 μg of PG $F_{2\alpha}$/0.2 ml, inhibitions were 39.1 ± 7.1 per cent ($P < 0.025$) (mean ± S.E.), 36.5 ± 4.2 per cent ($P < 0.005$) and 56.4 ± 3.7 per cent ($P < 0.005$) respectively. When PG $F_{2\alpha}$ was given at a dose of 50 μg/0.2 ml, 39.5 ± 5.4 per cent inhibition was obtained ($P < 0.01$) (Experiment II). There was no difference in the inhibition of vascular permeability with the two solvents. The dose–response relationship for the inhibition of vascular permeability is illustrated in Fig. 1.

Inhibition of [^3H]proline incorporation into collagen hydroxyproline. Table 2 shows the inhibitory effect of PG $F_{2\alpha}$ on [^{3}H]proline incorporation into collagen hydroxyproline of granuloma tissue. The specific radioactivity of hydroxyproline were decreased 45 per cent ($P < 0.005$) and 43 per cent ($P < 0.005$) of the control level, at doses of 50 μg/0.2 ml and 500 μg/0.2 ml of PG $F_{2\alpha}$, respectively.

Inhibition of [^3H]proline incorporation into non-collagen protein. As shown in Table 3, at a dose of 50 μg of PG $F_{2\alpha}$, incorporation of [^{3}H]proline into non-collagen protein is decreased about 57 per cent of the control ($P < 0.005$), almost the same level observed in [^{3}H]proline incorporation into collagen hydroxyproline (Table 2). An inhibitory effect on [^{3}H]proline incorporation into non-collagen protein was also observed at a dose of 500 μg of PG $F_{2\alpha}$ ($P < 0.005$) but there was not the statistically significant difference between the doses of 50 μg and 500 μg of PG $F_{2\alpha}$.

DISCUSSION

The results of the present work show that PG $F_{2\alpha}$, when administered locally into the granuloma pouch fluid, suppresses the vascular permeability (rate of inflammatory fluid production) of carrageenin granuloma as measured by the leakage of radioiodinated human serum albumin into the pouch fluid from local vascular beds.

Several aspects of the carrageenin granuloma pouch as used in the present work were described elsewhere by Tsurufuji and others [18]. The volume

Table 2. Effects of $PGF_{2\alpha}$ on [^{3}H]proline incorporation into collagen hydroxyproline

Treatment	No. of Rats	Specific activity (dpm/μg of Hyp)	% of Control
Control	8	1.02 ± 0.09	100
PG $F_{2\alpha}$ 50 μg	7	0.56 ± 0.07*	55.2
PG $F_{2\alpha}$ 500 μg	8	0.44 ± 0.04*	43.0

Results are given as mean ± S.E. Specific activity represents disintegrations per min per μg of hydroxyproline. Results marked with asterisk (*) show a statistically significant difference between PG $F_{2\alpha}$ treated group and control group.

* $P < 0.005$.

PG $F_{2\alpha}$ (50 μg or 500 μg per rat) was given locally into the pouch fluid, dissolved in 0.9% NaCl containing 1% EtOH in the volume of 0.2 ml. Control rats received 0.2 ml of the vehicle. Five min after the PG $F_{2\alpha}$ treatment, 10 μCi of [^{3}H]proline per 100 g body weight was injected subcutaneously in the abdominal region, and the rats were killed 30 min later.

Table 3. Effects of PG $F_{2\alpha}$ on [^{3}H]proline incorporation into non-collagen protein

Treatment	No. of Rats	Specific activity (dpm/μg of protein)	% of Control
Control	8	0.58 ± 0.05	100
PG $F_{2\alpha}$ 50 μg	7	0.33 ± 0.04*	57.5
PG $F_{2\alpha}$ 500 μg	8	0.30 ± 0.02*	52.7

Results are given as mean ± S.E. Specific activity respresents disintegrations per min per μg of protein. Results marked with asterisk (*) show a statistically significant difference between PG $F_{2\alpha}$ treated group and control group.

* $P < 0.005$.

PG $F_{2\alpha}$ (50 μg or 500 μg per rat) was given locally into the pouch fluid, dissolved in 0.9% NaCl containing 1% EtOH in a volume of 0.2 ml. Control rats received 0.2 ml of the vehicle. Five min after the PG $F_{2\alpha}$ treatment, 10 μCi of [^{3}H]proline per 100 g body weight was injected subcutaneously in the abdominal region, and the rats were killed 30 min later.

of the pouch fluid increased markedly during days 4–10 and then leveled off. Around day 7 after carrageenin injection, the pouch fluid was in a stage of active accumulation. The mechanism of active accumulation of the exudate in the granuloma pouch has not been clarified yet, but some chemical mediators of inflammation such as histamine, serotonin, kinins, PG E_1 and E_2 and some others may participate in the process of the exudat accumulation [19–21].

As to the effects of PG $F_{2\alpha}$ on vascular permeability, Cruckhorn and Willis [11] have reported that PG $F_{2\alpha}$ as well as PG E_1 and E_2 cause wheal and flare responses when injected intradermally into the human forearm, while in the rat skin it causes only a slight increase in vascular permeability. On the other hand, Willoughby [9] reported that PG $F_{2\alpha}$ antagonized the increased vascular permeability induced by intradermal injection of histamine, serotonin, bradykinin and lymph-node permeability factor in the rat. However, Kaley and Weiner [12] and Arora *et al.* [10] observed that PG $F_{2\alpha}$ failed to interfere with local vascular changes caused by PG E_1 in the rat skin. This agrees with the observation of Lewis *et al.* [13] who examined PG E_1 and E_2 in the bovine skin. According to their finding [10, 12, 13], our results suggest that active accumulation of the exudate around day 7 after carrageenin injection may be caused by some chemical mediators if any, other than PG E_1 and E_2, because PG $F_{2\alpha}$ has been shown to suppress vascular permeability of the chronic granuloma. To confirm this suggestion, we have assayed the content of PG E in the pouch fluid of carrageenin granuloma in rats by the radioimmunoassay method [22, 23] and found that the level of PG E on day 5 and 8 was 2.6 and 2.3 ng per ml of the pouch fluid, respectively. At this low level of PG E, it is rather doubtful that PG E participate in increasing vascular permeability in 7-day-old granuloma [4].

Collagen in granuloma tissue also continues to increase until at least 10 days after carrageenin injection [18]. Blumenkrantz and Sondergaard [24] demonstrated that collagen biosynthesis was increased by the addition of PG E_1 or PG $F_{1\alpha}$ in the incubation medium of 10-day-old chick embryo tibiae. We have examined the effect of PG $F_{2\alpha}$ on collagen and non-collagen protein biosynthesis in preformed chronic inflammatory tissue. As shown in Table 2, the incorporation of [^{3}H]proline into collagen hydroxyproline was decreased. And the incorporation of [^{3}H]proline into non-collagen protein was also inhibited by the PG $F_{2\alpha}$ treatment (Table 3). The inhibitory effect on collagen biosynthesis by PG $F_{2\alpha}$ treatment was not specific, but total protein biosynthesis in preformed chronic granuloma tissue was inhibited by the local administration of PG $F_{2\alpha}$. But the possibility remains that the degradation of newly synthesized protein has been occurred by PG $F_{2\alpha}$ treatment as PG $F_{2\alpha}$ was shown to labilize lysosomal membrane [25].

From the results of this investigation, we suggest that PG $F_{2\alpha}$ may play a part in anti-inflammatory activities. Anti-inflammatory activity of PG $F_{2\alpha}$ has already been reported by several investigators, mainly focused on acute inflammation [9, 10, 12]. The anti-inflammatory activity of PG $F_{2\alpha}$ on the development of chronic arthritis inflammation was also reported by Aspinal *et al.* [15]. We have found that PG $F_{2\alpha}$ has anti-inflammatory activity on preformed chronic granuloma tissue. The relatively high pharmacologic doses of PG $F_{2\alpha}$ required for these activities may cause the stimulation of the adrenal cortex. In heifers, Louis *et al.* [26] found that plasma glucocorticoid levels were increased and reached maximum at 30 min and leveled off at 2 hr after the intramuscular injection of PG $F_{2\alpha}$. In the preliminary experiment, the suppression of vascular permeability and [^{3}H]proline incorporation into acid insoluble protein by hydrocortisone administration (5 mg/kg) into the pouch fluid was not observed within 1 hr but 3 hr after the injection [5]. This suggests that a certain time lag was needed for the expression of the effectiveness of hydrocortisone. The present investigation reveals that the effect of PG $F_{2\alpha}$ is elicited during the 30 min after local administration of PG $F_{2\alpha}$. The supression of vascular permeability and the inhibition of [^{3}H]proline incorporation into protein caused by PG $F_{2\alpha}$ local administration might not be mediated by stimulation of the adrenal cortex.

PG $F_{2\alpha}$ has been reported to induce venoconstriction associated with increased vascular tissue levels of cyclic 3′:5′-guanosine monophosphate (cGMP) [27]. The suppression of vascular permeability in preformed chronic granuloma tissue caused by the local administration of PG $F_{2\alpha}$ might be due to constriction of the vascular bed in chronic granuloma tissue. Constriction of the vascular bed of preformed chronic tissue caused by PG $F_{2\alpha}$ local administration might result in a depression of blood flow and consequently the amount of [^{3}H]proline which is carried from the abdominal region might be less than in control rats. Thus the apparent beneficial effect on the inhibition of collagen and noncollagen protein biosynthesis caused by local administration of PG $F_{2\alpha}$ might be only a reflection of the amount of utilizable ^{3}H-labeled proline around chronic granuloma tissue. We are now investigating whether PG $F_{2\alpha}$ and cGMP are able to inhibit the synthesis of collagen and noncollagen protein of preformed chronic granuloma tissue by experiment *in vitro*.

Acknowledgement—We thank Miss Hiroko Kimura for her expert technical assistance.

REFERENCES

1. M. Fukuhara and S. Tsurufuji, *Biochem. Pharmac.* **18**, 475 (1969).
2. K. H. Shin, H. Nakagawa and S. Tsurufuji, *Chem. Pharm. Bull.* **23**, 1137 (1975).
3. W. C. Chang and S. Tsurufuji, *Eur. J. Pharmac.* **36**, 7 (1976).
4. W. C. Chang and S. Murota and S. Tsurufuji, *Biochem. Pharmac.* **25**, 2045 (1976).
5. S. Tsurufuji, K. Sugio and H. Endo, *Biochem. Pharmac.*, **26**, 1131 (1977).
6. T. J. Williams and J. Morley, *Nature, Lond.* **246**, 215 (1973).
7. S. Moncada, S. H. Ferreira and J. R. Vane, *Nature, Lond.* **246**, 217 (1973).
8. G. Thomas and G. B. West, *Br. J. Pharmac.* **50**, 231 (1974).
9. D. A. Willoughby, *J. Path. Bact.* **96**, 381 (1968).

10. S. Arora, P. K. Lahiri and R. K. Sanyal, *Int. Arch. Allerg.* **39,** 186 (1970).
11. P. Crunkhorn and A. L. Willis, *Br. J. Pharmac.* **18,** 49 (1971).
12. G. Kaley and R. Weiner, *Ann. N.Y. Acad. Sci.*, **180,** 338 (1971).
13. A. J. Lewis, P. W. Wells and P. Eyre, *J. Pharm. Pharmac.* **24,** 326 (1972).
14. G. DiPasquale, C. Rassaert, R. Richter, P. Welaj and L. Tripp, *Prostaglandins*, **3,** 741 (1973).
15. R. L. Aspinall, P. S. Cammarata, A. Nakao, J. Jiu, M. Miyano, D. E. Baker and W. F. Pautsch, *Adv. Biosci.* **9,** 419 (1974).
16. K. Juva and D. J. Prockop, *Analyt. Biochem.* **15,** 77 (1966).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
18. S. Tsurufuji, H. Nakagawa, T. Ohno and M. Fukuhara, in *Biochemistry and Pathology of Connective Tissue*, pp. 135–151. Igaku Shoin, Tokyo (1974).
19. H. Hayashi, I. Tasaki and M. Yoshinaga, *Nature, Lond.* **215,** 759 (1967).
20. A. L. Willis, *J. Pharm. Pharmac.* **21,** 126 (1969).
21. M. DiRosa, J. P. Giroud and D. A. Willoughby, *J. Pathol.* **104,** 15 (1971).
22. W. C. Chang, S. Murota and S. Tsurufuji, *Jap. J. Pharmac.* **25,** 219 (1975).
23. K. Ohuchi, H. Sato and S. Tsurufuji, *Biochim. biophys. Acta* **424,** 439 (1976).
24. N. Blumenkrantz and J. Sondergaard, *Nature New Biol.* **239,** 246 (1972).
25. R. Weiner and G. Kaley, *Nature New Biol.* **236,** 46 (1972).
26. T. M. Louis, J. N. Stellflug, H. A. Tucker and H. D. Hafs, *Proc. Soc. exp. Biol. Med.* **147,** 128 (1974).
27. E. W. Dunham, M. K. Haddox and N. D. Goldberg, *Proc. natn. Acad. Sci. U.S.A.* **71,** 815 (1974).