Br. J. Pharmac. (1975), 53, 75-83

STIMULATION OF THE HYPOTHALAMO–PITUITARY–ADRENAL AXIS BY COMPOUNDS FORMED IN INFLAMED TISSUE

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1 Rat paws were injected with carrageenin, and their subcutaneous tissue perfused 135 min later. These perfusates were injected intravenously into receptor rats in which they caused an attenuation of inflammatory responses.

2 The effect was not observed in adrenalectomized receptor rats nor in receptors with electrolytic lesions in the median eminence of the hypothalamus but persisted in adrenal-demedullated animals.

3 The active perfusates also induced eosinopenia in normal or adrenal-demedullated animals, but not in adrenalectomized rats, and produced an increase in blood corticosterone with a concomitant decrease in the amounts of adrenal ascorbic acid.

4 The active perfusates did not affect the responses of isolated preparations to histamine, bradykinin, prostaglandins and 5-hydroxytryptamine neither did they elicit changes of the arterial blood pressure in receptor animals.

5 The anti-inflammatory activity present in perfusates from inflamed paws seems to be formed slowly at the site of the developing inflammatory reaction, since perfusates collected 30-65 min after the injection of carrageenin were ineffective, as was plasma taken from donor rats at various time intervals after carrageenin injections.

6 It is suggested that the anti-inflammatory factor present in the active perfusates exerts its action by stimulation of the hypothalamo-pituitary-adrenal axis.

Introduction

Increased blood concentrations of corticosteroids after injections of endotoxins have been reported on several occasions (Melby, Egdahl & Spink, 1960; Moberg, 1971). Experimental and clinical observations have demonstrated that adrenal corticosteroids exert a marked anti-inflammatory activity (Cook & MacDonald, 1951; Ashton & Cook, 1952; Wyman, Fulton & Schulman, 1953; Moon & Tershakovec, 1954; Wyman, Fulton, Schulman & Smith, 1954; Germuth, 1956).

In the present paper the possibility was investigated as to whether local inflammatory processes could stimulate the adrenal secretion of corticosteroids which might limit the spread of the inflammatory reaction.

Methods

Male Sprague Dawley (Holtzman) rats weighing between 250-300 g were used. Unless otherwise indicated the animals were anaesthetized throughout the experiments with pentobarbitone sodium (30-40 mg/kg, i.p.). Surgery was performed under ether anaesthesia.

Production of oedema in rat paws

Carrageenin was dissolved in distilled water and 0.1 ml of the solution (0.5 mg/ml) injected into the subplantar area of one of the hind paws; the other was injected with 0.1 ml of 0.9% w/v NaCl solution (saline). The volumes of the paws up to the tibio-tarsal articulation were determined by plethysmography at varying time intervals with a modification of the apparatus described by Winder, Wax & Been (1957). Results are expressed as percentage increase in relation to the initial volume of the paw. Control values (saline-injected paws) were subtracted from values obtained in carrageenin-injected paws.

Double coaxial perfusion of rat paws

Simultaneous perfusions of the subcutaneous space of both hind paws of the rat were carried

out according to Garcia Leme, Hamamura & Rocha e Silva (1970). Tyrode solution kept between 5 and 10° C or at 37° C was used as the perfusion fluid; the flow rate was 1 ml/10 minutes. Perfusions lasted for 35 min and were performed at various time intervals after carrageenin or saline injections into the paws. The perfusates were assayed as indicated below. A group of animals was injected intravenously with Evans blue (20 mg/kg) (1% aqueous solution) 5 min before the perfusions and the amount of dye present in the perfusates estimated by spectrophotometry at 600 nm. Control values (dye present in perfusates from saline-injected paws) were subtracted from test values (dye present in perfusates from carrageenin-injected paws).

Tests on perfusates

Perfusates collected from donor rat paws treated with carrageenin, dextran or saline were injected intravenously (penis vein) into receptor animals (0.5 ml/rat). The following parameters were measured in the receptor rats: (i) oedema developing after an injection of carrageenin into a hind paw; (ii) dye leakage into the inflamed paws; (iii) number of blood eosinophils; (iv) serum corticosterone concentrations; (v) ascorbic acid concentrations in the adrenal glands; (vi) effect of perfusates upon arterial blood pressure. The assays were carried out in normal rats, bilaterally adrenalectomized rats, bilaterally adrenal-demedullated rats and rats with electrolytic lesions in the median eminence of the hypothalamus. The perfusates were also tested on the guinea-pig isolated ileum and rat uterus.

Blood eosinophils

Blood collected from the tails of non-anaesthetized receptor rats was diluted with Manners solution and the number of eosinophils counted in a Fuchs–Rosenthal chamber (Darmady & Davenport, 1963). Blood samples were collected before and 2 and 4 h after the injection of perfusates. Results are expressed as the percentage deviation from the initial counts.

Determination of serum corticosterone concentrations

Approximately 4 ml blood was collected from the aorta of anaesthetized rats through an opening in the abdominal wall and after clotting, centrifuged at 2,500 rev/min for 10 minutes. Corticosterone was extracted from 0.5 ml serum and estimated according to Guillemin, Clayton, Lipscomb & Smith (1959). Estimations were made 90 min after the intravenous injections of the perfusates or 60 or 180 min after paw injections of either carrageenin or saline. The same method was used to investigate the possible contamination of perfusates collected from donor rat paws with corticosterone.

Determination of adrenal ascorbic acid

The animals were kept isolated in cages for 24 h before the removal of the adrenal glands. At the time of the experiments they were anaesthetized, decapitated and both adrenal glands removed and weighed. Ascorbic acid was extracted and measured according to Mindlin & Butler (1938), 90 min after the intravenous injections of perfusates.

Bilateral adrenalectomy

The adrenal glands were removed under ether anaesthesia by the dorsal approach. The adrenalectomized rats were supplied with saline and water *ad lib.* and were used 48-56 h post-operatively. Within this period of time development of functioning accessory adrenocortical tissue does not occur.

Adrenal demedullation

The glands were exposed under ether anaesthesia and the medulla was expelled through a small incision in the capsule. The animals were used 48-56 h later.

Electrolytic lesion of the median eminence of the hypothalamus

Animals under ether anaesthesia were held in a stereotaxic apparatus (Johnson Sci. Instr. Co.). Through a hole drilled in the skull a stainless steel wire electrode was introduced into the median eminence, using the coordinates described by De Groot (1959). The electrode was isolated with the exception of its tip. The lesion was made by applying a 3 mA current for 10 seconds. The animals were used 36-40 h later. The effectiveness of the brain lesion was tested by the amount of water ingested in the 24 h period following surgery. Those animals with water intakes in excess of 100 ml in the first 24 h after the operation were used (McCann & Fruit, 1957).

Arterial blood pressure

Blood pressure in the carotid artery was measured with a mercury manometer. The perfusates were injected into a cannulated jugular vein.

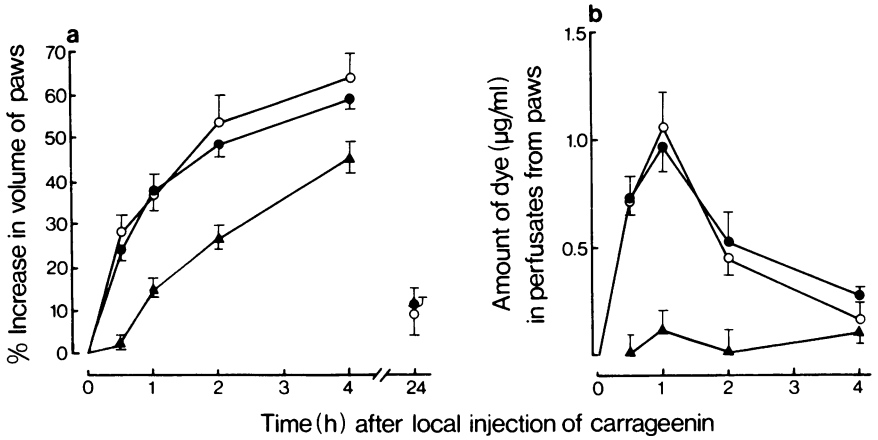

Figure 1 (a) Oedema formation in rat paws after the local injection of 50 μg carrageenin. (b) Dye (Evans blue, injected i.v.) concentration in perfusates from rat paws with carrageenin oedemas. (○) Controls; (▲) rats pretreated with 0.5 ml (i.v.) of a perfusate from paws of a donor rat with carrageenin oedema. Perfusate (5-10° C) collected 135-170 min after carrageenin injection. (●) Rats pretreated with perfusate from saline-injected donor rat paws. Each point is mean value; vertical bars show s.e. mean. Oedema experiments started with 26-40 rats in each group. At each time five rats were taken for perfusion experiments and subsequently killed. Perfusates administered to receptor rats immediately before paw injections.

Isolated preparations

The guinea-pig ileum was immersed in Tyrode solution in a 10 ml organ bath at 37° C, the rat uterus in de Jalón solution in a 5 ml bath at 28° C.

Drugs

Acetylsalicylic acid (Endosprin, Enila); ascorbic acid (E. Merck AG); Evans blue dye (E. Merck AG); bradykinin (BRS-640, Sandoz); carrageenin, sodium salt, mol wt. 60,000-100,000 (Merck, Sharp & Dohme); corticosterone (Sigma); 5-hydroxytryptamine (Serotonin, Mann Research Lab.); histamine diphosphate (Sigma); indomethacin (Merck, Sharp & Dohme); pentobarbitone sodium (Nembutal, Abbott); prostaglandins E_1 and E_2 (Upjohn); dextran, mol. wt. 60,000-90,000 (Nutritional Biochemical Corp.).

Results

The intravenous administration of 0.5 ml of a perfusate collected from a rat paw injected 135 min previously with carrageenin (active perfusates) attenuated the development of paw oedema induced by an injection of carrageenin in the receptor rats as well as exudation into an inflamed paw of dye previously administered by the intravenous route. Perfusates collected simultaneously from the saline injected control paw of the donor rats were ineffective (Figure 1). In these experiments the perfusion fluid was kept between 5-10° C. If it was kept at 37° C the activity was diminished.

When the perfusates were collected 30 min after the injection of carrageenin into the paw of the donor rat, no oedema reduction was observed in receptor animals (Figure 2). In these experi-

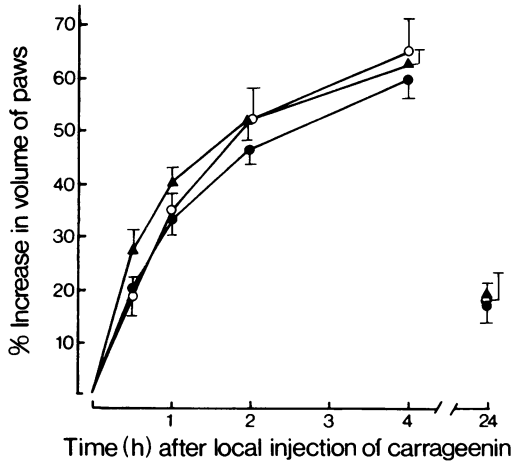

Figure 2 Oedema formation in rat paws after local injection of 50 μg carrageenin. (○) Controls; (▲) rats pretreated with 0.5 ml (i.v.) of a perfusate from paws of a donor rat with carrageenin oedema. Perfusate (5-10° C) collected 30-65 min after carrageenin injection. (●) Rats pretreated with perfusate from saline-injected donor rat paws. Means of 6 controls and 12 pretreated rats in each group. Vertical bars show s.e. mean. Perfusates administered to receptor rats immediately before paw injections.

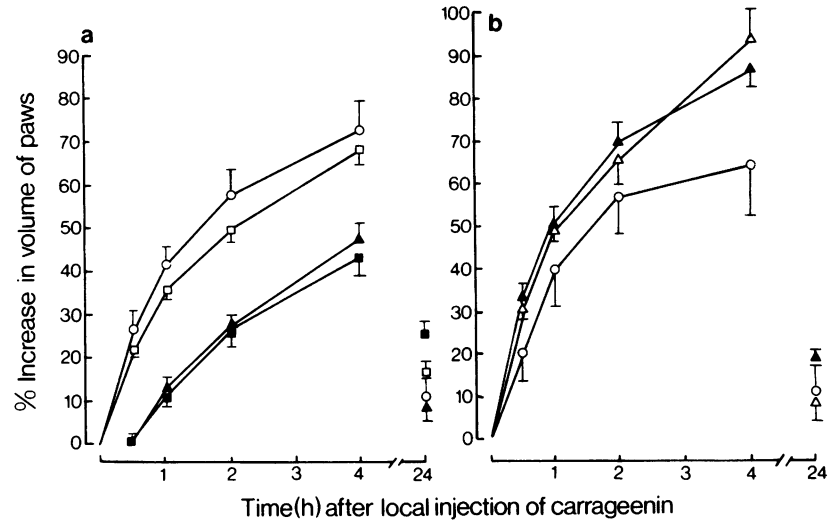

Figure 3 Oedema formation in rat paws injected with 50 μg carrageenin. (a) Results obtained in untreated rats (○); or in rats pretreated with 0.5 ml perfusates collected from donor rat paws injected with 50 μg carrageenin: intact (▲); adrenalectomized (□); adrenal-demedullated (■). (b) Results obtained in untreated rats (○) and in rats submitted to a lesion of the median eminence of the hypothalamus receiving (△) and not receiving (▲) 0.5 ml perfusates as in (a). Perfusates (5-10° C) collected 135-170 min after carrageenin injections in the donor animals and administered intravenously to receptor animals immediately before carrageenin. Each point is mean value (n = 7 for ○; 9 for ▲; 15 for □; 10 for ■; 5 for △ and ▲ in (b)). Vertical bars indicate s.e. mean.

Table 1 Changes in the number of circulating eosinophils in rats receiving 0.5 mg corticosterone or 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate).

	Blood eosinophils: % Deviation from the initial mean value	
Treatment	*2 h after injections*	*4 h after injections*
	Normal rats	
Corticosterone	−17.6 ± 5.5	−40.4 ± 3.8 (5)
Active perfusate	−33.7 ± 3.8	−48.3 ± 4.1 (5)
Inactive perfusate	+25.6 ± 8.5	+31.6 ± 5.2 (5)
Controls	+14.8 ± 12.4	+18.4 ± 9.9 (5)
	Adrenalectomized rats	
Corticosterone	−18.6 ± 2.9	−31.5 ± 3.6 (5)
Active perfusate	−0.7 ± 6.1	−0.6 ± 2.9 (5)
Inactive perfusate	+8.0 ± 5.0	+8.5 ± 6.3 (4)
Controls	+39.0 ± 16.4	+43.9 ± 17.8 (5)
	Adrenal-demedullated rats	
Corticosterone	−22.3 ± 4.2	−44.2 ± 3.5 (5)
Active perfusate	−33.8 ± 7.0	−52.4 ± 7.0 (5)
Inactive perfusate	+2.5 ± 4.7	+13.0 ± 7.9 (5)
Controls	+19.7 ± 18.5	+8.7 ± 6.2 (4)

Mean values with s.e. mean are given. Figures in parentheses indicate number of animals in each group. Corticosterone was given in oil-suspension (s.c.); perfusates (given i.v.) were collected between 135 and 170 min after carrageenin injections into the paw of the donor animals. The initial number of circulating eosinophils/mm^3 was 241 ± 25; 390 ± 62 and 373 ± 44 (mean ± s.e. mean) respectively in normal, adrenalectomized or adrenal-demedullated animals.

ments the perfusion fluid was kept between 5-10° C.

Similar results were observed when dextran (100 μg) was injected as an irritant in the donor rat paw and the perfusates collected were given intravenously to a receptor rat whose paw was injected with carrageenin.

Active perfusates also reduced carrageenin oedema in receptor animals which were bilaterally adrenal-demedullated but showed no effect in adrenalectomized rats or in rats with lesions in the median eminence of the hypothalamus (Figure 3). However, bilateral adrenalectomy and bilateral adrenal-demedullation did not influence the development of oedema when such rats were injected with carrageenin.

The intravenous injections of active perfusates, but not of those collected from saline-injected paws, produced a reduction in the number of circulating eosinophils in either normal receptor rats or in rats submitted to bilateral adrenal-demedullation. This action was not observed in bilaterally adrenalectomized animals. The eosinopenia was more intense than that observed after the subcutaneous injection of 0.5 mg corticosterone in oil suspension (Table 1). The number of eosinophils was counted 2 and 4 h after the injections of either the perfusates or corticosterone.

Increased concentrations of blood corticosterone (Table 2) and a decrease in the concentration of adrenal ascorbic acid (Table 3) were detected 90 min after the intravenous injections of active perfusates into normal rats. Perfusates collected from saline-injected paws of donor rats had no effect.

When instead of perfusates, plasma was collected from donor rats 30, 60 or 120 min after carrageenin and 0.5 ml injected intravenously into receptor animals, no attenuation of the carrageenin oedema was observed up to 4 h after the injection.

The active perfusates produced no arterial blood pressure changes in normal receptor animals; they did not elicit any response of the guinea-pig isolated ileum or rat uterus and did not interfere with the response of these tissues to histamine, bradykinin, prostaglandins E_1 and E_2 (guinea-pig ileum) or 5-hydroxytryptamine (rat uterus). No steroids could be detected in the active perfusates. The method employed easily detects 0.02 μg corticosterone/ml of the acid extract.

Subcutaneous injections of 1 mg corticosterone/rat (in oil suspension) 90 min before plantar administration of carrageenin, or intraperitoneal injection of acetylsalicylic acid (100 mg/kg) or indomethacin (10 mg/kg) 30 min

Table 2 Serum corticosterone concentrations in rats receiving 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate)

Treatment	*Corticosterone (μg/100 ml serum)*
Active perfusate	20.09 ± 2.22 (11) ($P < 0.01$)*
Inactive perfusate	9.75 ± 2.18 (8) ($P > 0.05$)*
Controls	10.28 ± 1.71 (7)

Estimations were made 90 min after intravenous injection of perfusates. Mean values with s.e. mean are given. Number of animals in each group are shown in parentheses. All animals were anaesthetized with pentobarbitone sodium. Perfusates were collected between 135 and 170 min after paw injections in the donor animals. * Student's *t* test, by comparison with control values.

Table 3 Adrenal ascorbic acid in rats receiving 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate).

Treatment	*Ascorbic acid (mg/100 g adrenal tissue)*
Active perfusate	282.87 ± 10.56 (7) ($P < 0.01$)*
Inactive perfusate	392.61 ± 28.41 (10) ($P > 0.05$)*
Controls	380.05 ± 10.28 (7)

Estimations were made 90 min after intravenous injections of perfusates. Mean values with s.e. mean are given. Number of animals in each group are shown in parentheses. All animals were anaesthetized with pentobarbitone sodium. Perfusates were collected between 135 and 170 min after paw injections in the donor animals. * Student's *t* test, by comparison with control values.

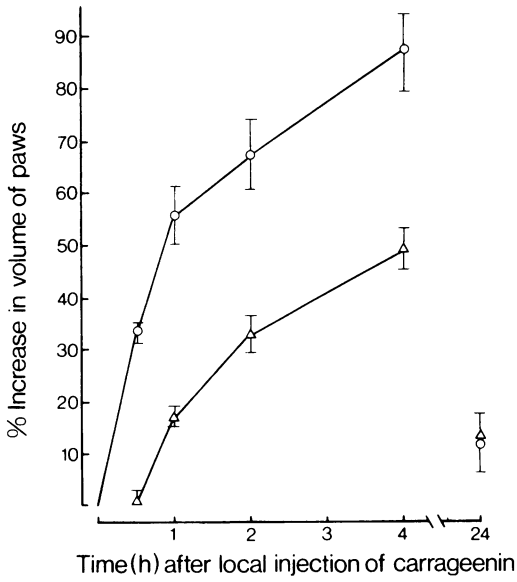

Figure 4 Effect of corticosterone (1 mg in oil, s.c.) on the development of carrageenin-induced rat paw oedema. Carrageenin (50 μg) injected locally 90 min after the steroid. (○) Controls; (△) corticosterone-treated rats. Each point is mean value (n = 6 for ○; n = 9 for △). Vertical bars show s.e. mean.

before carrageenin, reduced the resulting paw oedema to a similar extent as the active perfusates (Figures 4 and 5). In contrast to the active perfusates, acetylsalicylic acid and indomethacin were also effective in bilaterally adrenalectomized animals.

When carrageenin was injected into one of the hind paws of normal untreated rats and the other paw was injected with this irritant after a 135 min interval, the oedema caused in the latter was much smaller than that produced in the former. This attenuation of responses was not seen in adrenalectomized rats whose hind paws were injected with carrageenin keeping the 135 min interval between both injections (Figure 6). Furthermore 67% increase in the serum corticosterone concentration was observed in four normal rats 180 min after paw injections of carrageenin (15.0 ± 1.0 μg/100 ml serum; mean with s.e. mean) as compared to three normal rats injected with saline (9.0 ± 0.48 μg/100 ml serum). This increase was not seen 60 min after carrageenin injections.

Discussion

Our results show that perfusates collected from donor rat paws locally injected with carrageenin

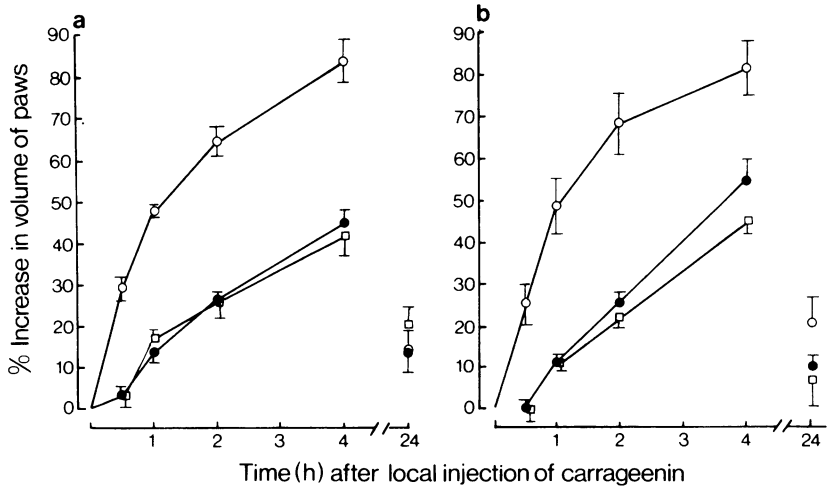

Figure 5 (a) Effect of acetylsalicylic acid (100 mg/kg, i.p.) or (b) indomethacin (10 mg/kg, i.p.) on the development of the carrageenin-induced rat paw oedema. Carrageenin (50 μg) injected locally 30 min after the anti-inflammatory drugs. (○) Untreated controls; (●) intact, drug-injected rats; (□) adrenalectomized drug-injected rats. Each point is mean value (n = 5). Vertical bars show s.e. mean.

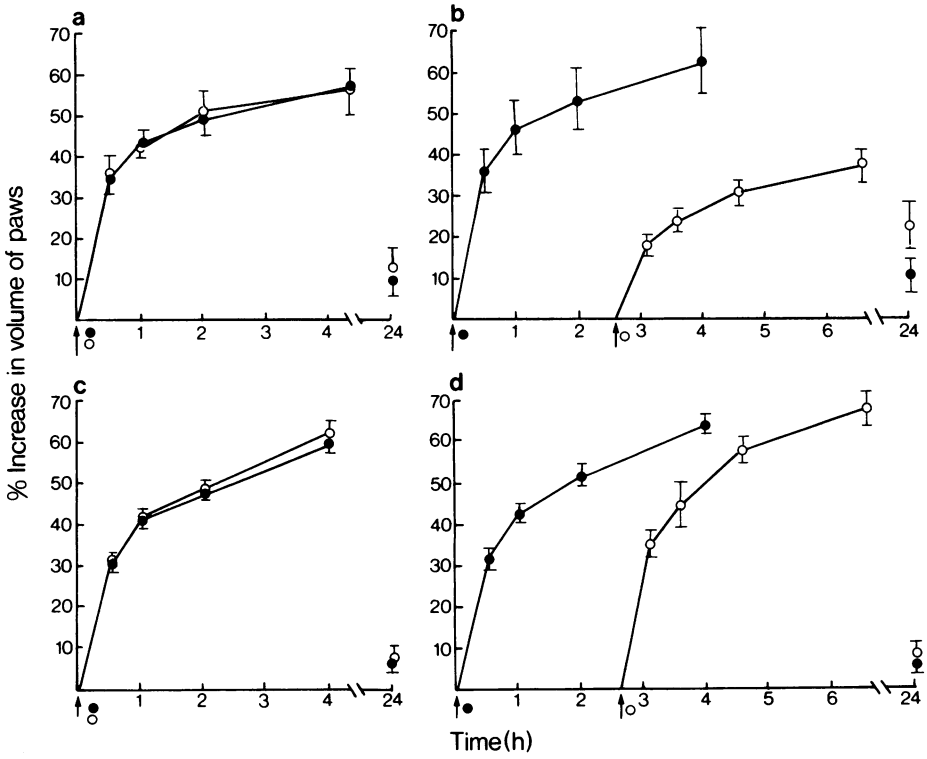

Figure 6 Oedema formation in rat paws after local injection of 50 μg carrageenin. (a) and (b) Results obtained in normal rats. (c) and (d) Results obtained in adrenalectomized rats. In (a) and (c) both hind paws were injected simultaneously with the irritant; in (b) and (d) right paws were injected 135 min after left paws. (○) Right paw; (●) left paw. Arrows indicate carrageenin injections. Each point is mean value (n = 6). Vertical bars show s.e. mean.

135 min previously can attenuate inflammatory responses in receptor rats. This effect was not observed in adrenalectomized receptor rats or in receptors with electrolytic lesions in the median eminence of the hypothalamus. It persisted in animals which had been adrenal-demedullated. The active perfusates also induced eosinopenia in normal or adrenal-demedullated animals, but not in adrenalectomized rats. Furthermore, they increased blood concentrations of corticosterone and produced a decrease in the adrenal ascorbic acid content.

The perfusates did not affect the responses of the isolated tissues to endogenous permeability factors such as histamine, bradykinin, prostaglandins and 5-hydroxytryptamine and did not elicit arterial blood pressure changes in receptor animals.

These results suggest that a factor was present in the active perfusates which could be transferred from donor to receptor animals, where it exerted an anti-inflammatory effect. The active factor probably stimulated the hypothalamo-pituitary-adrenal axis resulting in the release of adrenal corticosteroids (corticosterone). Catecholamines release from the adrenal glands did not play an important role in the phenomenon, as the perfusates were still active in adrenal-demedullated animals.

The formation of the anti-inflammatory substance or substances depended on the existence of a developing inflammatory reaction as shown by the fact that perfusates collected from rat paws injected with saline alone were ineffective. The anti-inflammatory activity detected in perfusates from inflamed paws seems to be formed slowly at the site of the developing inflammatory reaction, as perfusates collected between 30-65 min after the application of the noxious stimulus were not yet effective. Also, plasma taken from donor rats at various time intervals following carrageenin injections into the paws was devoid of anti-

inflammatory activity. This suggests that the factor responsible for the observed anti-inflammatory effect is concentrated in the inflamed tissues and is slowly released into the circulation.

It is unlikely that the factor in the perfusate from the carrageenin-injected paw is a metabolite of carrageenin, as perfusates from dextran-injected paws were similarly effective.

The mechanism of the anti-inflammatory effect described is different from that observed with acetylsalicylic acid or indomethacin, as these drugs were also active in adrenalectomized rats.

Furthermore, the injection of carrageenin into one of the hind paws of the rats, led to an attenuation of the response to this irritant injected in the other paw 135 min later. This attenuation was not observed in adrenalectomized rats. Our data therefore suggest the existence of a neuro-endocrine feed-back system during the development of acute inflammatory reactions: through local changes in the affected tissues, probably by cell disruption, protein breakdown, or enzymatic activation, a factor is formed which is released into the circulation and stimulates the hypothalamo-pituitary-adrenal axis resulting in the release of corticosterone. The effectiveness of this steroid in reducing oedema formation after carrageenin injection was observed in the present work. Germuth (1956) has suggested that the vaso-constrictive action of corticosteroids is their most important effect in counteracting inflammation. Moberg (1971) reported that the increase of plasma corticosterone resulting from injection of endotoxins was abolished in rats by electrolytic lesions of the median eminence of the hypothalamus or by a pharmacological blockade of the hypothalamic release of corticotropin-releasing factor. It has also been suggested that the anti-oedema activity of tetrahydrocannabinol was mediated by stimulation of the pituitary-adrenal axis since it was attenuated in adrenalectomized and in hypophysectomized rats (Sofia, Nalepa, Harakal & Vassar, 1973).

Endogenous anti-inflammatory factors have previously been detected in inflammatory exudates or plasma (Rindani, 1956; Di Pasquale & Girerd, 1961; Di Pasquale, Girerd, Beach & Steinetz, 1963; Robinson & Robson, 1964; Bonta & De Vos, 1969; Billingham, Robinson & Robson, 1969; Bonta, Bhargava & De Vos, 1970; Elliot, Ford-Hutchinson, Harford, Insley, Smith & Sturgess, 1973). Most of these anti-inflammatory factors were detected some days after the institution of an inflammatory response. From our present results it seems likely that the organism is also able to produce 'anti-inflammatory' factors at an earlier stage which help to limit the development of the inflammatory focus. These factors are produced by the inflamed tissue itself and stimulate the release of corticosteroids.

Garcia Leme, Hamamura, Leite & Rocha e Silva (1973) observed that after carrageenin injection in the rat's paw a dissociation in time exists between oedema formation and leakage of dye previously given intravenously: while the oedema developed slowly, reaching a maximum after 4-5 h, protein-dye-leakage was already maximal after 1 hour. It is possible that the adrenal corticosteroids are responsible for this dissociation by reducing protein-leakage through a vaso-constrictive action. Schayer (1963, 1964, 1967) assumed that the anti-inflammatory action of glucocorticoids was dependent on the antagonism of dilator effects exerted upon the microcirculatory bed in the affected tissue. In this theory the chief vaso-dilating agent was 'induced histamine'.

It should be remembered that since the work by Hench, Kendall, Slocumb & Polley (1949, 1950) it is known that several clinical and biochemical aspects of rheumatoid diseases are improved by the use of corticosteroids or adreno-corticotrophic hormone. Such agents might be reinforcing a naturally occurring situation.

We are greatly indebted to Drs J. Antunes Rodrigues and W.A. Saad for their help and suggestions; to Mr Rubens de Melo for technical assistance. This work was partly supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). One of the authors (E.E.S.S.) was on leave of absence from the School of Pharmacy, University of Rio Grande do Sul, Porto Alegre, RGS, Brazil.

Prostaglandins E_1 and E_2 were kindly supplied by Dr J.E. Pike, Upjohn, U.S.A.

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(Received July 16, 1974)