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Partial purification of the anti-inflammatory factor(s) in inflammatory exudate

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1. The carrageenin foot test was established as a sensitive and reliable assay procedure for determining the anti-inflammatory activity of inflammatory exudates.

2. Incubation alone at a temperature above 70° C or with pronase at 37° C destroyed the anti-inflammatory activity of exudate.

3. The anti-inflammatory component of exudate was partially precipitated by 50% ammonium sulphate.

4. A partial purification process was devised using Sephadex G-150 gel filtration and DEAE and CM cellulose ion exchange chromatography to obtain at least a 24 fold purification.

5. Measurements of 11-hydroxycorticosteroid levels indicated that steroids were not involved in the mechanism by which the exudate produced its anti-inflammatory effects.

There are many reports that irritant substances inhibit experimental inflammation (Laden, Blackwell & Fosdick, 1958; Buch & Wagner-Jauregg, 1962; Cygielman & Robson, 1963; Benitz & Hall, 1963; Robinson & Robson, 1964; Jori & Bernardi, 1966). It has also been suggested that the anti-inflammatory effects of some drugs used clinically could be ascribed to a similar counter-irritant action (Hollander, 1960).

On circumstantial evidence Laden, Blackwell & Fosdick (1958) suggested in explanation that at a site of injection of an irritant, a substance was released which could then exert anti-inflammatory effects in other parts of the body. Evidence that inflammatory exudates possessed such anti-inflammatory properties had already been given by Rindani (1956), and this was confirmed subsequently by DiPasquale & Girerd (1961) and DiPasquale, Girerd, Beach & Steinetz (1963). The activity was ascribed to a substance of extra-adrenal origin, not steroidal in nature, and which did not have ACTH-like activity. There was no evidence, however, that such an anti-inflammatory substance was present in the serum of animals in which an irritant had been injected, a rather surprising finding if this is to explain the anti-inflammatory effects produced by irritants at distant sites. Robinson & Robson (1964), using the cotton pellet test, essentially confirmed this work and produced some evidence for the presence of anti-inflammatory activity in the plasma of animals subjected to irritant action by the implantation of polyester sponges. The

fact that DiPasquale did not find such activity in the plasma may be ascribed to the capsule which forms around croton oil pouches which would prevent the passage of the anti-inflammatory substance into the blood. In our experiments using sponges, however, no capsule was apparent to impede the passage of such a substance into the blood.

More recently it was shown (Robinson & Robson, 1966) that exudate from polyester sponges also reduced the tensile strength of healing wounds, produced no increase in permeability to circulating trypan blue following intradermal injection, and did not inhibit formalin-induced foot oedema. This last finding led us to suggest that the anti-inflammatory effect was exerted specifically on the cellular as opposed to the permeability aspects of the reaction, and that it differed in this respect from the immediate anti-inflammatory effects seen on injection of an irritant.

The present investigation was therefore initiated to clarify some of these points. In the meantime, however, it had been shown (Winter, 1965) that the carrageenin foot oedema test was more sensitive than the formalin test, and we had made the additional observation that it was a highly satisfactory method for investigating the anti-inflammatory properties of exudate. This technique was therefore used in subsequent investigations, including the partial purification of the active constituents of the exudate.

Methods

Male Wistar rats were used throughout. The donor animals weighed between 150 and 250 g and were obtained from Animal Suppliers (London); the animals used in the foot oedema test weighed between 140 and 180 g and were bred at Guy's Hospital Medical School. The animals were maintained on Thompson cube diet and received water *ad libitum*. Adrenalectomized animals received a similar diet and 0.9% saline. Adrenalectomy was performed through a median dorsal incision as described previously (Robinson & Robson, 1964).

Preparation of exudate

Exudate was prepared by the method of Robinson & Robson (1966) with the exception that the exudate was routinely force dialysed at 5° C overnight (15–20 hr) before freeze-drying. Dried exudate was stored at −20° C until required with no apparent loss of activity.

Assay for anti-inflammatory activity

The carrageenin foot oedema test was used as described by Winter, Risley & Nuss (1962). 0.1 ml. of 1% carrageenin was injected into the rear right foot and the volume change measured plethysmographically in the unanaesthetized rat by a modification of the method described by Harris & Spencer (1962). In most of the experiments carrageenin kindly donated by Dr. Winter (Merck Institute) was used. This was originally supplied by Marine Colloids Inc., Rockland, Maine, U.S.A., and is labelled Viscarin Lot # RENJ 5043. In some of the later experiments a more potent Viscarin brand of carrageenin was used also supplied by Marine Colloids Inc. (donated by John Wyeth & Brother Ltd.).

Foot volumes were measured initially and 2.5, 5 and 24 hr after injecting the carrageenin, but since the reaction reaches a peak at about 5 hr all statistical analyses refer to measurements at this time. Material to be tested for anti-inflammatory activity was injected subcutaneously in the scruff of the neck immediately after the injection of the carrageenin.

Five animals were used in the control and each test group, and results are expressed as the mean increase in foot volume with time when compared with the initial uninjected volume of the foot. Animals treated with anti-inflammatory drugs therefore show a smaller increase in foot volume than those treated with saline. The significance of the difference between these groups was calculated by Student's *t* test as described by Bailey (1959).

Fractionation of inflammatory exudate

1. Force-dialysis (ultrafiltration)

Protein samples were subjected to force-dialysis in order to reduce the volume of water, and to remove small molecular weight material. Dialysis was carried out in Visking tubing (Hudes Merchandising Ltd.) at 4° C for a suitable period, so as to obtain a convenient volume of protein solution. By this method 100 ml. of protein solution could be reduced to between 2 and 5 ml. within 48 hr. The contents of the dialysis sac were then retrieved and the sac was carefully washed out with 1–2 ml. of distilled water to avoid loss of material. Sometimes slight precipitation of the protein occurred due to too complete a removal of the salt, but it could readily be redissolved by adding a small amount of sodium chloride.

2. Gel filtration

The pilot experiments were performed on a small column (60 × 2.5 cm) of Sephadex G-200 (Pharmacia), eluting with 0.9% sodium chloride at a flow rate of 20 ml./hr. This enabled up to 500 mg of exudate to be fractionated at one application.

Subsequently, larger columns (100 × 3.2 cm and 100 × 5 cm) of Sephadex G-150 were used, capable of separating 1–2 g of exudate. The columns were eluted with 0.5 M sodium chloride at flow rates of 40 ml./hr for the narrow column, and 65 ml./hr for the wider column. The eluant from the columns was collected in 5 or 10 ml. fractions and the protein content of each sample assessed approximately by its absorbance at 253 mμ, or at 300 mμ if the protein concentration was so large that it gave a full-scale reading.

3. Ion-exchange chromatography

Diethylaminoethyl (DEAE) cellulose. A column 2.5 cm in diameter and 45 cm long was used. This was eluted initially with 0.025 M phosphate buffer at pH 7.4, and subsequently with an ionic gradient produced by the addition of 0.5 M sodium chloride to a closed reservoir containing 500 ml. of phosphate buffer. Eluant fractions were collected and the protein distribution measured as described above.

Carboxymethyl (CM) cellulose. A similar technique to that used for DEAE cellulose was employed, except that the column was eluted with 0.02 M acetate buffer at pH 5 and an ionic gradient produced with 1.0 M sodium chloride.

4. Evaluation of the protein content of fractions

An approximate estimate of the protein content of the samples before and after chromatography was made by comparing their optical densities at 280 mμ. The amount of protein in 1 ml. of solution which had an optical density of 1.0 at this wavelength, with a light path of 1 cm, was defined as one optical density unit (O.D.U.) of material. This made it possible to assess the loss of material which occurred during column chromatography.

Steroid estimations

11-Hydroxycorticosteroid levels in exudate and serum were measured by a fluorimetric technique (McHardy-Young, Harris, Lessof & Lyne, 1967), a modification of the method originally described by Mattingly (1962). The estimations were performed by Miss C. Lyne of the Interdepartmental Laboratory, Guy's Hospital, using a technique currently used for the analysis of human blood levels of cortisol and cortisone.

Reliable estimations of levels between 10 and 100 μg of 11-hydroxycorticosteroids can be obtained, but figures below 5 μg% cannot be distinguished from background interference. Haemolysis of samples can produce a falsely high reading.

Results

The investigations fall chronologically into four categories:

(1) Establishment of the carrageenin test as a sensitive and reliable assay procedure for the anti-inflammatory factor(s).

(2) Pilot investigations to obtain preliminary information about the chemical nature of the active component.

(3) Partial purification of the active component by gel-filtration and ion exchange chromatography.

(4) Further investigations into the nature of the exudate.

Preliminary experiments on the carrageenin test

Time-response relationship

Figure 1 shows that 100 mg of undialysed freeze-dried exudate produces about 50% inhibition of foot oedema, the maximum effect occurring about 5 hr after the injection of carrageenin. This result was typical of many subsequent tests with dialysed and force dialysed exudate; serum from normal rats did not affect the inflammatory response.

Dose-response relationship

Because 100 mg/rat produced such a profound reduction, the effects of smaller amounts—25 mg/rat and 50 mg/rat—were also investigated. The result (Fig. 2) established a dose-response relationship for material which had been previously dialysed against a large volume of 0.09% saline.

Comparison of force dialysed exudate and serum

Three samples of material were tested for activity after force-dialysis. These were serum from normal intact animals, serum from animals bearing polyester sponges (referred to in future as " inflamed " serum) and exudate obtained from polyester sponge implants. Exudate produced a 65% inhibition ($P<0.001$) of the inflammatory reaction at 5 hr, whereas the effect produced by normal serum (17%) was not significantly different from that of the control animals ($P>0.05$). The effect produced by inflamed serum, however, was quite marked (-45%, $P<0.005$). This is in agreement with observations made using the cotton pellet test (Robinson & Robson, 1964).

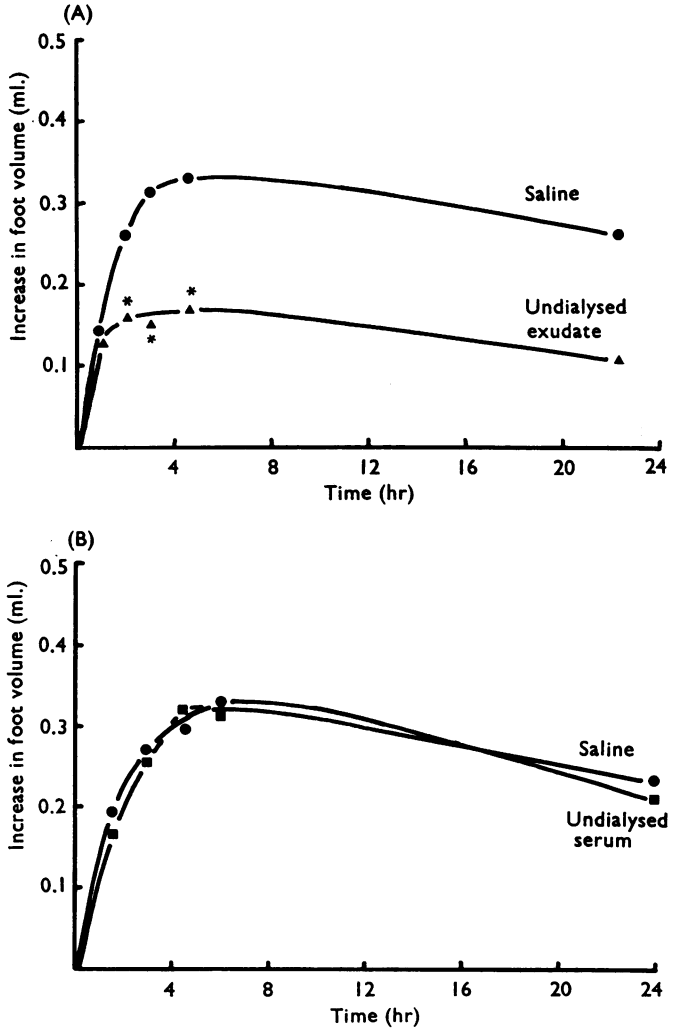

FIG. 1. Effect of 100 mg/rat (s.c.) of (A) undialysed inflammatory exudate (▲), and (B) 100 mg/rat (s.c.) of undialysed serum from normal rats (■) on the carrageenin-induced rat foot oedema. In both experiments comparison is made with saline-treated control animals (●), and points marked * indicate statistically significant difference from the control ($P<0.05$). Each point is the mean of five figures.

Pilot chemical investigations

Experiments both now and in the past (Robinson & Robson, 1966) have established that the anti-inflammatory factor does not appear to be dialysable. If the factor is associated with a protein, activity of the exudate should be progressively destroyed by exposure to increasing temperature, should be precipitable with ammonium sulphate, should be destroyed by the proteolytic enzyme pronase and should separate as a protein chromatographically.

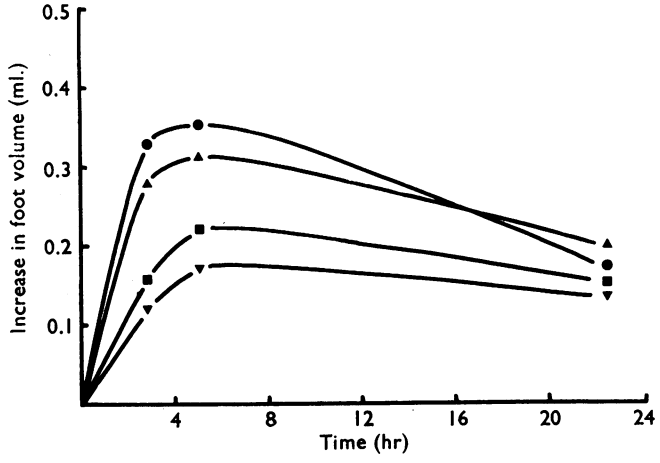

FIG. 2. Effect of different doses of inflammatory exudate on the increase in foot volume induced by injection of carrageenin. ●, Saline control; ▲, exudate 25 mg/rat; ■, exudate 50 mg/rat; ▼, exudate 100 mg/rat. At 3 hr and 5 hr both 50 mg/rat and 100 mg/rat produced significant inhibition of the inflammatory response ($P<0.005$). Each point is the mean of five figures.

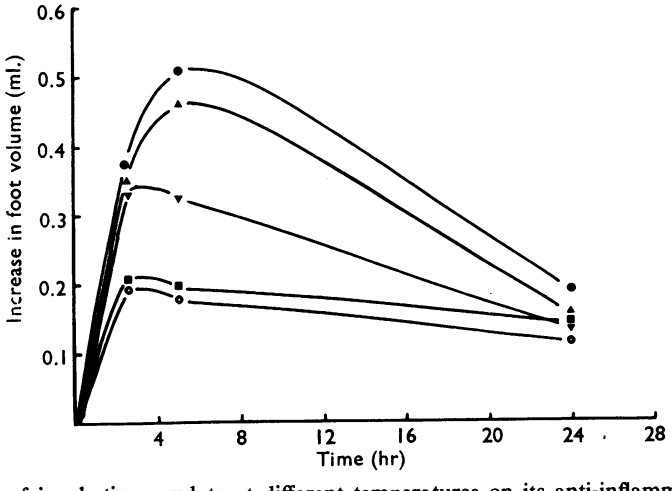

FIG. 3. Effect of incubating exudate at different temperatures on its anti-inflammatory activity (as shown by the inhibition of carrageenin-induced increases in foot volume). Incubation at 50° C (○) does not significantly alter the activity of exudate compared with incubation at 37° C (■) (65% inhibition $P<0.001$ compared with 63% inhibition $P<0.005$ at 5 hr). At 70° C (▼) activity is considerably reduced (36% inhibition at 5 hr, $P>0.5$) and at 100° C (▲) activity is destroyed (8% inhibition at 5 hr). ●, saline control.

Effect of temperature

Four samples of 500 mg of dialysed exudate were heated under reflux at either 37° C, 50° C, 70° C or 100° C for 5 min, and then cooled in a freezing mixture until used. The samples were then tested for anti-inflammatory activity by the carrageenin test in the usual way. The result is depicted in Fig. 3. Heating at 37° C and 50° C had no apparent effect on activity of the exudate, 100 mg/rat producing 63% ($P<0.005$) and 65% ($P<0.001$) anti-inflammatory effects respectively at 5 hr. Heating to 70° C, however, reduced activity to 36% ($P>0.05$), and heating to 100° C reduced it to 8% ($P>0.5$).

Ammonium sulphate precipitation

In an attempt to separate the active component from other inactive proteins, the exudate was subjected to 50% ammonium sulphate precipitation at a temperature below 10° C. Approximately half of the total protein present was precipitated by this technique, so that 50 ml. of exudate gave 900 mg of precipitate, 30 ml. of serum gave 1,500 mg, and 37 ml. of "inflamed" serum gave 1,400 mg. When tested for anti-inflammatory activity at a dose of 100 mg/rat the precipitated exudate produced a 37% inhibition ($P<0.005$) and the precipitated "inflamed" serum a 38% inhibition ($P<0.005$), but normal serum did not produce a significant effect (11%, $P>0.2$). These effects are less than those obtained with 100 mg of each of the crude materials, which would imply that 50% precipitation does not remove all the active material and thus would not be suitable as a purification procedure. The technique nevertheless provides some evidence that the activity is associated with proteins.

Enzymic digestion (pronase)

In order to determine whether the activity of the exudate is associated with a protein, it was tested after incubation with the proteolytic enzyme pronase. Incubation was carried out for 24 hr in phosphate buffer at pH 7.4. The results which are given in Fig. 4 show that the anti-inflammatory activity of the exudate is considerably reduced after incubation with pronase.

Sephadex G-200

The results indicated that the active component may be a protein of high molecular weight, so an attempt was made to purify it by passing it down a column of Sephadex G-200.

Figure 5 shows that there are four distinct peaks in the absorbance at 300 mμ following the elution of dialysed exudate (500 mg) from the column. The samples corresponding to these were pooled as indicated, dialysed against distilled water and freeze-dried. Each pool of material was divided into five equal doses because the amount of exudate separated (500 mg) represents the equivalent of one assay using 100 mg/rat.

Both Pool 2 and Pool 3 produced significant anti-inflammatory effects, indicating that the active component was distributed somewhere in this region. Repeated experiments with G-200 and G-150 (a gel which according to the manufacturer gives a more distinct separation between the albumins and globulins) have confirmed this result.

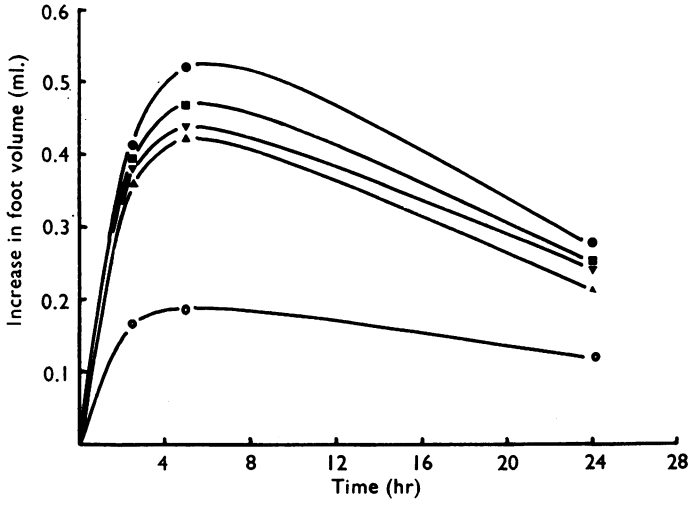

FIG. 4. Comparison of the activity of exudate in buffer solution and exudate which had been incubated with pronase in buffer solution in inhibiting the increase in foot volume induced by carrageenin injection. Control results were obtained for buffer solution, saline and pronase in buffer solution. At 5 hours exudate in buffer solution (O) produced a large anti-inflammatory effect (65% inhibition compared with saline-treated controls (●) $P \ll 0.001$); and 61% compared with phosphate buffer-treated controls (■) $P \ll 0.001$). In the presence of pronase the activity of exudate was markedly reduced (▲) producing only 18% anti-inflammatory effect ($P<0.02$) when compared with the saline control and 9% ($P>0.2$) when compared with the buffer control: the effect of pronase alone in buffer solution (▼) was not significantly different from either the saline control (−10%, $P>0.1$) or the buffer control (−7%, $P>0.2$).

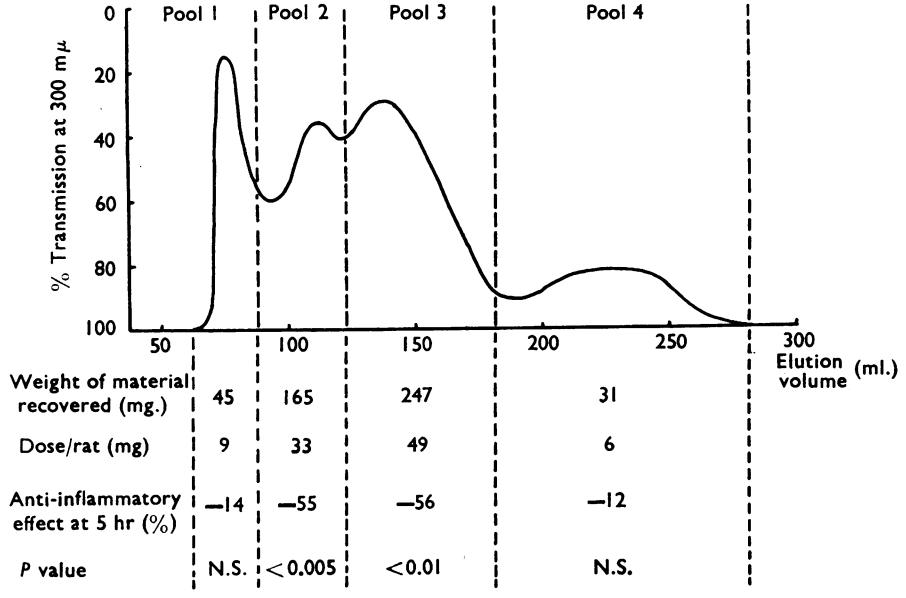

FIG. 5. Protein distribution (absorbance at 300 mμ) following Sephadex G-200 separation. The dotted lines indicate the pools into which the separated material was divided, and below the graph are the anti-inflammatory effects produced by each sample (measured as inhibition of carrageenin-induced foot oedema at 5 hr compared with saline-treated control animals).

Purification of the exudate

Sephadex G-150

Following the partial separation of the active component using a small column of Sephadex G-200, further separations were performed using larger columns of G-150, which were capable of separating 2 g of material.

Table 1 shows the anti-inflammatory activity of two different samples of exudate separated using G-150; the poolings are similar to those described for the G-200 column. A typical division of the separated protein into pools is shown in Fig. 6A. Pool 2 in both experiments showed some activity, but Pool 3 appeared to contain more, and was therefore used for further purification. The finding of more activity in Pool 3 may simply have been related to the greater quantity of Pool 3 material injected into each rat (50–52 mg compared with 20–24 mg of Pool 2 material), and undoubtedly some of the active material was lost by not including all or part of Pool 2 for further separation.

The peptides and other small molecular weight material (referred to in the G-200 separation as Pool 4) previously shown to be inactive were not tested for activity.

Normal serum showed a very similar protein distribution to that of the exudate, but similar poolings of the material showed insignificant anti-inflammatory activity (Table 1).

Sephadex G-150 therefore seems to provide a good first step in removing inactive components; 51 mg/rat (mean figure) of Pool 3 producing between 54 and 61% inhibition of the carrageenin oedema. This degree of inhibition compares favourably with the original exudate (compare Figs. 1, 2 and 3), yet was obtained using only half the weight of materials.

DEAE ion-exchange chromatography

By use of DEAE ion-exchange chromatography it is possible to separate proteins according to their basic and acidic properties, which may in turn provide a useful means of distinguishing different proteins.

Figure 6B shows a characteristic protein distribution following application of 500 mg of Pool 3 material from a G-150 Sephadex separation. The hatched area in Fig. 6A shows the actual material used. The first peak (DEAE Pool 1) constitutes that material which is eluted by 0.025 M phosphate buffer at pH 7.4 (basic

TABLE 1. *Distribution of anti-inflammatory activity following separation of exudates and serum by Sephadex G-150*

		Inhibition of response at 5 hr (%)	*P* value	Dose (mg/rat)
Pool 1	Exudate (a)	2	N.S.	13
	Exudate (b)	6	>0·6	14
	Serum	12	>0·1	18
Pool 2	Exudate (a)	41	<0·005	24
	Exudate (b)	38	<0·005	20
	Serum	9	>0·2	24
Pool 3	Exudate (a)	61	<0·001	52
	Exudate (b)	54	⩽0·001	50
	Serum	9	>0·2	50

The figures indicate the percentage inhibition of the carrageenin-induced foot oedema at 5 hr for two samples of exudate and one of serum when compared with saline-treated control animals. Pools 1, 2 and 3 refer to the various protein peaks shown in Fig. 6A.

proteins). The subsequent protein distribution was obtained by eluting the column with a progressively increasing ionic gradient. Using poolings of material as indicated it was possible to investigate the distribution of anti-inflammatory activity. A loss of protein approaching 20% occurred during separation and this was taken into account when calculating the doses of separated material required for a single assay. The exact loss was calculated knowing the number of optical density units

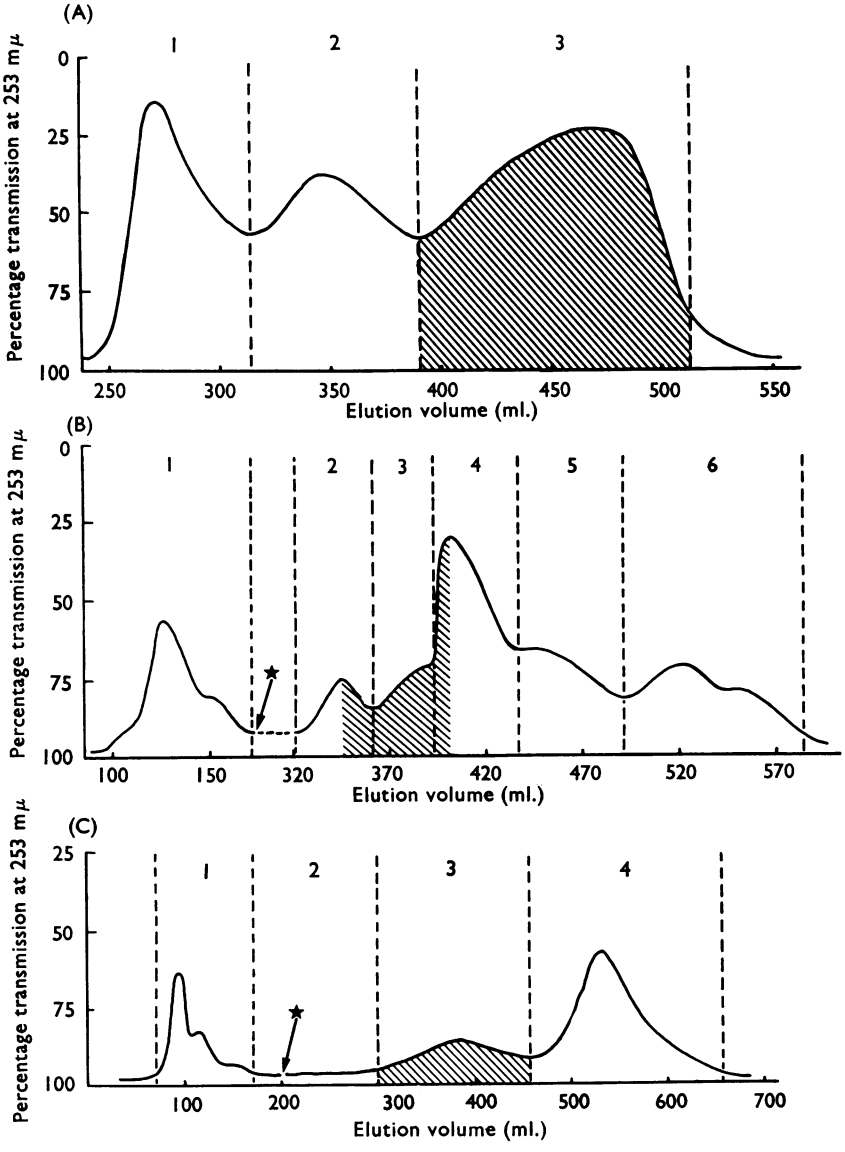

FIG. 6. Comparison of the protein distribution of exudate following:
(A) Sephadex G-150 separation of force dialysed exudate;
(B) DEAE cellulose separation of Pool 3 from Sephadex G-150;
(C) CM cellulose separation of Pool 2, 3, 4 (hatched area) from DEAE cellulose. The dotted lines define the pools of material which were tested for anti-inflammatory activity. The hatched areas indicate where the major portion of the anti-inflammatory activity was found, and hence the material which was subjected to further separations.
The asterisk indicates the point at which the ionic gradient was applied.

of the material applied to the column and that of the eluted samples. Thus the total amount of material assayed for anti-inflammatory activity was equivalent to that used in previous assays of whole exudate—that is 500 mg (405 O.D.U.). In Table 2 the relative anti-inflammatory effects of similar poolings from three independent DEAE separations are compared. In all three cases activity was distributed around Pools 2, 3 and 4. Pool 6 was not tested because this did not always separate as a distinct band.

One way of expressing the purification achieved by this separation is in terms of specific activity. This is defined as the percentage inhibition of oedema at 5 hr divided by the number of optical density units of material administered to each rat.

TABLE 2. *Distribution of anti-inflammatory activity following separation of Sephadex G-150 Pool 3 material on a DEAE cellulose column*

		Inhibition of response at 5 hr (%)	*P* value	Dose/rat mg.	Dose/rat O.D.U.	Specific activity*
Pool 1	a	35	<0·001	4	10	3·5
	b	29	>0·1	8	9	3·2
	c	20	>0·1	6	7	2·9
Pool 2	a	44	<0·001	4	4	11·0
	b	40	<0·05	5	3	13·3
	c	21	>0·1	2	3	7·0
Pool 3	a	68	⩽0·001	5	5	13·6
	b	43	<0·05	8	3·5	12·3
	c	61	<0·001	5	5	12·2
Pool 4	a	39	<0·005	26	13	3·0
	b	45	<0·025	25	18	2·5
	c	41	<0·005	21	14	2·9
Pool 5	a	24	<0·02	9	8	3·0
	b	26	>0·1	10	7	3·7
	c	28	<0·05	7	5	5·6

The figures show the percentage inhibition of the carrageenin-induced foot oedema at 5 hr (and the statistical significance, *P* value) for three independent separations a, b and c, compared throughout with saline treated control animals. Pools 1–5 refer to the various protein peaks following DEAE chromatography (see Fig. 6B). The quantity of material administered to each rat is shown both as the number of mg and the number of optical density units (O.D.U.) (see **Methods**).

* Specific activity is defined as the percentage inhibition of oedema at 5 hr divided by the number of optical density units of material administered to each rat. The figures thus give an indication of the activity of the fractions when related solely to protein content.

TABLE 3. *Comparison of the activities of exudate at each step of the purification procedure*

Sample	Dose/rat mg.	Dose/rat O.D.U.	Anti-inflammatory effect (%)	Specific activity*	
Dialysed exudate	100	81	61	0·75	Average=0·78
	100	81	65	0·81	
Sephadex G-150 Pool 3	52	38	61	1·61	Average=1·60
	50	34	54	1·59	
DEAE Pool 3	5	5	68	13·6	Average=12·7
	8	3·5	43	12·3	
	5	5	61	12·2	
CM Pool 3	2	2·4	43	17·9	Average=18·8
	3	3·0	56	18·7	
	2·6	2·3	44	19·1	

The anti-inflammatory activity (as given by 5 hr inhibition of the carrageenin oedema) of the most active pools after each stage of the purification procedure, together with the quantity of material (expressed as number of mg and number of optical density units O.D.U.).

* Specific activity is defined as the percentage inhibition of oedema at 5 hr divided by the number of optical density units of material administered to each rat. These figures show the good correlation which exists between the activities of different samples at each stage of the purification procedure, and the progressive concentration of activity which occurs with each step.

This provides a good indication of the activity of the fractionated exudate in relation solely to its protein content.

CM cellulose

Owing to the spread of activity obtained following DEAE chromatography, a pool of material was used for further separative technique which included part of Pool 2, all of Pool 3 and part of Pool 4 (the hatched area in Fig. 6B).

This material was applied to a column of CM cellulose and a typical separation is shown in Fig. 6C, together with the relevant poolings of material subsequently used for anti-inflammatory testing. Pool 1 represents material not retained on the column at the pH used, Pool 2 the small amount of protein removed initially on applying the gradient, and Pools 3 and 4 the protein removed with increasing ionic gradient. Three separations were performed by this technique and the various pools tested for anti-inflammatory activity.

In all three experiments activity was greatest in Pool 3 (Table 3). In the first, a single dose of 2 mg/rat (2.4 O.D.U./rat) produced a 43% inhibition of the carrageenin-induced oedema, in the second, 3 mg/rat (3 O.D.U./rat) produced a 56% inhibition and in the third, 2.6 mg/rat (2.3 O.D.U./rat) produced a 44% inhibition. In all three, these values were highly significant ($P<0.001$). Although the percentage anti-inflammatory activity varies from 43 to 56% inhibition, the specific activity (% effect/O.D.U.) remains remarkably constant (see Table 3).

Steroid estimations

A possible explanation of the effects observed with exudate is the presence of anti-inflammatory steroids bound to protein. The activity of the exudate could not be due to the presence of anti-inflammatory steroids, however, because the total 11-hydroxycorticosteroids present in two samples of exudate and serum from donor animals were less than 3–4 μg%, compared with 50–69 μg% present in serum from normal animals.

Discussion

Preliminary experiments

The cotton pellet test which has been used in the past to investigate the anti-inflammatory properties of inflammatory exudate (Robinson & Robson, 1964, 1966, 1967) is uneconomic in that it occupies 4 days and requires 2 g of freeze-dried exudate for a single test, and then gives an inhibition of only 30–40%. Also used previously was the formalin-induced foot oedema, but this was shown to be totally insensitive to exudate. The carrageenin test was therefore tried because it had been shown by other workers (Winter, Risley & Nuss, 1962) to be a sensitive and reliable assay procedure for most anti-inflammatory drugs and because it gives a significant result within 5 hr. Furthermore, of a number of screening tests, this test is the least influenced by unspecific factors such as ganglion blockade, diuresis, hypotension and vasodilatation (Garattini, Jori, Bernardi, Carrara, Paglialunga & Segre, 1965). In our hands the test provided an excellent technique for assaying the anti-inflammatory activity of inflammatory exudate, a single subcutaneous injection of 100 mg/rat producing a 50–65% inhibition of the oedema (Fig. 1A) and as little as 50 mg/rat producing a statistically significant effect (Fig. 2). Despite this increased sensitivity, serum from normal non-adrenalectomized rats still produced an

insignificant anti-inflammatory effect (Fig. 1B and Table 1); yet serum from adrenalectomized animals bearing polyester sponges was shown to possess significant anti-inflammatory properties (see Table 1). Correlating this with the levels of 11-OH corticosteroids found in samples of serum from intact animals (50–69 μg%) and from adrenalectomized animals (3–4 μg%), there is thus good evidence that the anti-inflammatory activity measured in our test is not of steroidal origin. This confirms the observations of Robinson & Robson (1964) and of DiPasquale and his co-workers (1963).

Pilot chemical investigations

Robinson & Robson (1966), using the cotton pellet test, reported that the anti-inflammatory activity of exudate was unaffected by dialysis against 0.9% saline or a large volume of tap water. In the present work we have shown, using force-dialysis, that activity as indicated by the carrageenin foot test is still retained within the dialysis sac.

Circumstantial evidence of the protein nature of the active anti-inflammatory material was obtained from the observations that activity was progressively destroyed by subjecting to increasing temperature, that activity was destroyed by incubation with the proteolytic enzyme pronase, and that the active material could be precipitated with ammonium sulphate.

These three pieces of information, although each alone not conclusive, when taken together provide good evidence that the anti-inflammatory activity found in exudate is associated with a protein. The position of the anti-inflammatory factor following Sephadex G-200 separation supports such a hypothesis and enables characterization still further, placing it in molecular size between the γ-globulins of which Pool 2 (Fig. 5) is predominantly composed, and the albumins which account for most of Pool 3. Pool 1 represents the macroglobulins totally excluded by the column and Pool 4 the peptide and other small molecular weight material.

Purification

The ability of the purification process to concentrate the active anti-inflammatory component of exudate is summarized in Table 3. It is now possible to produce similar activity with as little as 3 mg of purified material as was obtained originally with 100 mg of crude exudate—that is an increase in activity of more than 30-fold. This exaggerates slightly the concentrating ability of the procedure, and probably the best indication is provided by the specific activity figures calculated at each stage. These figures relate the percentage inhibition of oedema at 5 hr to the number of optical density units (protein content) of exudate administered to each rat. Specific activity thus increases from 0.78 for crude exudate to 18.8 for the purest material obtained, a purification factor of approximately 24. Although more concentrated, this material is by no means pure; and following starch gel electrophoresis, for example, three bands are produced, while after acrylamide gel electrophoresis at least ten bands are apparent. Furthermore we do not know whether the anti-inflammatory factor represents only a small fraction or nearly all of the remaining 2–3 mg of material.

Qualitatively the purification procedure has added little, except confirmation of the information already presented.

Source of anti-inflammatory factor

The origin of the anti-inflammatory factor is unknown but the presence of anti-inflammatory activity in serum of animals in which there is an inflammatory reaction suggests two main alternatives. First, the anti-inflammatory factor may be released at the inflammatory site either by damaged cells or by invading polymorphs and mononuclear cells, and the activity found in the blood stream may merely represent the overflow of material from this site, which has found its way there via the blood and lymphatic system. Second, it may be travelling from its site of synthesis (perhaps the liver) to the inflammatory site; the accumulation of the anti-inflammatory fact at the inflammatory site being most probably due to the specific exudation which follows the increase in permeability of venular and capillary membranes. It is known, for example, that radio-iodinated albumin and glycoprotein are specifically sequestered at an inflammatory site (Houck & Jacob, 1966).

The additional evidence of its protein nature supports the hypothesis of an hepatic origin of the anti-inflammatory factor, because the majority of serum proteins are synthesized in the liver. Furthermore, during inflammatory conditions a characteristic rise in certain serum proteins (for example, fibrinogen, α- and β-globulins, glycoproteins) has been reported (Darcy, 1964; Glenn, Bowman & Koslowske, 1968). Sarcione & Bogden (1966) have shown that after implantation of polyvinyl sponges (or other inflammatory stimuli) there is an increased synthesis by the liver of an α_2-globulin. This substance is absent from normal serum, but shows a specific rise following acute inflammatory reactions, the increase being proportional to the extent of the inflammatory lesion.

Mode of action

So far we have little evidence regarding the mode of action of the anti-inflammatory factor and we can only say how it is not producing its effect. Previously, because of its inactivity by the formalin test it was concluded that the anti-inflammatory factor produced its effect exclusively on the cellular as opposed to the oedematous portion of the reaction (Robinson & Robson, 1966). However, the anti-inflammatory factor is highly active against carrageenin oedema, a fact at variance with this inference. Formalin-oedema is notoriously insensitive to the newer anti-inflammatory compounds, indomethacin and flufenamic acid, neither substance producing inhibition of the reaction (Winter, 1965); yet both substances, like the anti-inflammatory factor, inhibit carrageenin oedema. It has been suggested that in carrageenin oedema the release of kinin is more important that that of histamine and 5-hydroxytryptamine (Van Arman, Begany, Miller & Pless, 1965) which are, on the other hand, relatively more important in formalin oedema. The mode of action of all three substances, indomethacin, flufenamic acid, and the anti-inflammatory factor we have studied, could depend on their interfering with the release, synthesis or actions of kinins.

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