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FURTHER STUDIES ON THE ANTI-INFLAMMATORY FACTOR FOUND AT A SITE OF INFLAMMATION

BY

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In a previous paper (Robinson & Robson, 1964) it was reported that implantation of polyester sponges in adrenalectomized rats bearing cotton-wool pellets caused less tissue to be deposited on the pellets compared with results with animals in which only pellets had been implanted. A similar anti-inflammatory effect was also found if fluid obtained from such sponges was injected into other rats bearing cotton-wool pellets. This observation agrees with that of Di Pasquale, Girerd, Beach & Steinetz (1963). It was suggested that the substance(s) contained in the fluid might play an important role in the defence reactions of the body in limiting and ultimately terminating the inflammatory reaction.

Investigations have since been performed which fall into two classes. Firstly those which provide a technical improvement in the production of the exudate, or give more information about the anti-inflammatory effects observed in the cotton pellet test; and secondly those which extend observations on the biological activity of the exudate. In the first group freeze-drying was introduced to simplify the storage of the exudate without loss of activity. In addition the effects of duration of implantation of the sponges and of dialysis on the activity of the exudate were investigated. In the second group additional biological tests were performed in order to obtain more precise information about the nature of the anti-inflammatory activity. They include the effect of exudate on rat foot oedema, on capillary permeability and on the rate of wound healing.

METHODS

Male Wistar rats obtained from Animal Suppliers (London) were used throughout. In the cotton pellet test and the rat foot oedema test, animals weighing 150 to 250 g were used, but in the wound healing experiments rats weighing not less than 200 g were used. The animals were maintained on cube diet 41B and received water *ad libitum*. Adrenalectomized animals received a similar diet and 0.9% saline which had been coloured with a few drops of methylene blue.

Injections were given either subcutaneously or intraperitoneally, except in the rat foot oedema test where the intramuscular route was also used.

Adrenalectomy, when required, was performed during ether anaesthesia through a median dorsal skin incision; a slit was made over the anterior pole of each kidney, and the adrenal glands were carefully removed by means of curved forceps. The slits in the body wall were sutured with cotton, and the skin flaps were closed with 12 mm suture clips. When implantation of pellets and/or sponges was required this was always done immediately after adrenalectomy.

Preparation of sponge exudate

Implantation of sponges

Polyester sponge obtained from the Defiance Rubber Co. (Croydon) as 0.25 in. sheet was cut to the required size by means of a guillotine. In the case of donor animals this was always 20 × 50 mm. Before implantation the sponges were sterilized by heating to 150° C for 2 hr. They were then implanted under the skin of the back through a median dorsal skin incision and the skin flaps were closed with metal suture clips. Adrenalectomy was always performed immediately before implantation.

Collection of sponge exudate

After the period of implantation, the animals were killed, the sponges together with any adhering tissue were dissected out, and the fluid was squeezed out manually. The fluid was centrifuged at about 2,000 rev/min for 30 to 40 min in order to remove blood and tissue cells. Occasionally a clot of fibrin appeared in the fluid and this was removed. In the majority of the experiments the fluid was then freeze-dried. This was done to provide a method of storage without loss of activity. In earlier experiments the exudate had been injected into the recipients within a few hours of collection from the donors.

Freeze-drying of sponge exudate

The apparatus was of a type commonly used in the technique of freeze-drying. It consisted essentially of a glass tube of about 700 ml. capacity which was open to the atmosphere at the top. It was filled with about 300 ml. of acetone, and sufficient "Cardice" (solid carbon dioxide) to reach two-thirds of the way up the flask. This tube was jacketed by another glass tube, the base of which terminated in a ground glass fitting. This enabled one or, by use of a manifold, three round-bottomed flasks to be attached. An outlet at the top of the jacket connected the flasks through to a "Speedivac" two-stage rotary pump which provided a vacuum of down to 0.002 mm Hg. In order to speed the process of drying, the exudate was frozen to the walls of two or three round-bottomed flasks; the thinner the layer produced, the more rapid the drying process. In general, complete dryness was obtained within 2 to 3 hr. Phosphorus pentoxide incorporated into the system assisted in this process. The dry powdered exudate was placed in a screw-top bottle, weighed and stored in the deep freeze (−10° C) until required.

Dialysis of sponge exudate

Dialysis provided a technique whereby the exudate could be divided into two fractions, the dialysate consisting of molecules with a molecular weight of less than 15,000 and the residue of molecules larger than this. It was not used as a routine procedure but merely to investigate the nature of active principle of the exudate.

Two dialysis methods were used, firstly against a large volume of 0.9% saline, and secondly against running tap water. In both cases the exudate was placed in 1 in. diameter Visking collodion membrane, and dialysis was performed in a refrigerator at 4° C for 24 hr. After this period the residue was freeze-dried, and in the case of dialysis against 0.9% saline the dialysate was also freeze-dried. These samples were subsequently tested for anti-inflammatory activity by the cotton pellet test.

Tests for biological activity

Cotton pellet test

Anti-inflammatory activity was measured by a modification of the method of Meier, Schuler & Desaulles (1950), the details of which were given in the previous publication (Robinson & Robson, 1964), which also gives details of the statistical treatment of results.

Rat foot oedema

Oedema was induced in the rat foot by injection of 0.1 ml. of 3% formol-saline into the plantar aponeurosis. Volume changes were then measured by plethysmographic recording, using the method described by Buttle, D'Arcy, Howard & Kellett (1957) as modified by Harris & Spencer (1962). Three independent measurements of foot volume were made on each occasion and the mean was

recorded. Measurements were made at the time of injection of the formol-saline and at various subsequent time intervals (for example, 2, 4, 6, 24 . . . to 96 hr). Drugs were administered either at the time of injection of the formol-saline or up to 20 hr previously. Groups of not less than five animals were used and not more than three groups were tested at any one time.

Results are expressed as the increase in foot volume with time 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 difference between the treated and control foot volumes thus provides an indication of drug action.

Test for increased capillary permeability

The method used was essentially that described by Spector (1951) in which the leakage into the skin of an intravenously administered protein-bound dye was assessed visually after intradermal injection of the test substance. 1 ml. of 1% Trypan blue was injected slowly into the tail vein of a rat. After 10 min the animal was anaesthetized with ether and the hair was removed from the abdomen by means of electric clippers. Intradermal injections of both histamine acid phosphate (25 to 100 μg) and sponge exudate (5 to 30 mg) were then made into the skin of the abdomen in a volume not greater than 0.1 ml. and the animal was allowed to recover. After 20 min the animal was killed with ether, and the skin was removed from the abdomen. The skin was then pinned fur-side down on a cork board and the injection sites were examined. Substances which cause an increase in capillary permeability produce local leakage of the dye or "blueing" around the injection site. Histamine serves as a control since it is known to produce this effect.

Measurement of the rate of wound healing

Description of apparatus. The apparatus used was a refinement of that described by Charney, Williamson & Benhart (1947). It consisted essentially of a fixed Perspex block and a similar movable one, the wound being clamped longitudinally between them so that the wound edge was at right angles to the direction of movement of the block (Fig. 1). Suitable guides ensured movement of the block in the correct direction. These have been omitted in Fig. 1 for clarity. Tension was applied to the wound by addition of mercury at a constant rate to a polyethylene container which was attached by a thread over a small pulley to the movable block. The mercury was added at a rate of about 3 ml./min (approximately 40 g) from two 50 ml. all-glass, Luer-fitting syringes connected in parallel to a Palmer constant injection apparatus. When sufficient mercury had

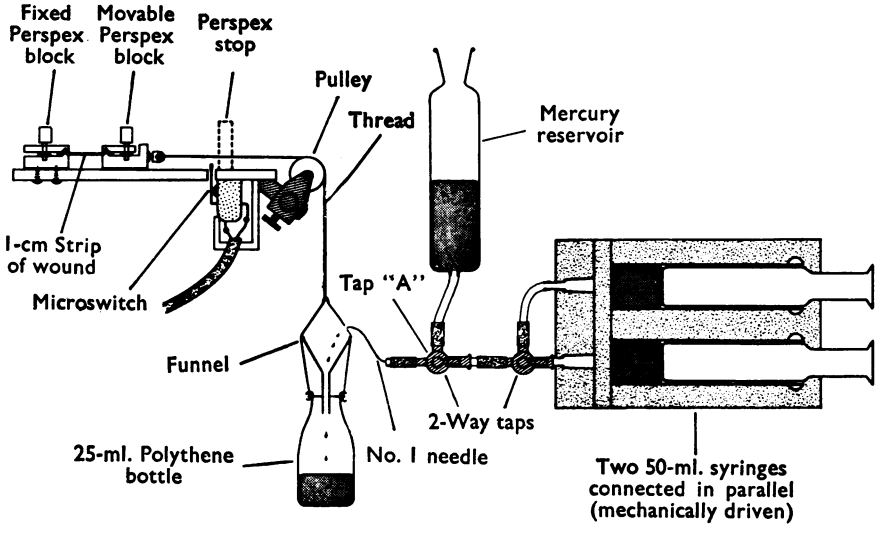

Fig. 1. Apparatus for the *in vitro* measurement of the tensile strength of healing skin wounds.

been added to the container to break the wound, the movable block slid under the weight of the mercury towards a Perspex stop. This prevented the container from falling to the bench, and at the same time caused a microswitch to be depressed, switching off the constant injection apparatus and thus stopping the flow of mercury. A small overflow of about 0.5 g occurred each time, but this was negligible compared with the total weight of mercury added (100 to 200 g); it was due to a syphoning action of the mercury in the out-flow needle from the syringe.

The breaking strain of the wound was assessed by unscrewing the polyethylene container and weighing it to the nearest gram. The weight of the movable block, the thread, the funnel and the screw cap of the container were ignored since they were constant, and in any case small compared with the total weight of mercury.

The mercury was poured back into the reservoir and the polyethylene bottle reconnected to the apparatus. There was sufficient mercury in the syringes to perform seven to 10 estimations of wound strength, after which the syringes were recharged by adjusting tap "A" to allow mercury to run from the reservoir back into the syringes. When the syringes were full the tap was returned to its original position so that the apparatus was ready for further estimations. The syringe barrels were lubricated at intervals with liquid silicone.

Production of wounds. The animals were anaesthetized with ether, and the hair was removed from their backs by means of electric clippers. Three independent incisions each 1 to 2 cm long were then made with a sharp scalpel along the length of the back. A single 12 mm suture clip was used to close each wound, the area around the wound was swabbed to remove any blood, and the wound was sprayed with "Nobecutane" plastic dressing. The use of this substance not only prevented contamination of the wound, but also deterred other animals within the same cage from attempting to remove the suture clips. As a result more consistent breaking strain measurements were obtained.

Adrenalectomy if required was performed through the central incision. Once the experimental procedure had been established it was found that the suture clips could be safely removed after only 1 day. This was done during ether anaesthesia. Splitting of wounds using this technique occurred very rarely, and then only in those animals which had been treated with high doses of drugs which inhibited the rate of wound healing.

Preparation of wound strips for measurement of tensile strength. A technique was used in which drugs were given on the day of wounding and on the four subsequent days. On the sixth day the animals were killed with ether and skin was removed from the back in a single piece, taking care not to place any strain on the wound edges. The sheet of skin was pinned fur-side down on a cork board, and with the aid of a wooden template a 1 cm wide strip of skin was cut from each of the three wounds. The tensile strength of each of these strips was measured by the method already described. The strips waiting to be measured were kept moist by placing them on a saline-soaked swab. The effects of drugs were recorded as the percentage reduction in the tensile strength of 1 cm wide strips of wound from treated animals compared with those from saline-treated or untreated controls.

RESULTS

The results are described under the two headings mentioned in the introduction, namely, experiments providing additional information using the cotton pellet test, and other tests used to detect biological activity.

Experiments employing the cotton pellet test

Freeze-drying. Exudate was obtained from sponges which had been implanted in twenty adrenalectomized donor animals for 4 days; 80 ml. of fluid was obtained, which after freeze-drying gave 3.17 g of dry powder (equivalent to 4.0 g/100 ml.).

Two groups of five adrenalectomized rats of similar mean body weight were used to test the activity of the fluid. Cotton pellets were implanted in each group, and daily

TABLE 1

THE EFFECT OF FREEZE-DRYING ON THE ANTI-INFLAMMATORY ACTIVITY OF SPONGE EXUDATE

Results (means and standard errors) and body weights represent the averages for ten animals. The mean body weight of animals in each group at the outset of the experiment was 222 g. Controls received daily injections of 2 ml. of 0·9% saline intraperitoneally, and the treated animals received intraperitoneally 100 mg of freeze-dried sponge exudate reconstituted in 2 ml. of sterile distilled water. Both the donor and recipient animals were adrenalectomized

Treatment	Final animal weight (g)	Increase in dry weight of pellet (mg)	Effect (%)	P
Saline	215	8·48±0·58		
Sponge exudate (freeze-dried)	213	6·15±0·38	−27·5	<0·001

injections of reconstituted sponge exudate were given to one group, whilst the other received 2 ml./rat/day of 0.9% saline. The freeze-dried exudate was reconstituted when required for reinjection so that each animal received daily injections of 100 mg dissolved in 2 ml. of sterile distilled water. This is approximately equivalent to its original concentration.

Table 1 shows that marked anti-inflammatory activity was still present in the sponge exudate after freeze-drying, 100 mg daily producing a 27.5% reduction of tissue deposition ($P<0.001$). In this experiment the freeze-dried exudate was only stored for 1 week but in some subsequent experiments it was stored for 30 to 40 days still without apparent loss of activity.

Duration of implantation of sponges. The presence of apparently unaltered anti-inflammatory activity in freeze-dried fluid enabled other experiments to be performed. Of particular interest was the effect of length of time of implantation of the sponges on the anti-inflammatory activity of the resulting exudate. Forty donor rats were adrenalectomized and had sponges implanted. After 1, 2, 4 and 8 days ten animals were killed, and the sponge exudate was collected, centrifuged and freeze-dried. The dry powder on each occasion was stored at −10° C for a period ranging from 24 to 31 days. Table 2 shows the amount of dry powder obtained from the exudate of ten animals on each of the four days. Anti-inflammatory activity of the exudate was then investigated by the cotton pellet test. On each of the 4 days of the test, one-quarter of the total freeze-dried exudate obtained from each of the donor groups of animals was reconstituted in 10 ml.

TABLE 2

THE EFFECT OF DURATION OF IMPLANTATION OF POLYESTER SPONGES ON THE DRY WEIGHT AND ACTIVITY OF SPONGE EXUDATE

Ten adrenalectomized rats were used in each group of donor animals, and anti-inflammatory activity was assessed by the cotton pellet method using groups of five adrenalectomized recipients, The anti-inflammatory activity of fluid obtained on Day 8 is not significantly less than that obtained on Day 4 ($0·1>P>0·05$) nor is the activity of fluid obtained on Day 4 signif cantly different from that obtained on Day 2 ($0·4>P>0·3$)

Duration of implantation (days)	Weight of dry powder (g)	Concentration (g/100ml.)	Anti-inflammatory effect (%)	P
1	1·86	4·64	−16·2	>0·1
2	1·97	4·92	−38·8	<0·001
4	1·94	4·85	−43·9	<0·001
8	1·91	4·76	−31·2	<0·005

TABLE 3

THE EFFECT OF DIALYSIS OF SPONGE EXUDATE ON ITS ANTI-INFLAMMATORY ACTIVITY

Results represent the means of five animals together with the standard errors, The mean body weight of the animals in each group at the outset of the experiment was 227 g in (1) and 164 g in (2). Both donor and recipient animals were adrenalectomized

	Treatment	Final animal weight (g)	Increase in dry weight of pellet (mg)	Effect (%)	P
1	Saline	219	9·96±0·94		
	Residue	200	7·71±0·51	−22·6	<0·05
	Dialysate	221	9·69±0·68	− 2·6	Insignificant
2	Saline	174	7·98±0·55		
	Residue	170	5·94±0·41	−25·6	<0·01

of sterile distilled water, and each of the five recipient rats received 2 ml. of the resultant fluid. Controls received 2 ml. of 0.9% saline.

A change in anti-inflammatory activity was apparent which reached a maximum of 44% on Day 4 (Table 2). However, statistical analysis showed this value to be insignificantly higher than the figures obtained on Day 2 ($P>0.3$) or on Day 8 ($0.1>P>0.05$). Table 2 also shows that the change of activity between Day 1 and the other days was not related to the dry weight of sponge exudate obtained on each of the days.

Dialysis. The results of two experiments in which sponge exudate had been dialysed are given in Table 3. In both cases anti-inflammatory activity, as revealed by the cotton pellet test, has been shown to reside in the nondialysable fraction. In the first experiment 40 ml. of sponge exudate obtained from ten adrenalectomized donors was dialysed against 60 ml. of 0.9% saline for 24 hr. Freeze-drying gave 2.34 g of dry residue and 0.53 g of dry dialysate. When testing for anti-inflammatory activity these amounts were each divided into four equal portions. Each portion was dissolved in 10 ml. of sterile distilled water, and each recipient rat received 2 ml. daily. The daily doses per rat of residue and dialysate amounted therefore to 120 and 27 mg respectively.

Three groups of five adrenalectomized rats were used, of which one group received the residue, one group the dialysate, and the remaining group served as a saline-treated control. The residue in this experiment produced a 22.6% reduction in tissue deposition ($P<0.05$) whereas the dialysate produced an insignificant effect (only 2.6% reduction) (Table 3).

Since dialysis against a fixed volume of fluid only provides a partition of the smaller molecules it was decided to dialyse against running tap water, and thus to eliminate the smaller molecules as effectively as possible. 40 ml. of sponge exudate after 24 hr dialysis provided 1.52 g of freeze-dried exudate, that is, four daily injections of 76 mg into each of five rats. In this instance a 25.6% reduction of tissue deposition occurred when tested for activity ($P<0.01$) (Table 3). This confirms that the active principle remains in the residue and is thus either a large molecule, or is bound to one.

Other tests for biological activity

Rat foot oedema. The effect on the development of formalin-induced foot oedema in the rat of freshly obtained sponge exudate, freeze-dried sponge exudate and sponge implants was investigated. Five experiments were performed in which the exudate was

given both to intact and to adrenalectomized animals in doses ranging from 20 to 200 mg of freeze-dried powder or the estimated equivalent of freshly obtained material. The exudate was administered by the intramuscular, intraperitoneal or subcutaneous route. In none of the experiments was a significant reduction of the formalin-induced oedema observed when compared with saline-treated control animals following a similar dose schedule.

In the initial experiment 20 mg of freeze-dried exudate given intramuscularly in 0.2 ml. of sterile distilled water (that is, at twice its original concentration) did not produce a significant action compared with controls receiving 0.9% saline. Hydrocortisone acetate, 5 mg, however, produced a significant inhibition of the oedema, observed both 6 and 24 hr later. The result of this experiment, which can be regarded as typical, is shown in Fig. 2. Increasing the dose and concentration of exudate to 100 mg in 0.5 ml. of distilled water was equally ineffective compared with, on this occasion, 3.6% saline to take account of the increased tonicity. Hydrocortisone acetate, 5 mg, again produced significant inhibition of the oedema at 4, 6 and 24 hr.

In an attempt to increase the dose still further, injections of larger volumes of exudate were made intraperitoneally and subcutaneously. Both 2 ml. of fresh sponge exudate (equivalent to 100 mg of freeze-dried powder) and 50 mg of hydrocortisone acetate were ineffective by the intraperitoneal route. However, by the subcutaneous route 50 mg of hydrocortisone acetate produced its characteristic inhibition of oedema, significant at 4, 6 and 24 hr; but sponge exudate was still ineffective, even in the combined dosage of 2 ml. (100 mg) 18 hr before the formol-saline and 2 ml. (100 mg) on injection of the formol-saline. This latter observation was also found for adrenalectomized animals.

Yet further confirmation of the ineffective nature of sponge exudate against rat paw oedema was obtained in one experiment when sponges were implanted subcutaneously

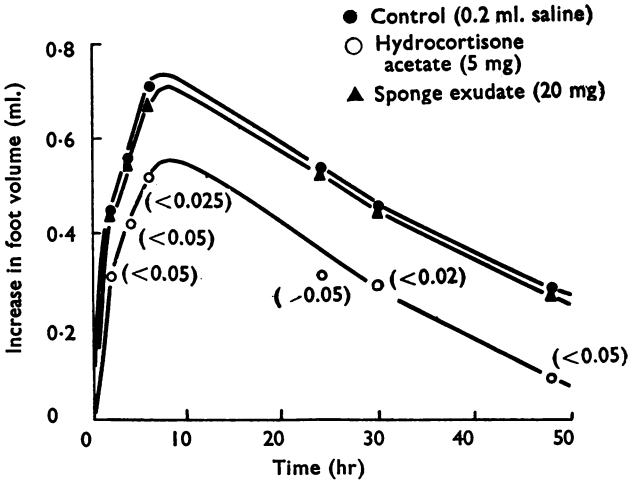

Fig. 2. The effect of intramuscular injections of hydrocortisone acetate (5 mg) and sponge exudate (20 mg) on the increase in rat foot volume following injection of formol-saline. The abscissa is time in hours and the ordinate increase in foot volume (ml.). The numbers in brackets indicate the statistical significance (*P* value) of the difference between the hydrocortisone-treated and control animals.

into adrenalectomized rats 4 days before injection of the formol-saline. No reduction of the oedema was observed compared with that of adrenalectomized control animals.

Capillary permeability. Using the leakage of a protein-bound dye as an indication of increased capillary permeability it was shown in two separate experiments that, whereas histamine acid phosphate in doses as low as 25 μg was extremely effective in producing " blueing " of the skin, injections of sponge exudate were ineffective in doses ranging from 5 to 30 mg. 30 mg is the maximum quantity of exudate which could be injected in 0.1 ml. of distilled water, but in any case ability to increase capillary permeability in a concentration greater than this could hardly be regarded as of physiological significance. The exudate used in one of the experiments was shown to possess significant inhibitory properties in both the cotton pellet test (−19%) and the wound healing test (−19%).

Tensile strength of skin wounds. A preliminary experiment was done to determine the optimum time at which tensile strength measurements should be made. Wounds were made in five groups of animals, and tensile strength measurements were performed 3, 5, 6, 7 and 8 days after wounding (Fig. 3). As a result of this, all subsequent experiments were designed so that tensile strength was measured 5 days after wounding. This was found to be the shortest period at which wound strengths were sufficiently high to show the inhibitory action of drugs. Also, to avoid variation due to body weight (Calnan & Fry, 1962), the mean weights of each group of animals were equalized at the outset of the experiment, and only those animals above 200 g were used (Fenton & West, 1963).

Table 4 shows the effect on tensile strength measurements of treatment of groups of nine adrenalectomized animals with sponge exudate (100 mg/rat/day reconstituted in 2 ml. of sterile distilled water) or hydrocortisone acetate (2 mg/rat/day in 2 ml. of 0.9% saline) compared with saline-treated control animals (2 ml./rat/day). Subcutaneous injections were made in the scruff of the neck (the wounds were placed longitudinally in the middle of the back). Five of the nine animals used for each group also had cotton

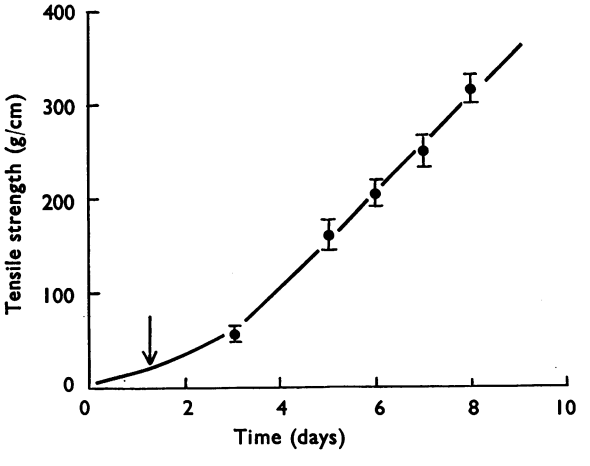

Fig. 3. The effect of time (abscissa, days) on the tensile strength of healing skin wounds (ordinate, g/cm). Points indicate the means of fifteen results together with the standard errors. Suture clips were removed at the arrow.

TABLE 4

THE ACTION OF SPONGE EXUDATE AND HYDROCORTISONE ACETATE ON THE TENSILE STRENGTH OF SKIN WOUNDS AND DEPOSITION OF TISSUE ON COTTON PELLETS

Cotton pellet weights are the means of twenty results (from five animals) and tensile strength measurements are the means of twenty-seven results (from nine animals). Means are expressed with the standard errors. The numbers in parentheses after the treatment indicate the daily dose

	Cotton pellets			Wounds		
Treatment	Increase in dry weight of pellet (mg)	Effect (%)	P	Tensile strength (g/cm)	Effect (%)	P
Saline (2 ml)	6·86±0·63			171±7		
Exudate (100 mg)	5·40±0·44	−21·3	>0·05	121±5	−28·9	<0·001
Hydrocortisone (2 mg)	4·64±0·27	−32·4	<0·005	93±6	−45·7	≪0·001

pellets implanted subcutaneously, and the inhibitory action of these substances on tissue deposition is indicated in Table 3. Hydrocortisone acetate produced a 32% reduction of tissue deposition ($P<0.005$) and a 46% reduction of tensile strength ($P\ll0.001$), whilst sponge exudate reduced tissue deposition by 21% ($P>0.05$) and caused a 29% reduction of tensile strength ($P<0.001$).

In a second independent experiment (Table 5) the inhibitory effect of sponge exudate on wound healing was confirmed, and a similar result was noted for sponge implants. 100 mg/rat/day of freeze-dried exudate reconstituted in 2 ml. of sterile distilled water in this experiment produced a 19% reduction ($P<0.005$) and sponge implants produced a 29% reduction ($P<0.001$). Two sponges were used, each measuring $5\times10\times50$ mm, and were placed longitudinally under the skin on either side of the wounds. Again there were nine animals in each group. An independent assay of the freeze-dried exudate for anti-inflammatory activity by the cotton pellet test revealed a 19% reduction of tissue deposition using a similar dose ($P<0.02$).

TABLE 5

THE ACTION OF SPONGE EXUDATE AND SPONGE IMPLANTS ON THE TENSILE STRENGTH OF SKIN WOUNDS IN ADRENALECTOMIZED RATS

The tensile strength measurements are the means and standard errors of twenty-seven results from nine animals, The exudate was shown by the cotton pellet test to produce a 19% reduction of tissue deposition ($P<0·02$) in an independent experiment

Treatment	Tensile strength of wound (g/cm)	Effect (%)	P
Saline	227±15		
Sponge implants	161±10	−29·1	<0·001
Sponge exudate (100 mg)	183±13	−19·1	<0·005

DISCUSSION

Studies employing the cotton pellet test

In the previous publication the possible labile nature of the anti-inflammatory factor found at a site of inflammation was mentioned (Robinson & Robson, 1964). The technique of injecting exudate into the recipient animals, within a short while of collecting from the donor animals, although effective, was not really satisfactory as studies became more complex. However, freeze-drying has provided a successful answer to this problem apparently without loss of activity. This agrees with the findings of Di Pasquale & Girerd (1961), who used exudate from croton oil-induced pouches.

As a result it was possible to study the effect of duration of implantation of the sponges on the anti-inflammatory activity of the exudate. It is interesting that the volume of fluid, and the total weight of solid from 1 to 8 days after implantation, varied little ; yet the anti-inflammatory activity altered markedly, ranging from 16% after 1 day to 44% at 4 days. This indicates firstly that the protein exudate and fluid formation occur early in the inflammatory reaction, secondly that they are not related to the anti-inflammatory activity of the exudate, and furthermore that the anti-inflammatory effect in the recipients is not merely that of an irritant (being simply related to the total weight of injected material). It seems likely that in this type of experiment the acute inflammatory response is essentially over by the fourth day, by which time the anti-inflammatory factor is helping to return the site to normal (Robinson & Robson, 1964).

The observation that following dialysis of the exudate the activity is retained within the dialysis membrane is also interesting. It means that the active factor is either a protein-bound small molecule, or is a protein of molecular weight greater than about 15,000. This is the maximum molecular size that will pass through the pores of the collodion membrane.

Studies on capillary permeability

The rat foot oedema test was used to determine whether injections of sponge exudate or implantation of polyester sponges modified the oedematous, as opposed to the cellular aspect of the inflammatory reaction. The results strongly suggest that sponge exudate does not significantly alter the formation of oedema fluid, and that this is true regardless of the dose or route of administration. Hydrocortisone, however, produced its characteristic inhibitory action, as shown previously by Kellett (1959). The tenfold difference in dose required to produce an effect by the subcutaneous as opposed to the intramuscular route also agrees with the findings of Kellett (1959). It is presumably related to differences in the rates of absorption and metabolism.

The failure of sponge exudate to inhibit the increase in permeability at an inflammatory site elsewhere in the body is an observation at variance with the result of Laden, Blackwell & Fosdick (1958). These authors showed that the volume of oedema fluid produced in the pleural cavity after injection of an irritant substance could be markedly reduced by injection of an irritant into the knee joint. They further showed that this effect was still present in adrenalectomized and in hypophysectomized animals, and that enclosing the knee joint in a plaster cast (to prevent it from swelling) did not alter the systemic anti-inflammatory effect. The discrepancy between these two sets of results probably lies in the difference in the nature of the two inflammatory sites at the time at which measurements were made. In all our experiments exudate obtained after a period of 4 days was used, and the pharmacologically active components of such exudate would be expected to differ greatly from those present in exudate obtained within a matter of hours, up to 15 hr in the work of Laden *et al.* (1958). Many of the substances present in early inflammatory exudates (for example histamine, 5-hydroxytryptamine and bradykinin) are known to increase capillary permeability (Spector & Willoughby, 1963). It could be that a local increase in permeability at one site inhibits the permeability increase elsewhere in the body. This hypothesis is given weight by the recent observation of Horakova & Muratova (1965) that systemic injections of bradykinin, 5-hydroxytryptamine

and histamine inhibit rat foot oedema induced by a local injection of these same substances. Like the results of Laden *et al.* (1958) the phenomenon was unaffected by hypophysectomy or adrenalectomy, or in addition by administration of corticotrophin or section of the main nerve to the foot. It seems reasonable to suggest that injection of an irritant, as in the experiments of Laden *et al.* (1958), releases permeability-increasing substances and thus produces a systemic anti-inflammatory effect.

This hypothesis also explains the inhibition of polyvinylpyrrolidone- or kaolin-induced rat foot oedema obtained by Buch & Wagner-Jauregg (1962) after intraperitoneal injection of oedema-producing substances; and the inhibition of a carrageenin-induced tail pouch abscess after intraperitoneal injection of carrageenin or diatomaceous earth (Benitz & Hall, 1963). It does not, however, explain the absence of an anti-inflammatory action in our experiments on injection of sponge exudate or implantation of polyester sponges. This can be reconciled in two ways. Firstly Horakova & Muratova (1965) showed that intraperitoneal injections of permeability-increasing substances did not inhibit the rat foot oedema induced by injection of formalin. Thus, in our experiments using formalin-induced oedema, even if permeability factors were present, they would not produce an action. Secondly, it was shown, by the absence of dye-leakage from the blood stream following intradermal injection of sponge exudate, that permeability-increasing substances were not in any case present.

It is probable, therefore, that a completely different mechanism of action is involved in the anti-inflammatory effects observed, and in order to obtain more information about this mechanism the experiments on wound healing were performed.

Studies on the rate of wound healing

Daily injections of 100 mg per rat of freeze-dried sponge exudate inhibited the rate of wound healing, as shown by tensile strength measurements, by 20 to 30%; and sponge implants produced a similar action.

It is very intriguing that there is agreement between this result and that found in the cotton pellet experiment, a comparison made more interesting by the chemical and histochemical similarity between the two reactions (Edwards, Pernokas & Dunphy, 1957). In both cases granulation tissue ingrowth is a common feature and fibroblasts are the dominant cell, although the origin of the fibroblast is very much a matter of conjecture. Two possible sources have been suggested, namely local multiplication of pre-existing cells and morphological differentiation of blood cells. Either way inhibition of their formation could explain the action of sponge exudate, and it is pertinent that O'Driscoll, Beach & Steinetz (1964) have recently demonstrated inhibition of blood-borne cells in endotoxin-induced lung inflammation in the mouse, by the parenteral injection of inflammatory exudate obtained from croton oil-induced pouches in the rat. They have shown that destruction of the lung parenchyma and invasion by polymorphonuclear leucocytes (as shown by lung weight) are both inhibited, and that such invasion is not inhibited by injections of rat serum.

This implies that inhibition of a cellular component is an essential feature of the systemic anti-inflammatory action of inflammatory exudate. However, evidence has been presented by Houck & Jacob (1961) which strongly suggests that inflammatory sites produce systemic anti-inflammatory effects by an action at the subcellular level. They

showed that, associated with skin wounds in rats, there was a local loss of dermal collagen which was accompanied by a smaller decrease in the insoluble collagen at dermal sites distant to the wound. Such collagen is thought to be formed by polymerization of a monomeric substance which is secreted by fibroblasts (Peacock, 1962). The reduced level of collagen found by Houck & Jacob (1961) in previously normal skin distant from the site of injury can, therefore, be explained in two ways. Firstly, by a reduction in the number of fibroblasts in the skin, though this seems unlikely, since they were dealing with normal, not inflamed, skin. Secondly, by reduced secretion of collagen by the fibroblasts already present, that is, an effect at the subcellular level. The reduction of tissue deposition in the cotton pellet test, and the reduction of tensile strength following implantation of polyester sponges could equally be attributable to such a loss of collagen.

It seems probable therefore, both from our experiments and from those of other workers, that injection of irritant substances can produce systemic anti-inflammatory effects by two distinct means. Firstly an action on the oedematous portion of the reaction, by local production at the site of injection of the irritant of substances which increase capillary permeability. Secondly by reducing the cellular component of the reaction either by a direct action on the cells involved or by an indirect action on some subcellular substance (or both). The anti-inflammatory action of sponge implants appears to fall into the second category, but its mode of action is still obscure.

SUMMARY

1. Experiments were performed to improve the method of production of exudate obtained from polyester sponges implanted into rats, and to provide further information about the nature of the anti-inflammatory effect it produces.

2. Freeze-drying was shown to provide a means of preserving the exudate without loss of activity.

3. Dialysis revealed that the active principle was retained within the dialysis sac.

4. Biological investigation of the exudate showed that it did not modify formalin-induced foot oedema in the rat, nor did it induce leakage of a protein-bound dye from the blood when injected intradermally.

5. A method of measuring tensile strength of skin wounds in the rat was used to assess the rate of healing.

6. Daily injections of exudate into rats with healing skin wounds were shown to retard the rate of healing.

7. The significance of these results is discussed and possible modes of action of the exudate are suggested.

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