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A NEW ANTI-INFLAMMATORY TEST, UTILIZING THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO

BY

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Existing methods for the detection of anti-inflammatory activity involve the use of experimental animals. These methods depend on the production of an experimental inflammation in the animal and the reduction of this inflammation by repeated doses of an anti-inflammatory agent. Such methods in common use include " the formalin foot test " (Selye, 1949), " the cotton pellet test " (Meier, Schuler & Desaulles, 1950), " the B.C.G. test " (Long & Miles, 1950), "the ultra-violet erythema test " (Wilhelmi & Domenjoz, 1951), " the granuloma pouch test " (Selye, 1953), " the pleural exudate test " (Holtkamp, Wang & Doggett, 1958), and various modifications of these methods (Buttle, D'Arcy, Howard & Kellett, 1957; Bush & Alexander, 1960; Tanaka, Kobayashi & Miyake, 1960; Newbould, 1963; and Meli, Smith & Wolff, 1964). In addition, various capillary permeability tests utilizing the spread of dyes through tissue have been introduced (Bacharach, Chance & Middleton, 1940; Seifter, Baeder & Begany, 1949) and also tests involving the anaphylactic reaction (Smith & Humphrey, 1949; Ungar, Damgaard & Hummel, 1952).

The present study describes an inflammatory response induced on the chorio-allantoic membrane of the eight-day-old chick embryo, and its reduction by known anti-inflammatory agents, both steroidal and non-steroidal. These agents, all of which were effective in this test, included betamethasone, hydrocortisone, phenylbutazone, chloroquine, indomethacin and sodium salicylate.

METHODS

A localized inflammatory reaction was induced on the chorio-allantoic membrane of the chick embryo by the implantation of a sterile filter paper disc, followed by re-incubation *in situ* for 4 days.

Eggs

Fertile eggs of a cross strain were used (Sudanese bantam–English White Leghorn). These were incubated at 36–37°; a moist atmosphere was maintained by placing dishes of water on alternate shelves in the incubator; the eggs were turned twice daily. Since the ambient temperature was very high throughout the year, it was possible that incubation commenced even before the eggs were collected. For this reason, eggs were always placed in the laboratory incubator on the day that they were laid. This is contrary to general practice in more temperate climates where eggs are often stored for several days before incubation is commenced. In preliminary tests, the initial incubation period varied between 8 and 12 days.

Discs

Filter paper discs, of diameter 13 mm, were stamped out of No. 1 Whatman filter paper, using a No. 8 cork borer. Any disc with rough or uneven edges was discarded; the discs were washed in distilled water to remove fragments and then sterilized in Universal screw-topped bottles, approximately 50 discs/bottle, by autoclaving (15 lb/sq. in./20 min). One disc was implanted in each egg. In preliminary experiments, four smaller discs, each approximately 5 mm diameter, were implanted in each egg. Subsequent experiments showed, however, that no greater response was achieved by the use of the smaller discs; furthermore, the movement of the embryo tended to cause the discs to overlap on the chorio-allantoic membrane and the separate areas of granulation tissue to merge together into an indistinguishable mass. Various grades of filter paper were tested; the amount of granulation tissue produced was maximal when Whatman No. 1 filter paper was used.

Technique

On the day of implantation, the eggs were candled to determine the position of the air sac and the embryo (Fig. 1a). A triangle, with sides approximately 18–20 mm, was marked on the shell where the chorio-allantoic membrane was best developed (Fig. 2a). Areas with large blood vessels were avoided to obviate possible haemorrhage. Using a dental drill fitted with a straight hand-piece and a diamond impregnated steel disc, 22 mm in diameter, the sides of the marked triangle were drilled, taking care not to pierce the underlying shell membrane. In one corner of this large triangle a second smaller triangle was drilled, with sides of approximately 5 mm. A small slit was drilled in the shell over the air sac. A mixture of molten paraffin wax and Vaseline was painted over the drilled surfaces to prevent fragments of shell from falling on to the membrane when it was later exposed. Thereafter aseptic technique was used for the implantation of the disc.

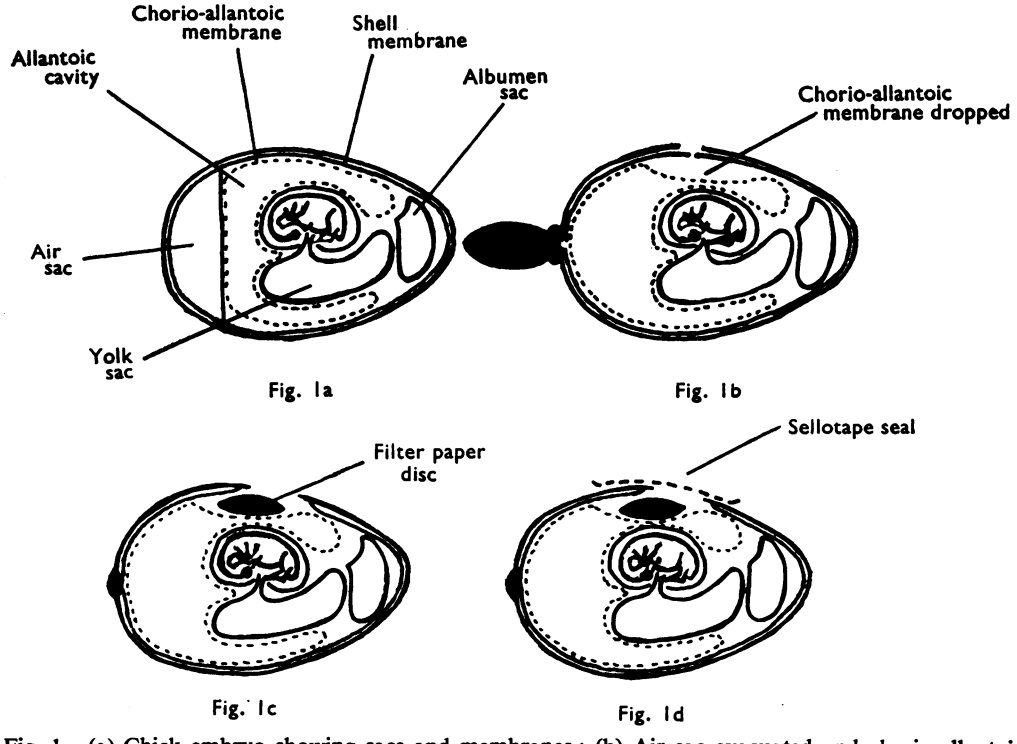

Fig. 1. (a) Chick embryo showing sacs and membranes; (b) Air sac evacuated and chorio-allantoic membrane dropped; (c) Filter paper disc placed on the chorio-allantoic membrane; (d) Triangular opening through shell and shell membrane sealed with Sellotape.

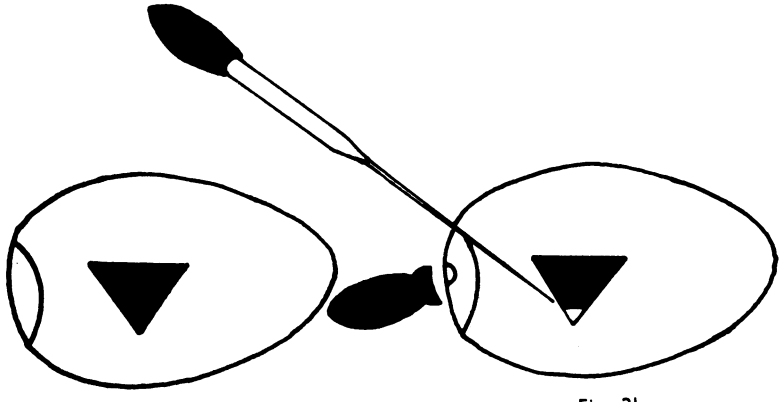

Fig. 2a

Fig. 2b

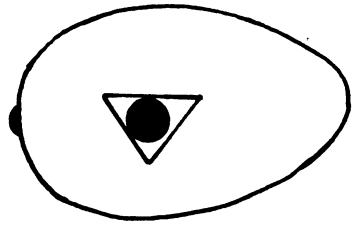

Fig. 2c

Fig. 2. (a) Triangle with sides 18–20 mm marked on shell where the chorio-allantoic membrane is well developed; (b) Technique for dropping the chorio-allantoic membrane; (c) Filter paper disc in position on the dropped chorio-allantoic membrane.

The technique used for dropping the chorio-allantoic membrane was a modification of that described by Beveridge & Burnet (1946). The egg was mounted on a stand, with the drilled area of shell uppermost; a straight Hagedorn's needle was gently inserted under one corner of the smaller triangle of shell and this triangle was raised and removed, care being taken to avoid puncturing the shell membrane during this procedure. A drop of sterile saline was placed on the exposed shell membrane and a small slit made in this membrane (and not through the closely adjacent chorio-allantoic membrane) by gentle downward pressure with the Hagedorn's needle. By means of a rubber teat, suction was applied to the hole drilled over the air sac and the chorio-allantoic membrane fell away from the shell membrane, drawing in the drop of saline (Figs. 1b and 2b). The shell and shell membrane circumscribed by the larger triangle were then removed, and the sterile filter paper disc inserted and carefully lowered on to the exposed membrane (Figs. 1c and 2c). The opening in the shell was sealed with Sellotape and the hole over the air sac region sealed with molten paraffin wax (Fig. 1d). The eggs were then reincubated at 37° for 4–6 days, without turning. At the end of this period, the chorio-allantoic membrane was exposed by cutting around the long circumference of the egg with a pair of curved scissors; the chorio-allantoic membrane remained in the top half of the shell, together with the filter paper disc (Fig. 3). The membrane was gently eased out of the shell using forceps and placed in a dish containing normal saline. The filter paper disc and the underlying portion of thickened membrane were dissected out; this underlying granulation tissue was well circumscribed and was easily distinguished from the remaining surface of the membrane. Surplus moisture was removed with blotting paper and the disc, together with the underlying granulation tissue, was placed on a plastic spotting tile and dried overnight at 55°. The dry disc plus granulation tissue was subsequently weighed.

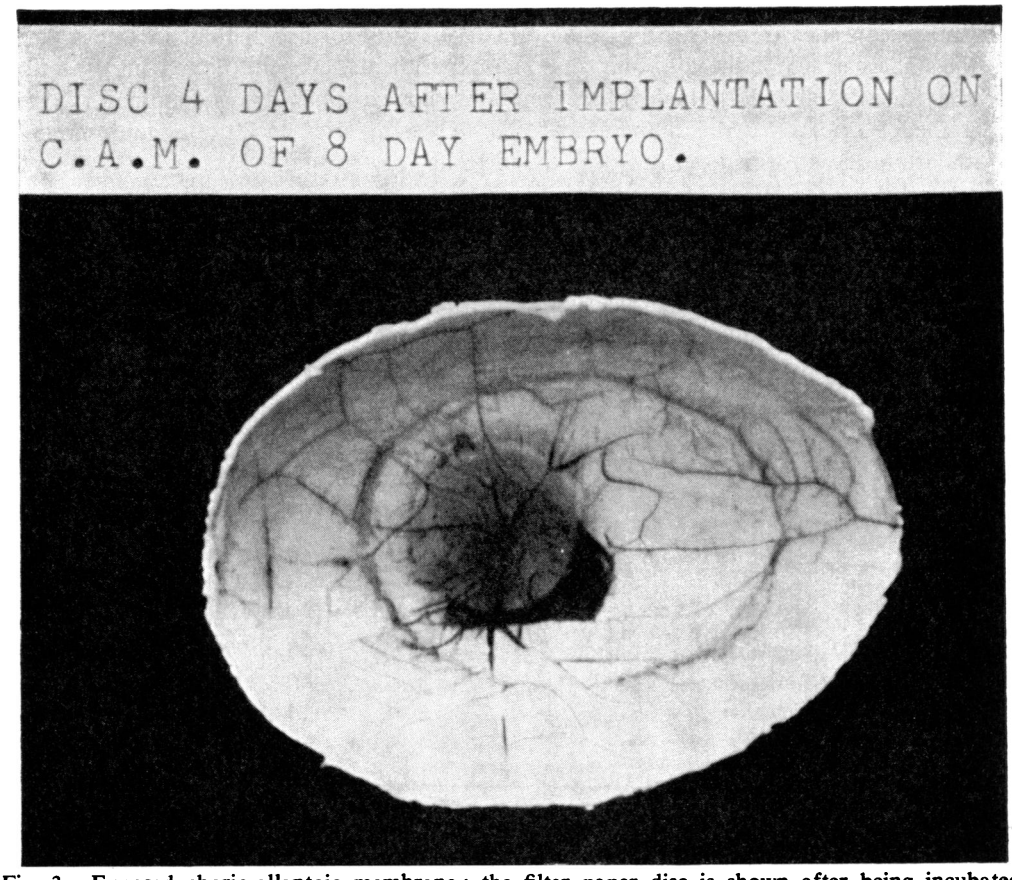

Fig. 3. Exposed chorio-allantoic membrane; the filter paper disc is shown after being incubated on the membrane for 4 days at 37°. The embryo was incubated for 8 days before the implantation of the disc.

In order to assess the actual amount of granulation tissue present, the weight of the dried filter paper disc was subtracted from the total weight of the dried disc plus granulation tissue. The mean weight of 50 dried discs had previously been determined as 10.71 ± 0.04 mg.

Drugs

The drugs examined were: betamethasone, hydrocortisone acetate, phenylbutazone, chloroquine sulphate, indomethacin and sodium salicylate. The dose volume used was 0.025 ml. which was the amount required to saturate the disc. The volume was measured by means of a 0.25 ml. graduated glass syringe, fitted with a No. 26 hypodermic needle. Soluble drugs were prepared with 5% gum tragacanth in an attempt to localize the drug on the disc, and were applied immediately after the implantation of the disc on the membrane. Insoluble drugs were suspended in saline and 0.025 ml. of the drug suspension was dropped on to the surface of each disc immediately prior to implantation. The disc was then placed on the membrane so that the drug impregnated face was adjacent to it. Discs in control eggs were treated with 0.025 ml. of either diluent.

RESULTS

The presence of a filter paper disc on the chorio-allantoic membrane of an 8-day chick embryo, when incubated at 37° for 4 days, produced an inflammatory reaction

on the membrane. An area of granulation tissue was formed which became attached to the undersurface of the disc and, in some experiments, even infiltrated into the fibres of the disc itself. Histological examination showed that this inflammatory tissue contained fibroblasts and inflammatory cells. Over a series of 20 tests the amount of granulation tissue formed per disc was found to be very constant (Fig. 4).

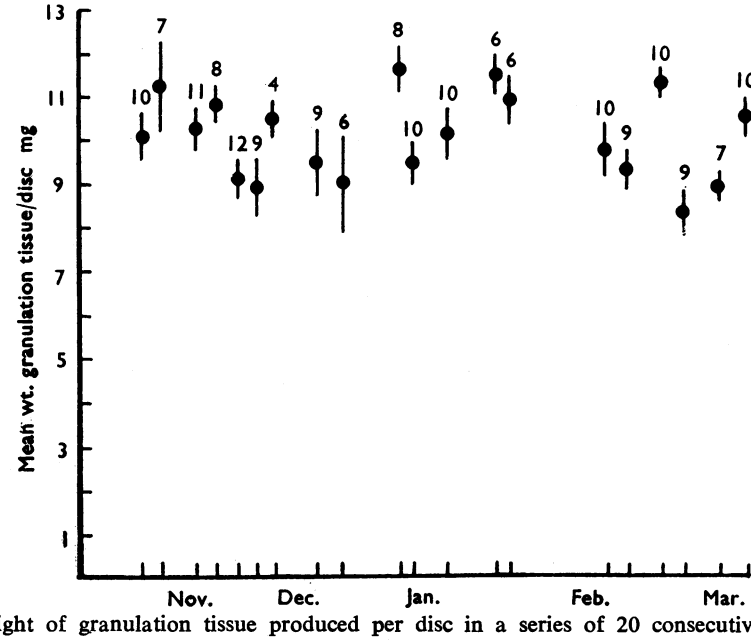

Fig. 4. Weight of granulation tissue produced per disc in a series of 20 consecutive experiments performed over a period of 5 months. Each point represents the mean result of an experiment; the number of eggs used is shown above the point. The vertical lines represent the standard errors of the means.

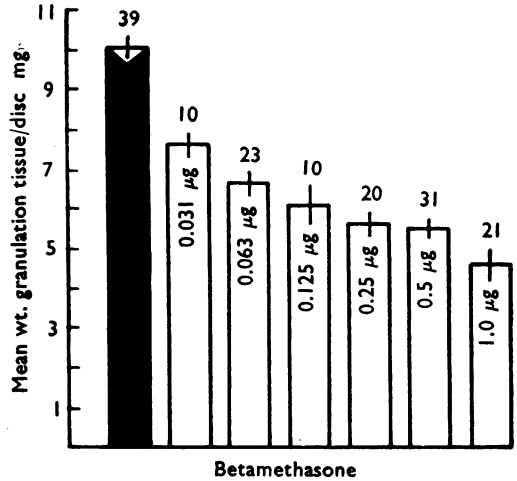

Fig. 5a

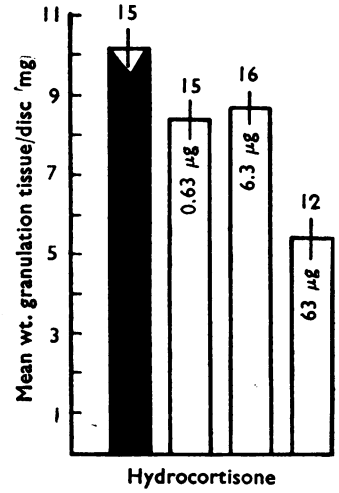

Fig. 5b

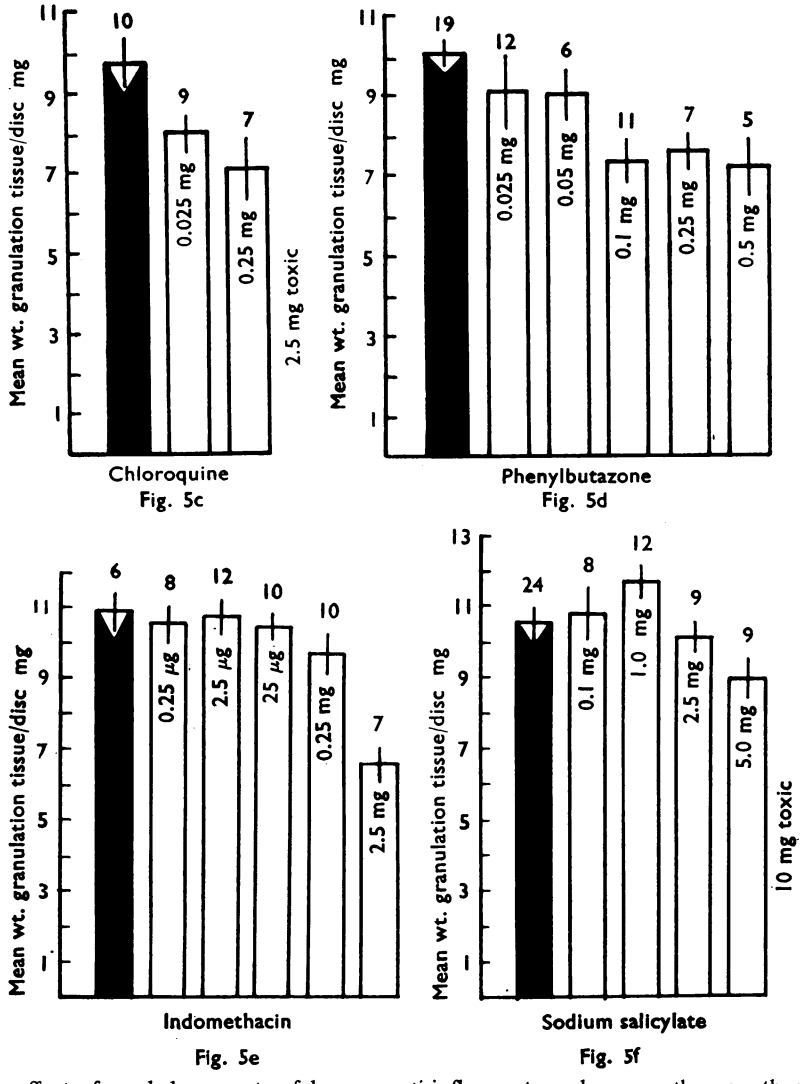

Fig. 5. The effect of graded amounts of known anti-inflammatory drugs on the growth of granulation tissue on the under-surface of the implanted disc. Each column represents the mean weight of granulation tissue per disc; the black columns represent the control values. The vertical lines represent the standard errors of the means, and the figures above each column the number of eggs used.

Increasing the primary incubation period from 8 to 10 or 12 days did not produce any significant increase in the weight of the granulation tissue formed beyond that of the basic 8-day period. Furthermore, an increase in the duration of the post-implantation period from 4 to 6 days did not increase the amount of granulation tissue formed beyond that occurring after the initial 4 days. For routine tests, therefore, it was decided to use the shorter incubation times—that is, an 8-day pre-implantation period and a 4-day post-implantation period.

All the drugs used reduced the amount of granulation tissue growing on the undersurface of the implanted disc, and this reduction was related to the amount of drug used. Betamethasone was the most potent of the drugs examined; it caused significant inhibition of granulation tissue at doses as low as 0.063 μg. (Fig. 5a). Although assessment of the results was always calculated from the dry weight of the removed disc, it was possible to observe macroscopically at an early stage—that is, on dissection of the disc from the chorio-allantoic membrane—that the drug had reduced the amount of granulation tissue, since discs impregnated with betamethasone were thin and pale in colour because they were practically devoid of any vascular involvement. This effect was graded according to the amount of betamethasone impregnated in the disc (Fig. 6).

Hydrocortisone was less active than betamethasone; it produced a significant reduction of inflammation when discs were impregnated with 0.63 μg of the steroid (Fig. 5b). It was also possible to observe this effect by visual observation of the discs. Chloroquine showed significant anti-inflammatory activity at 0.025 mg (Fig. 5c), phenylbutazone at 0.1 mg (Fig. 5d), and indomethacin at 2.5 mg (Fig. 5e), but with sodium salicylate a significant reduction in granulation tissue was only observed at near toxic doses of 5.0 mg (Fig. 5f).

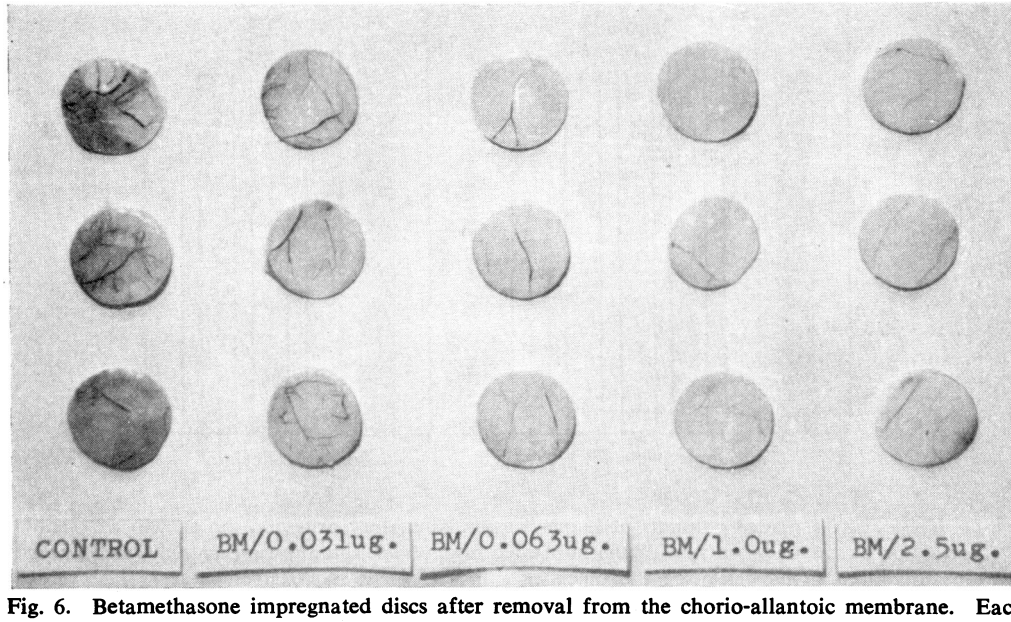

Fig. 6. Betamethasone impregnated discs after removal from the chorio-allantoic membrane. Each disc was implanted on the membrane of an 8-day old embryo and incubated in position for 4 days at 37°. The amounts of the steroid impregnated are shown. Observe that the reduction in amount of granulation tissue formed is proportional to the amount of betamethasone impregnated in the disc.

Routinely, all embryos were examined and, in some cases, the drugs produced recognizable changes in these embryos. This was especially noted with the corticosteroids, the embryos being smaller than those of the control eggs and very oedematous (Fig. 7). Sodium salicylate, at doses of 5.0 mg or 10 mg/disc produced petechial

haemorrhages on the surface of the embryo and similar changes to these were produced by indomethacin (2.5 mg/disc). As far as could be determined, chloroquine and phenylbutazone did not cause any adverse effects on the embryo at the doses used, although, as with the other drugs, it was possible to kill the embryo if the amount of the drug placed on the disc was sufficiently large.

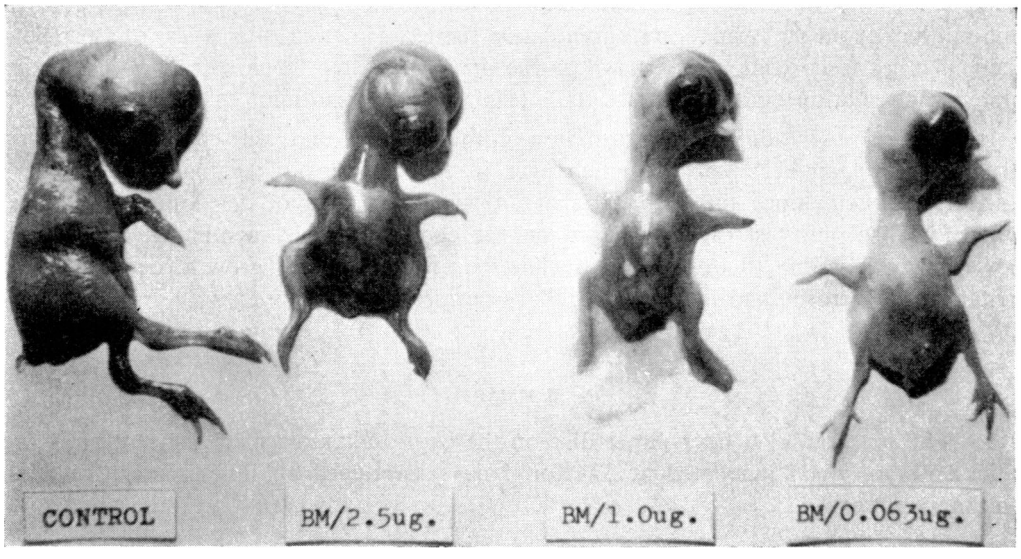

Fig. 7. Embryos removed from eggs in which betamethasone impregnated discs had been implanted on the chorio-allantoic membrane and incubated for 4 days at 37°. The embryos were 8 days old at the time of implantation. The amounts of betamethasone impregnated in each disc are shown. Observe that the embryos are smaller than those of the control eggs and are oedematous.

DISCUSSION

Although there are many existing methods for assessing anti-inflammatory activity, there is no single test which is outstandingly suitable for large scale screening of new compounds. This present test has specific advantages over methods involving the use of laboratory animals ; in particular, it is comparatively cheap to perform and requires little laboratory space ; the techniques involved are simple to perform and only small quantities of the drugs are required. The test is sensitive to both steroidal and non-steroidal agents and, in the current study, the results placed the relative potencies of the drugs in an order similar to that found in clinical practice.

One disadvantage of the test is that the slope of the dose-response curve appears to be inherently shallow and therefore the test may not be suitable for precise biological assay. However, this test has been designed specifically for the routine screening of new compounds at fixed dose levels, such as would be likely to occur in an industrial project and, in this respect, the test provides a simple and inexpensive method of routinely dealing with large numbers of compounds in a relatively short space of time. It may well be

necessary, at a later stage, to utilize other methods to investigate more precisely the potency of active anti-inflammatory compounds revealed by this simple initial screening test.

As yet no experiments have been carried out to determine whether the test is entirely specific for assessing anti-inflammatory activity. The drugs diffuse into the general circulation of the embryo, as is evidenced by the changes induced in the embryo itself. High doses may, therefore, exert a fallacious effect by acting systemically on the embryo and not locally on the formation of granulation tissue. However, this is less of a problem than in other tests using animals, where the drug may have to pass through the entire systemic circulation before it affects the site of the inflammation.

In this test, much additional information on the toxic or other side-effects of the tested drug may be gained by observing changes in the embryos. Teratogenic effects are unlikely to occur since the embryo is at an advanced stage of development (8 days) when the disc plus test drug is placed on the chorio-allantoic membrane. It should, however, be possible to observe such effects as retardation of growth, oedema, local irritation or necrosis, and increased capillary permeability.

SUMMARY

1. The presence of a filter paper disc on the chorio-allantoic membrane of an 8-day chick-embryo, when incubated at 37° for 4 days, produced an inflammatory reaction on the membrane.

2. This inflammatory response was significantly reduced when betamethasone (0.063 μg), hydrocortisone (0.63 μg), chloroquine (0.025 mg), phenylbutazone (0.1 mg), indomethacin (2.5 mg) or sodium salicylate (5 mg) was impregnated into the disc prior to implantation.

3. This response was graded according to the amount of drug used.

4. Specific effects of the drugs on the embryos were observed.

5. The test, being economical of materials and laboratory space, is suitable for the routine screening of potential anti-inflammatory compounds at fixed dose levels.

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