EFFECT OF ACTH, CORTISONE, SPHINGOMYELIN, SPHINGOSINE AND PHENERGAN (ANTIHISTAMINE) IN INHIBITING THE SKIN REACTION IN CATTLE SENSITIZED TO *TRICHOMONAS FOETUS* ANTIGEN

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(With 9 Figures in the Text)

INTRODUCTION

In cattle, circulating antibody against *Trichomonas foetus* becomes fixed in and sensitizes the skin and the subcutaneous areas immediately below the skin. Sensitization may follow natural infection or vaccination of the animal with antigen (Kerr & Robertson, 1941; Kerr, 1944).

Sensitization is also produced passively in the infant calf by the ingestion, during the first day of life, of colostrum containing antibody (Kerr & Robertson, 1946). Direct evidence of the presence of antibody in the skin was published in 1949 (Kerr, McGirr & Robertson, 1949).

Sensitization is shown by the formation of a circumscribed local oedema when the antigen or hapten is introduced intradermally. In animals sensitized either by natural infection or by vaccination with *Trichomonas* antigen, the swelling appears very rapidly and the test time for reading it is 30 min. after the injection. At 60 min. many animals already show some dispersal of the oedema. The regular time for the reading has therefore been established at 30 min. Very occasionally in testing inhibiting agents which have been injected intramuscularly the peak of the accumulation of fluid may be delayed somewhat.

Measurements are made as in the tuberculin test in cattle in terms of the difference between the caliper measurement of the skin at the site of the injection and that of the undisturbed skin nearby. The specific test fluid in all this work, except where definitely stated to the contrary, was the ethylene glycol extract of freeze-dried *Trichomonas* bodies prepared according to the method of Dr Morgan and Dr Feinberg which will be published in due course. The extract was dialysed and finally freeze-dried. It is soluble in saline and has no obvious antigenic properties. It appears to be a hapten and has the specific character of the serological variety of the strain used in making it. There are two serological varieties, 'Belfast' and 'Manley', distinguished from one another by the hapten (Kerr & Robertson, 1945).

It has been found that the testing fluid can be used repeatedly in the same animal even on the same day. It does not cause any desensitization or other observable change in the immune reactions.

There is a great deal of variation in the capacity of different animals to fix antibody in the skin. Some animals do not produce good skin reactions and do not

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retain the sensitivity as long as others. Some, while keeping a constant or nearly constant skin sensitivity, are much more readily desensitized than others. Some animals become very highly sensitized and are much more difficult to desensitize.

All the really effective inhibiting agents, if used in adequate doses do, however, clearly inhibit the skin reaction to *Trichomonas* testing fluid even in the most highly sensitized animals.

I. THE WHITE BLOOD CORPUSCLE RESPONSE AND THE INHIBITION OF THE SKIN REACTION AFTER THE INJECTION OF THE SUBSTANCES

Methods

The animal is sensitized by the intramuscular injection of *Trichomonas* antigen. It may take several injections or courses of injections to establish sensitivity. When the animal is ready for experiment the skin sensitivity is tested before the injection of the inhibiting substance. As soon as the test has been read the animal is given the inhibiting substance to be tested in a single injection. The intramuscular route is generally used, but in certain cases the injection is intravenous. The activity of the drug is then judged by the response to suitably spaced intradermal injections of the specific test fluid. Every skin measurement noted refers to a fresh injection of the specific test fluid.

Blood samples are taken for differential white blood corpuscle counts from the jugular vein, twice on the day before the injection of the drug and also immediately before the injection and then at suitable intervals after it (see Figs. 1–9). If the substance tested causes no serious systemic reaction the W.B.C. changes can be taken as directly due to its action, but when the substance is badly tolerated and causes pronounced disturbances of any kind the counts may have a different significance.

The animals used in this work were all quiet and tame and so accustomed to being handled that no disturbance was occasioned by the experimental procedure itself.

Control experiments in which animals were injected with inactive substances showed no changes in the differential W.B.C. counts. Graphs of counts after injection of saline and of arachnis oil containing calciferol have been published (Kerr *et al.* 1949).

Experimental results

Adrenocorticotropic hormone

The small amount of ACTH at our disposal was used for comparison with other substances. While it gives an acute response both in the W.B.C. effect and in desensitization, it seems to vary a good deal from sample to sample. Further, as several physiologically active principles appear to be present in it, we felt that it did not afford a very suitable base-line reaction for comparison. Its use was therefore discontinued.

The ACTH samples used were of Armour's U.S.A. standard product. Injections were intramuscular and gave no local or other immediate reaction.

K 10. 22. *i.* 51. Highly sensitized animal with very well-marked skin reaction to test fluid. Heifer, 5 cwt. Not in calf but ovulating regularly.

400 mg. ACTH injected intramuscularly

Skin response before injection	8·0 mm.
Skin response at 1 hr.	11·0 mm.
Skin response at 4½ hr.	6·5 mm.
Skin response at 6 hr.	1·0 mm.
Skin response at 24 hr.	7·0 mm.

A very good desensitization. The inhibiting effect did not come into play until between 4 and 6 hr. after injection.

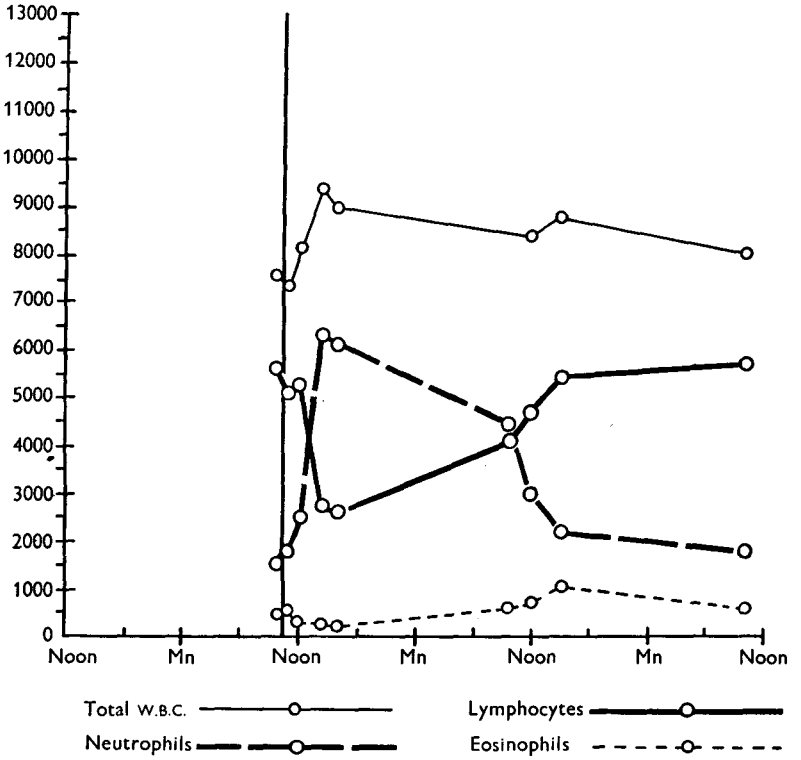

Fig. 1. ACTH 400 mg. intramuscularly. Total differential W.B.C. counts. Pronounced lymphopoenia at 4½ and 6 hr. (K 10, 22. i. 51.) The vertical line in this and subsequent figures indicates the time of the injection described in the text.

A rise in sensitivity is sometimes seen in highly sensitized animals after the injection of a substance which has some desensitizing action. Its significance is not at present clear. The rapid return of sensitivity at 24 hr. is characteristic of the action of ACTH.

The cellular response in this heifer was very marked. At 4½ hr. there was a considerable lymphopoenia which persisted at 6 hr., the number of lymphocytes being only 50 % of the starting count. At 24 hr. the lymphocytes were still very few and the original count was not restored until 29 hr. The number of neutrophils rose markedly between 4½ and 6 hr. and accounted for the rise in the total count in spite of the great drop in lymphocytes. This experiment, both as regards the cell response and the desensitization, is a typical example of the action of ACTH, used in an adequate dose of an active sample, in a highly sensitized animal (Fig. 1).

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P 6. 30. *v.* 51. Non-gravid cow. Full-grown animal. This animal had been recently sensitized and showed a good skin response equal to an increase of 6 mm., but was fairly easily desensitized.

300 mg. of ACTH belonging to a different batch from that used for K 10 was injected intramuscularly.

Skin response before injection	6·0 mm.
Skin response at 2 hr.	2·5 mm.
Skin response at 6 hr.	2·5 mm.
Skin response at 24 hr.	4·0 mm.
Skin response at 48 hr.	6·0 mm.

This was a significant inhibition of the skin reaction with a smaller dose. The cell response showed a very slight drop in the lymphocytes to 90% of the starting figure. The total count rose considerably, the increase being due to the very marked run up of the neutrophils. The persistent rise of these cells through the period from injection to 6½ hr., and the slight but also persistent drop in the lymphocytes is in the same direction as the much sharper reaction in K 10 with the larger dose.

K 7. 5. *vi.* 51. Heifer, 4 months in calf. Steady skin response of 5·5 mm. to the *Trichomonas* test fluid. The animal was fairly easily desensitized. The dose used was small, 265 mg. Armour's standard, of which 175 mg. was from the same batch as that used for P 6 and 95 mg. was from another batch.

Skin response before injection	5·5 mm.
Skin response at 2 hr.	2·0 mm.
Skin response at 6½ hr.	1·0 mm.
Skin response at 24 hr.	5·0 mm.
Skin response at 48 hr.	5·5 mm.

This was a good desensitization, beginning as in P 6 at 2 hr. and reaching the peak at 6½ hr. At 24 hr. the sensitivity had returned and the reaction at 48 hr. was the same as that before injection. W.B.C. counts: lymphocytes dropped to 82% of the starting figure and the neutrophils rose considerably. In 24 hr. the lymphocytes had declined a little further, while the neutrophils were returning to their pre-injection number. At 30 hr. the W.B.C. counts were practically the same as those made before the injection.

In these examples the powerful but very transitory inhibition of the skin response in a highly sensitized animal was shown with a full dose of an active sample. The less acute effects with the smaller doses were what might have been expected, although the response did not run quite parallel with the amounts used. Differences between animals and between samples have to be taken into account in work of this kind.

Merck's cortisone acetate

In our early experiments in 1948 with cortisone, the substance used was Upjohn's lipo-adrenal-cortex preparation. This was an oily product containing all the active hormones which have an oxygen atom attached at C_{11}.

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The response to this substance has already been described (Kerr *et al.* 1949). It suffices here to say that with a dose of 50 ml. (375 mg.) the lymphocytes dropped at 5½ hr. to 55 % of the starting count. The lymphopoenia was of short duration, and at 7 hr. the count was already 74 % of the original figure. The pre-injection state of the lymphocytes was practically restored at 24 hr., though it took another 24 hr. before the neutrophils, which had increased rapidly in number up to 5½ hr., had returned to the pre-injection level.

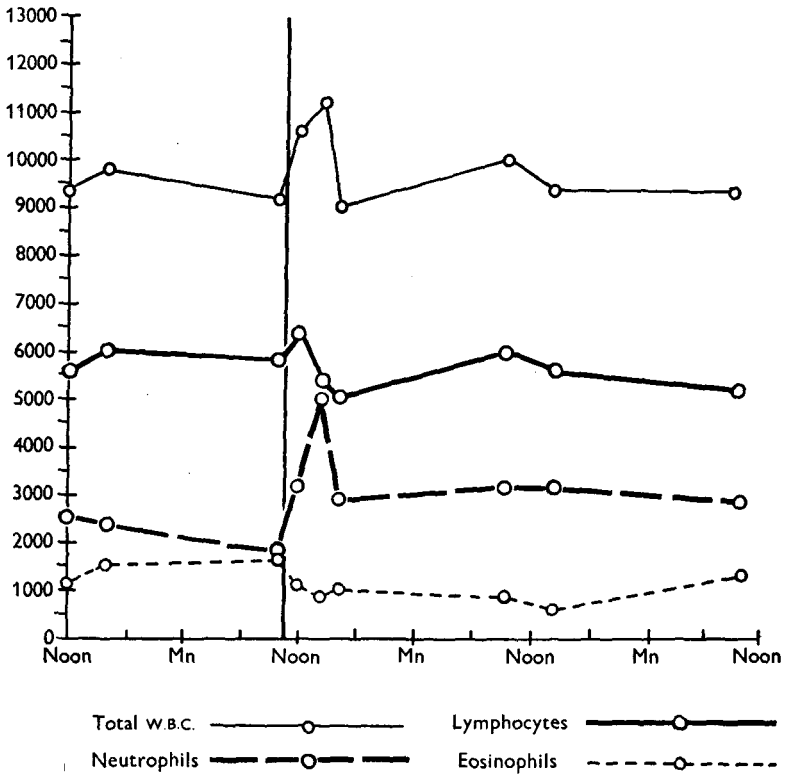

Fig. 2. Cortisone acetate 300 mg. intramuscularly. Total differential W.B.C. counts. Slight drop in lymphocytes. The dose is below the full reacting level. (K 12, 16. i. 51.)

With a dose of 100 ml. (750 mg.) of the Upjohn preparation the neutrophils rose very greatly and the lymphocytes dropped to 70 % of the starting figure at 5 hr. and were still only 73 % at 11 hr. Desensitization was not quite complete with this dose but was definite. The skin response was 8·0 mm. before injection and 3·5 mm. at 7½ hr. At 24 hr. the inhibition was beginning to disappear and the skin reaction was 4·5 mm. At 48 hr. the sensitivity had returned and the reading was 7·0 mm.

The substance used in the recent experiments was Merck's cortisone acetate.

K 12. 16. *i*. 51. Virgin heifer. 4 cwt. Lightly sensitized; easily desensitized. The skin reaction ranged between 3·5 and 5·5 mm. It was decided to use a small dose to establish the lower limit of the reaction.

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300 mg. was given intramuscularly.

Skin response before injection	3·5 mm.
Skin response at 2 hr.	2·5 mm.
Skin response at 6½ hr.	2·5 mm.
Skin response at 24 hr.	1·5 mm.
Skin response at 48 hr.	3·5 mm.

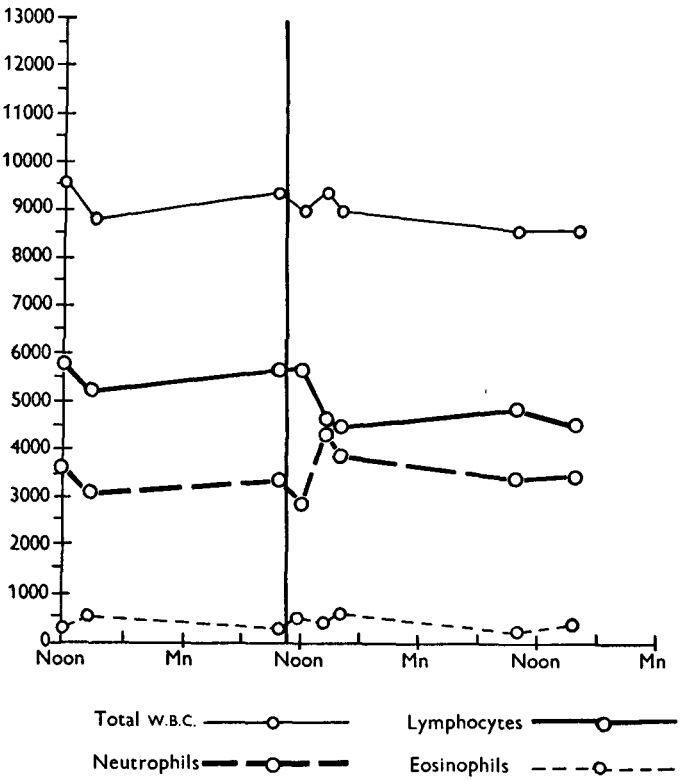

Fig. 3. Cortisone acetate 800 mg. intramuscularly. Total differential W.B.C. counts. Decline in lymphocytes to 78 % of the starting figure. (K 9, 28. v. 51.)

The reduction in sensitivity was thus slight. The lymphocytes dropped to 86 % of the starting figure by 6½ hr., and the neutrophils rose rapidly from the time of injection to 4½ hr. and then began to decline. This is an example of a minimal reaction showing that the dose was below the fully effective level (Fig. 2).

K 9. 28. *v.* 51. Virgin heifer. Well vaccinated, and had a skin response of 4–5 mm. This animal belonged, however, to the easily desensitized group. 800 mg. of cortisone acetate (Merck) was injected intramuscularly. Before injection the skin response was 4·5 mm., with no change at 2 hr. after it. At 6½ hr. the skin reaction was 1·0 mm. and at 24 hr. 0·5 mm. There was a complete desensitization of the skin which persisted at 48 hr. and at 72 hr. The reaction began to return in a minor degree after the fourth day. The lymphocytes declined to 78 % of the starting count. There was a rise in the neutrophils, but the changes in the W.B.C. counts were of the restricted, rather mild type that seems to be characteristic of

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the action of Merck's cortisone acetate. This animal showed with what appeared to be an adequate dose a long desensitization and a restrained W.B.C. reaction (Fig. 3). Another animal, E 7*c*, was selected. It was very well sensitized, had a persistent, high skin reaction, and was among the more difficult animals to desensitize.

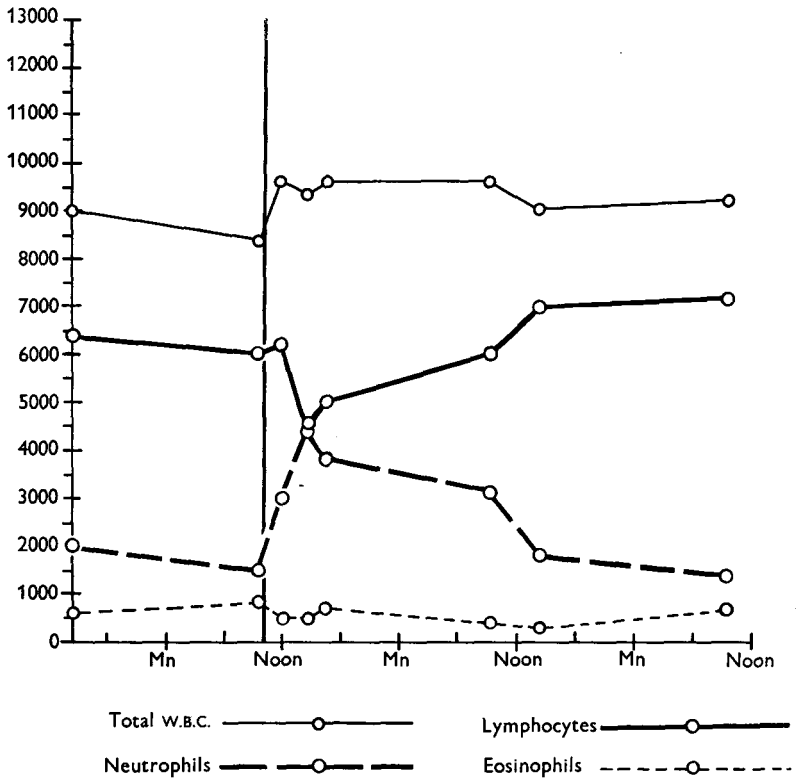

Fig. 4. Cortisone acetate 600 mg. intramuscularly. Total differential W.B.C. counts. Lymphocytes decline to 75 % of pre-injection figure. (E 7*c*, 16. i. 51.)

E 7c. 16. *i*. 51. 4½ cwt. 15 months old.

600 mg. cortisone acetate (Merck) injected intramuscularly.

Skin response before injection	8·5 mm.
Skin response at 2 hr.	5·5 mm.
Skin response at 6½ hr.	4·0 mm.
Skin response at 24 hr.	3·5 mm.
Skin response at 48 hr.	2·0 mm.
Skin response at 54 hr.	4·0 mm.
Skin response at 72 hr.	4·0 mm.

After 5 days the sensitivity corresponded to an increase of 7 mm. in the skin measurement. This was a good desensitization, and the long duration of the effect is seen again. There was a well-marked reduction in the lymphocytes to 75 % of the starting count at 4½ hr. and a slight rise to 81 % of that figure at 6½ hr. The neutrophils rose steadily from the time of injection until 4½ hr. and then began to decline (Fig. 4).

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On the fifth day after this injection of 600 mg., when the sensitivity had returned, a bigger dose of 1·1 g. was given to this animal intramuscularly. The skin response before the injection was 7·0 mm. There was little change at 2 hr., but at 6½ hr. the skin reaction was 2·0 mm. During the next 2 days it was 4·0 mm., and on the third and fourth days after the injection it was 2·5 and 1·5 mm. After this the sensitivity began to return, and the response was 5·0 mm. on the eighth day and 7·0 mm. on the ninth day. There was here again a long period during which the skin response was markedly reduced. Lymphocytes showed a slight rise at 2 hr., which is a not infrequent effect of desensitizing agents, followed by a drop to 83 % of the starting figure. At 4½ hr. the lymphocytes rose a little above the pre-injection figure, but at 24 hr. they were again low at 86 % of the original number. The neutrophils rose at 4½ hr. but after that began to decline. It would appear that on repeating the dose the desensitization response was repeated and, indeed, increased in accord apparently with the larger dose used, but, while the W.B.C. reaction was more prolonged and the lymphopoenia reappeared at 24 hr., it did not increase in intensity. Variation in the rate of absorption of Merck's cortisone acetate administered intramuscularly probably accounts for some differences in the cell response.

The characteristic action of Merck's cortisone acetate appeared to be a relatively prolonged and effective inhibition of the skin reaction with a definite but not excessive lymphopoenia. 300 mg. was below the adequate dose, but 600–800 mg. were in the effective range for these animals.

Sphingomyelin and sphingosine

Sphingomyelin is a substance obtained from the brain of cattle. A study of the reaction of W.B.C., particularly lymphocytes, to the injection of sphingomyelin was made by Tompkins (1946) as part of a wider research on the action of the ether-soluble fraction of brain lipoids. She described a lymphopoenia in rabbits which was so similar to that observed by us in cattle that we considered it worth while to explore the action of sphingomyelin in sensitized animals. We were led to do this because we had found that the lymphopoenia occurring at parturition, and also as a result of further injection of antigen into sensitized animals, was associated with desensitization of the skin (Kerr *et al.* 1949). At that time we had not been able to obtain any samples of cortisone, but the similarity of the W.B.C. response produced by sphingomyelin to that produced by the pituitary-adrenal complex of hormones was remarked on by Tompkins (1946).

Crude sphingomyelin used intravenously in cattle sensitized to *Trichomonas* antigen effectively inhibited the skin reaction. The duration of the inhibition varied from 24 to 48 hr. The cell response consisted always of a marked lymphopoenia, sometimes but not always accompanied by a rise in neutrophils. As with ACTH and cortisone the peak of the W.B.C. reaction occurred usually between 5 and 7 hr. after injection but was sometimes delayed.

The sphingomyelin used in the 1948 experiments was made in the laboratory of the Ministry of Agriculture, Northern Ireland, at Stormont by J. L. McGirr using

Levene's (1916) method. No sample was pure in the sense that it contained only sphingomyelin, but the early samples had a consistent action and effectively inhibited the skin reaction in sensitized animals. Later samples showed great variation and instability and work was suspended.

In a study of sphingomyelin made in 1951, Fisher, Harington and Long obtained for the first time *N*-acetylsphingosine in the crystalline state. This preparation diminished the tuberculin sensitivity of guinea-pigs sensitized by infection with BCG.

A small amount of *N*-acetylsphingosine was kindly placed at our disposal by Sir Charles Harington.

Two animals chosen for these experiments, E 7*c* and K 8, were highly sensitized to *T. foetus* antigen and gave marked responses to intradermal injection of the testing fluid.

The first, E 7*c*, was a bullock, 2 years of age, weight 6 cwt. On the 2 days before the injection of sphingosine the skin test reading was equal to an increase of 9·0 mm. One gram of *N*-acetylsphingosine, in the form of a white powder, was ground up in 20 ml. of saline and the resulting suspension was injected intramuscularly.

E 7*c*. 16 *x*. 51.

Skin response before injection	9·0 mm.
Skin response at 1½ hr.	8·0 mm.
Skin response at 6½ hr.	4·5 mm.
Skin response at 24 hr.	5·0 mm.
Skin response at 30 hr.	6·0 mm.
Skin response at 48 hr.	9·0 mm.

Desensitization was not complete, but the drop in the skin reaction from 9·0 to 4·5 mm. is certainly significant in an animal of this type and represents quite a high degree of inhibition of the skin reaction. The duration of a reduced skin sensitivity through the next day is also important. The desensitization had disappeared in 48 hr. The W.B.C. reaction showed a drop in the total count until 5 hr. after injection, but the numbers had returned to the original level at 6½ hr., and remained very steady until the end of the count period at 48 hr. The lymphocytes dropped in number to 78·7 % of the starting figure at 5 hr. and, after a brief increase at 6½ hr., were 75·7 % of the pre-injection figure at 9 hr. The lymphocytes remained rather low through the subsequent period, but approached the starting count at 48 hr. (Fig. 5). There was a moderate rise in neutrophils which reached its maximum at 9 hr.

The second animal, K 8, a 2½-year-old heifer, not in calf, weight 6 cwt., was a highly sensitized animal with skin response of 9·0 mm., but in general it was somewhat less difficult to desensitize than E 7*c*. 1·1 g. of sphingosine ground up in saline was injected intramuscularly.

K 8. 18. *x*. 51.

Skin response before injection	8·5 mm.
Skin response at 5½ hr.	5·0 mm.
Skin response at 7½ hr.	6·0 mm.
Skin response at 24 hr.	5·0 mm.
Skin response at 29 hr.	2·5 mm.
Skin response at 31 hr.	3·5 mm.
Skin response at 48 hr.	6·5 mm.
Skin response at 96 hr.	8·5 mm.

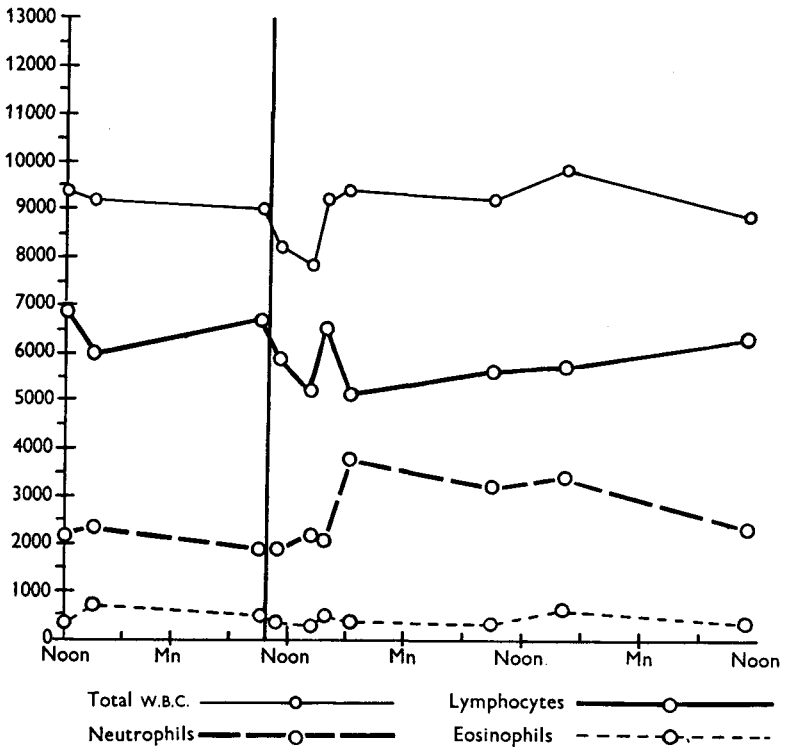

Fig. 5. Sphingosine 1 g. intramuscularly. Total differential W.B.C. counts. Lymphocytes drop in number. (E 7*c*, 16. x. 51.)

Desensitization was very good, although it developed slowly and was most pronounced at 29 hr. At 48 hr. the sensitivity was returning, and at 96 hr. the original reaction was found once more.

The W.B.C. response is shown in Fig. 6. There was no lymphopoenia during the first 7½ hr.; indeed, the numbers rose during this period, and only at 9½ hr. was there any sign of a diminution in number in comparison with the starting figure. At 24 hr. the lymphocytes were 83·8% of the pre-injection count. The decline continued to 76·0% at 26 hr. and there was little difference at 31 hr.

The neutrophils rose slightly above the lymphocytes from 24 hr. to 31 hr. At 48 hr. the lymphocytes were rising again and the neutrophils declining. It should

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be noted that the period of the most pronounced inhibition of the skin reaction coincided with the period of lymphopoenia.

With so few examples it is not possible to make any general statement, but it is quite clear that *N*-acetylsphingosine has retained much of the activity of crude sphingomyelin.

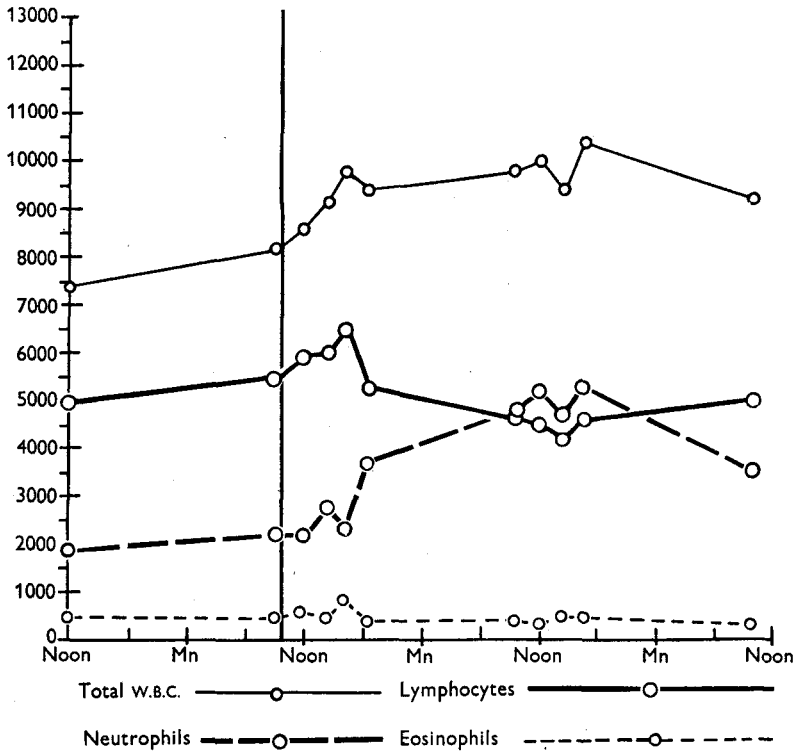

Fig. 6. Sphingosine 1·1 g. intramuscularly. Total differential W.B.C. counts. Decline in lymphocytes takes place after 24 hr. They are 76% of the pre-injection number at 29 hr. (K 8, 18. x. 51.)

It was considered advisable to relate the activity of sphingosine in these two highly sensitized animals to that of cortisone. They were therefore each injected with 1 g. of Merck's cortisone acetate after the experiments described above.

E 7*c*. 24. *x*. 51. 1·0 g. of cortisone acetate was injected intramuscularly 8 days after the experiment with sphingosine.

Skin response before injection	10·5 mm.
Skin response at 3 hr.	10·5 mm.
Skin response at 7½ hr.	9·0 mm.
Skin response at 24 hr.	5·0 mm.
Skin response at 29 hr.	5·0 mm.
Skin response at 31 hr.	4·5 mm.
Skin response at 48 hr.	9·5 mm.

The inhibition of the skin reaction was again not complete but was definitely significant. It was of much the same order as that shown with sphingosine in this

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animal. The W.B.C. reaction is shown in Fig. 7. There was a drop in the number of lymphocytes to 80·0 % of the pre-injection figure at 5½ hr. At 7½ hr. they had risen again and were very nearly at the original level at 24 hr. after the injection. At 29 hr. the lymphocytes were at 86·5 % of the starting count and remained at this level at 31 and 48 hr. The neutrophils rose somewhat during the first 24 hr., but the reaction was slight. The cellular response with cortisone was very mild in this animal.

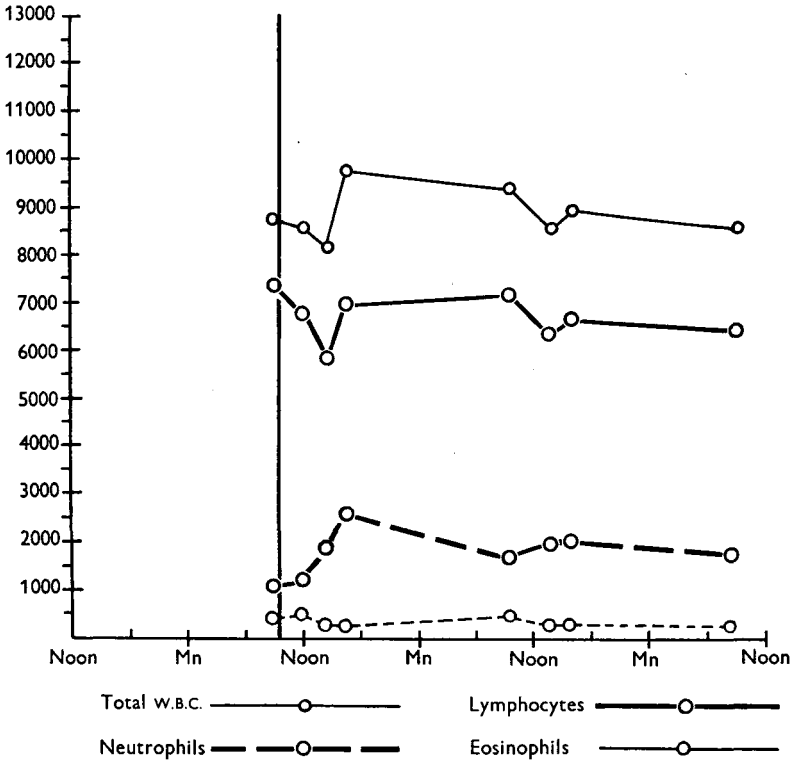

Fig. 7. Cortisone acetate 1 g. intramuscularly 8 days after 1 g. of sphingosine. Total differential W.B.C. count. Mild cellular reaction in this animal. Lymphocytes decline to 80 % of starting figure at 5½ hr. At 29 and 31 hr. they are 86·5 % of the pre-injection number. (E 7c, 24. x. 51.)

K 8. 30. *x*. 51. 1·0 g. of cortisone acetate was injected into this animal 12 days after the sphingosine experiment.

Skin response before injection	8·0 mm.
Skin response at 3 hr.	5·0 mm.
Skin response at 7 hr.	2·5 mm.
Skin response at 24 hr.	2·5 mm.
Skin response at 31 hr.	3·5 mm.
Skin response at 48 hr.	4·0 mm.

Desensitization was very good, an increase of 2·5 mm. being just below what we consider to be the level of a positive response. The degree of inhibition in this animal was again of the same order as that produced by sphingosine and, though

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the effect was somewhat more rapid and of longer duration with cortisone, these differences might be due to the more suitable physical state of the cortisone.

The W.B.C. response is shown in Fig. 8. It illustrates the drop in the number of lymphocytes to 70 % of the starting count and, after a very brief rise at 7 hr., the number remained low until 48 hr. The neutrophil count rose from the time of the injection, and had not subsided at 48 hr.

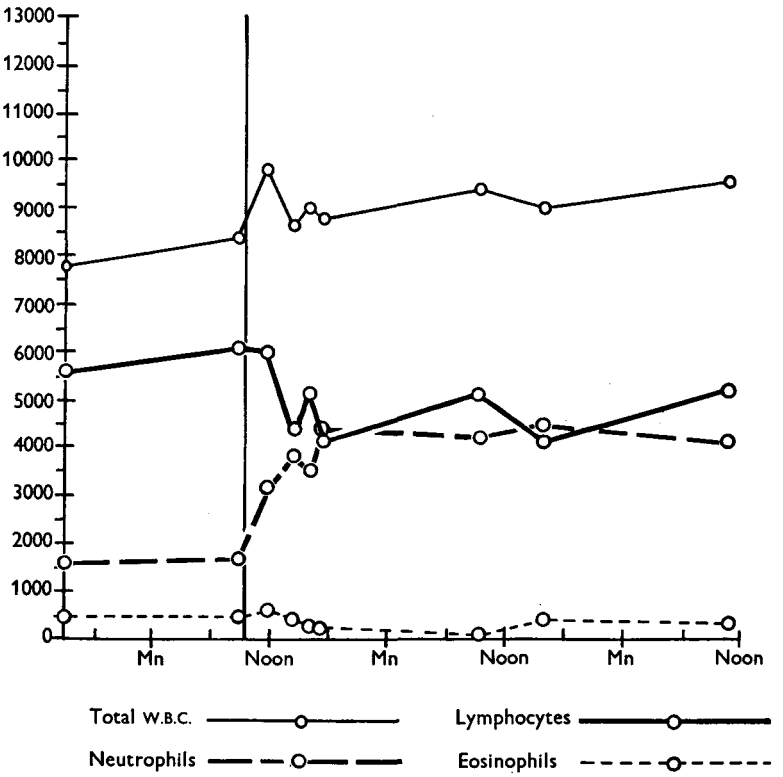

Fig. 8. Cortisone acetate 1 g. intramuscularly 12 days after 1·1 g. of sphingosine. Lymphocytes decline to 70 % of the starting figure and number remains low for 48 hr. (K 8, 30. x. 51.)

Cortisone acetate and sphingosine seem to be of much the same potency when judged on their capacity to inhibit the skin reaction in highly sensitized animals.

The W.B.C. reaction is characterized by a lymphopoenia which seems to coincide with the period of the greatest degree of inhibition of the skin response.

Phenergan

It seemed interesting to compare the action of a substance of quite a different type, such as an antihistamine, with those already discussed. The synthetic antihistamine, phenergan, was chosen. Intravenous injections were adopted as they seemed to cause less disturbance of the total W.B.C. count than did intramuscular injections.

The drug is not well tolerated in effective doses, which lie between 500 and 750 mg. The intensity of the systemic reaction varies, but even when severe it passes off very rapidly and never seems to last for more than about 10 min. This, however,

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makes it much less useful in a study involving the W.B.C. count, as there is a possibility that the drug may cause effects due to the systemic disturbance.

In the early experiments the animals always showed a lowering of skin sensitivity, but the practice at that time was to test at 6–7 hr. and again at 24 hr. The inhibition was obviously of brief duration and at 6–7 hr. was less complete than was expected. Tests made earlier after the injection showed a much more acute effect.

E 14. 29. *ix.* 49. 750 mg. phenergan injected intravenously. Slight systemic reaction passing off in 10 min.

Skin response before injection	6·5 mm.
Skin response at 6½ hr.	3·5 mm.
Skin response at 24 hr.	6·5 mm.

H 6. 20. *vii.* 50. 500 mg. phenergan injected intravenously. Acute but brief systemic reaction.

Skin reaction before injection	11·0 mm.
Skin reaction at 15 min.	0·5 mm.
Skin reaction at 1 hr.	1·0 mm.
Skin reaction at 6½ hr.	0·0 mm.
Skin reaction at 24 hr.	3·0 mm.
Skin reaction at 48 hr.	5·0 mm.

E 8*c* 17. *vii.* 51. 500 mg. phenergan injected intravenously. Acute systemic reaction of short duration. Animal chewing cud in 10 min.

Skin reaction before injection	9·0 mm.
Skin reaction at 15 min.	0·5 mm.
Skin reaction at 1 hr.	1·0 mm.
Skin reaction at 6½ hr.	1·5 mm.
Skin reaction at 24 hr.	6·0 mm.

There was nothing very characteristic in the W.B.C. response to this drug. The total counts changed considerably, but the different kinds of leucocyte had a tendency to increase and decrease together, and there was little or none of the striking drop in lymphocytes and rise in neutrophils characteristic of the response to ACTH, cortisone, sphingomyelin and sphingosine.

Fig. 9 illustrates the W.B.C. changes after the injection of phenergan into E 8*c*, described above. At 1 hr. the total counts had risen from 7200 to 8800 per cu.mm., and the lymphocytes and neutrophils both rose in number. At 4½ hr. all the cells had declined together to give the low total of 6600 per cu.mm. and there was little change at 6½ hr. Phenergan, unlike ACTH, cortisone, sphingomyelin and sphingosine, generally does not produce the prolonged lymphopoenia with its peak usually at about 5–7 hr. after the injection.

The most striking and important thing in the reaction to phenergan is the very early inhibition of the skin response. Our interpretation is that the circulating antihistamine antagonizes the effect of the combination of antigen and antibody at the site of the injection of the specific test fluid. This inhibiting effect is most powerful when the drug is most concentrated where the test fluid is injected.

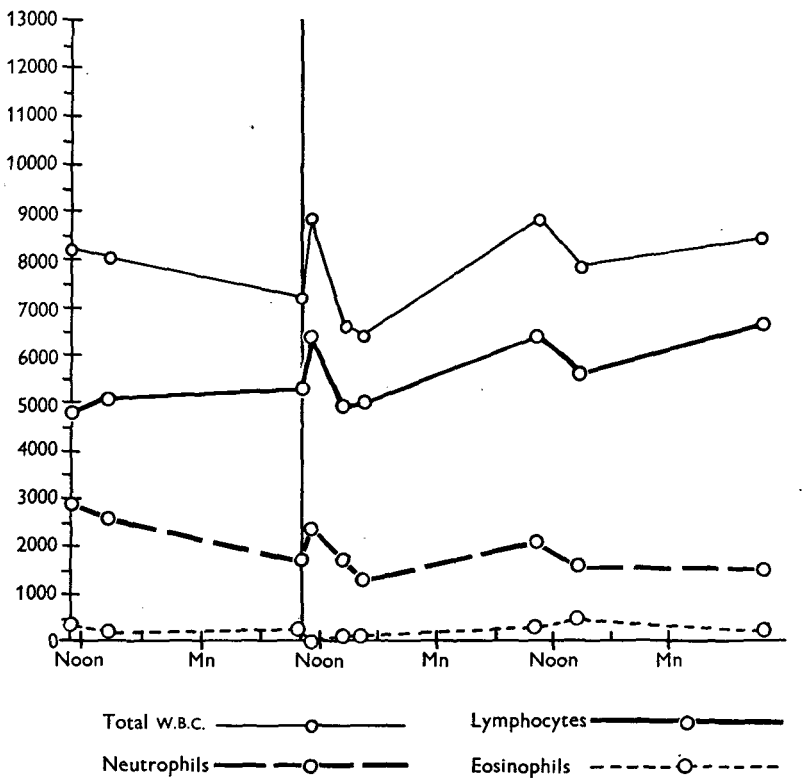

Fig. 9. 500 mg. Phenergan intravenously. Total differential W.B.C. counts. The different types of leucocytes move together and there is no definite lymphopoenia. (E 8c, 17. vii. 51.)

II. THE EFFECT OF THE INHIBITING SUBSTANCES WHEN MIXED *IN VITRO* WITH THE REAGENTS PRODUCING THE SKIN REACTION

In the following pages the local inhibition of the skin reaction is considered, the substances under test being added *in vitro* to the material provoking the skin reaction.

The basic experiments used as the pattern for some of the following work were those described by Cooke, Barnard, Hebald & Stull (1935) and later used by Maunsell (1946). In this work the sera of untreated persons sensitive to pollen when mixed *in vitro* with the appropriate antigens and then injected intradermally into normal volunteers produced the characteristic skin reactions.

In our experiments the prerequisites were to have reagents (antiserum and antigen) which, injected singly, excited no effective degree of reaction, while the antigen-antiserum mixture gave rise to a measurable swelling. In cattle this type of experiment had to be carried out with rabbit anti-*Trichomonas* serum because we were not able to produce an active mixture of bovine anti-*Trichomonas* serum and antigen.

The method of using a mixture of antigen and antiserum, made *in vitro*, to produce a skin reaction in normal animals proved less valuable than was at first hoped, and after the series of experiments now reported it was no longer used. The reason we

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abandoned the method was that many animals were sensitive to rabbit serum alone.

In the work described below, in which an inhibiting substance was tested by being included in the antiserum-antigen mixture, the fact that the animal was sensitive to the antiserum alone as well as to the antiserum-antigen combination did not invalidate the experiment, since the action of the desensitizing agent was strictly non-specific and equally effective against the sensitivity to serum as against sensitivity to the antigen-antiserum complex (see Tables 4 and 5 dealing with phenergan).

Experimental results

A titration of an antigen-antiserum mixture in a normal animal is illustrated in Table 1 (K 6).

Table 1. *Intradermal injection of antigen and rabbit antiserum into normal calf, 2½ months old (K 6)*

Tube	Antiserum (ml.)	Antigen (mg.)	Saline (ml.)	Mixture injected	Increase in skin measurement in mm. After 30 min.	Increase in skin measurement in mm. After 60 min.
1	0·1	—	—	Serum	1·0	1·0
2	0·05	0·1	—	Serum antigen	4·0	5·0
3	0·05	0·08	—	Serum antigen	5·0	7·0
4	0·05	0·06	—	Serum antigen	3·0	3·0
5	0·05	0·04	—	Serum antigen	4·0	3·5
6	0·05	0·02	—	Serum antigen	4·0	4·0
7	0·05	0·01	—	Serum antigen	3·0	4·0
8	0·05	0·005	—	Serum antigen	2·5	3·0
9	0·05	0·002	—	Serum antigen	2·0	2·0
10	—	—	0·1	Saline	0·5	0·5
11	—	0·1	0·1	Saline antigen	0·5	1·0
12	—	0·01	0·1	Saline antigen	0·5	1·0
13	—	0·001	0·1	Saline antigen	0·5	0·5

Antiserum: rabbit, anti-'Manley', heated. Antigen: 'Manley'. Ultrasonic. Dialysed. Centrifuged. Expressed as the soluble fraction of dried product in saline after centrifuging. The solution was nominally made up on basis of 4 mg./ml. but after centrifuging it was less concentrated. Quantities shown were injected intradermally in 0·1 ml.

The antiserum used was rabbit anti-*Trichomonas*, strain 'Belfast' 78III. The antigen solution was prepared from *Trichomonas*, strain 'Belfast', exposed alive to ultrasonic radiation for 1 min. This broke up the bodies. The material was then dialysed against distilled water at 0° C. and freeze-dried. It was taken up in saline immediately before use and centrifuged. Antigen treated in the Waring Blendor apparatus for 1–2 min., then dialysed and freeze-dried, can be used instead of that exposed to ultrasonic radiation. These two preparations were used as being more soluble than the crude freeze-dried *Trichomonas* bodies.

In the example shown (K 6), the immune rabbit serum injected alone gave an increase in the skin measurement of only 1 mm. The titration series of antigen mixed with antiserum produced at the best combination, which was 0·08 mg. of

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antigen to 0·05 ml. of serum, the considerable increase of 5·0 mm. at 30 min. and 7·0 mm. at 60 min. The saline alone showed an increase of only 0·5 mm. which became 1·0 mm. at 60 min.; these are quite insignificant responses to injection.

Experiments with cortisone acetate (Merck)

The animals available when these experiments with cortisone were made were all sensitive to the rabbit serum. They ranged in age from 6 months to 2 years and were in perfect health and condition.

The cortisone at our disposal was in the form of cortisone acetate (Merck) in a saline suspension containing 25 mg./ml. It contained also benzyl alcohol and 'suspending substances' of unknown character. It was the same product with which the desensitizing experiments described in the earlier part of the paper were carried out and was a fully active preparation.

The cortisone when injected alone intradermally was tolerated pretty well in amounts from 0·0625 up to 0·5 mg. in saline. The strongest reaction caused was equal to an increase in the skin measurement of 2 mm. In some animals it caused no measurable reaction at all. When the inoculum included a serum to which the animal was sensitive the cortisone slightly enhanced the action of the serum, and in no instance was the reaction diminished as compared with that produced by the serum alone.

Table 2. *Intradermal injection of cortisone, antigen and rabbit antiserum (strain 'Belfast') into red calf L–G, about 2 years old*

Tube	Antiserum (ml.)	Cortisone (mg.)	Antigen (mg.)	Saline (ml.)	Mixture injected	Increase in skin measurement after 30 min. (mm.)
1	0·05	—	—	0·15	Serum	3·5
2	0·03	0·125	0·1	—	Serum cortisone antigen	8·0
3	0·03	0·125	0·08	—	Serum cortisone antigen	10·0
4	0·03	0·125	0·02	—	Serum cortisone antigen	6·0
5	0·03	0·125	0·005	—	Serum cortisone antigen	6·0
6	—	—	—	0·1	Saline	0·5
7	0·03	0·125	—	—	Serum cortisone	4·0
8	—	0·125	—	0·1	Saline cortisone	0·5
9	—	0·125	0·1	—	Antigen cortisone	1·5
10	—	—	0·1	0·1	Antigen saline	1·5
11	0·03	—	0·1	—	Serum antigen	6·5

Antigen: 'Belfast'. Blendor. Antigen is expressed as the soluble fraction of the dried product after centrifuging. The solution was nominally made up on basis of 4 mg./ml., but after centrifuging it was less concentrated. The reagents were put into tubes and well mixed, cortisone first then serum, and finally antigen. Quantities shown were injected in 0·1 ml.

Table 2 shows the type of reaction and the effect of cortisone included in the antigen-antiserum mixture. It will be seen that the contents of tube 1 gave an increase of 3·5 mm. as the response to 0·05 ml. of antiserum, and those of tube 7,

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containing antiserum and cortisone, gave an increase of 4 mm. Readings against tubes 2, 3, 4 and 5 show that there was no inhibition of reaction from the presence of cortisone as compared with the effect of the antiserum and antigen alone in tube 2.

Cortisone injected with the antigen intradermally into a sensitive animal did not reduce the reaction to the antigen.

Table 3. *Cortisone-antigen titration in sensitive animal* (*H* 6, *highly sensitized heifer* 20 *months old*)

Tube	Cortisone (mg.)	Antigen (mg.)	Saline (ml.)	Mixture injected	Increase in skin measurement after 30 min. (mm.)
1	—	—	0·1	Saline	3·5
2	0·125	—	0·1	Saline cortisone	8·0
3	0·125	0·1	—	Cortisone antigen	11·0
4	0·125	0·01	—	Cortisone antigen	12·0
5	0·125	0·005	—	Cortisone antigen	13·0
6	—	0·01	0·1	Saline antigen	9·0
7	—	0·001	0·1	Saline antigen	10·5

Antigen: 'Belfast'. Blendor. 2 mg./ml. Quantities shown were injected in 0·1 ml.

Table 3 illustrates this. The animal in question, H 6, was in a highly reactable state, and even saline by itself produced a response. Saline and cortisone produced quite a marked reaction as shown against tube 2. The specific reaction produced by the contents of tubes 3–5 containing cortisone was not reduced. Against tubes 6 and 7 is shown the reaction with antigen alone.

As a control, a series of intradermal injections was made into a normal animal not sensitized to *Trichomonas* antigen. These were exact repetitions of those used in Table 3. There was no measurable reaction at all.

In order to elucidate the cortisone results and to provide another set of controls, a series of tests of this nature was made with phenergan. Phenergan is a synthetic antihistamine drug. Its effect when injected into the animal has been described above. It forms a clear watery solution.

The action of phenergan in this type of experiment is in striking contrast with the results recorded for cortisone. Table 4 gives the details of the experiment. The antiserum used (tube 1) itself produced an increase of 4·5 mm. in the skin measurement. Saline alone and saline-phenergan (tubes 2, 3 and 5) gave negligible reactions of 0·5–1 mm. In marked contrast to the antiserum alone, antiserum and phenergan together in two concentrations (tubes 4 and 6) resulted in an increase of only 1·0 mm. Antiserum with three different concentrations of antigen produced very substantial swellings amounting to increases of 7·0, 8·0 and 6·0 mm. (tubes 7, 8 and 9).

The addition of phenergan in these mixtures, adjusted so as to retain the same amount of antibody and antigen in the inoculum, inhibited the reaction completely, and an increase of only 1·0 mm. was produced by each mixture (tubes 10, 11 and 12). We conclude that, unlike cortisone, phenergan can directly inhibit the

reaction in the skin. We observed a certain degree of turbidity in all the tubes containing phenergan and antiserum, with or without antigen.

N-acetylsphingosine was also used in a local test in a highly sensitized animal, K 10, to find out if there was any inhibition of the skin reaction when the substance was used in conjunction with the specific testing fluid and injected intradermally.

Table 4. *Intradermal injection of phenergan, antigen and rabbit antiserum (strain 'Belfast') into normal animal 5–6 months old*

Tube	Antiserum (ml.)	Phenergan (mg.)	Antigen (mg.)	Saline (ml.)	Mixture injected	Increase in skin measurement after 30 min. (mm.)
1	0·05	—	—	0·05	Saline serum	4·5
2	—	—	—	0·1	Saline	0·5
3	—	0·25	—	0·1	Saline phenergan	1·0
4	0·05	0·25	—	—	Serum phenergan	1·0
5	—	0·125	—	0·1	Saline phenergan	1·0
6	0·05	0·125	—	—	Serum phenergan	1·0
7	0·05	—	0·1	—	Serum antigen	7·0
8	0·05	—	0·08	—	Serum antigen	8.0
9	0·05	—	0·06	—	Serum antigen	6·0
10	0·05	0·125	0·1	—	Serum antigen phenergan	1·0
11	0·05	0·125	0·08	—	Serum antigen phenergan	1·0
12	0·05	0·125	0·06	—	Serum antigen phenergan	1·0

Antigen: 'Belfast'. Ultrasonic. Not dialysed. Quantities shown were injected in 0·1 ml.

Phenergan was included in the experiment as a reagent of known activity.

Saline and phenergan gave no skin response.

Saline and sphingosine gave no response.

Testing fluid (specific hapten) and phenergan gave no response. Sphingosine and specific hapten gave a marked response equal to an increase in skin measurement of 11·0 mm. Saline and the specific testing fluid showed a marked response equal to an increase of 11·5 mm.

Table 5 shows the details of the experiment.

It is clear from the above that whereas phenergan inhibits the skin reaction in a sensitized animal when introduced along with the specific testing fluid, sphingosine has no such local inhibiting reaction. In its lack of a direct local inhibiting action on the skin response, sphingosine resembles cortisone.

DISCUSSION

Certain conclusions can be drawn from the work described above. It seems clear that the junction of antibody and antigen *in vitro* produces a substance which when injected intradermally into a normal animal is capable of inducing the local oedema characteristic of the skin reaction in sensitized animals. The action of this combination in normal animals can be neutralized or inhibited by adding an anti-

histamine drug, phenergan, to the mixture *in vitro*. It seems reasonable to conclude that when the specific testing fluid is injected into the skin of a sensitized animal the fixed antibody reacts with the antigen (or hapten) probably to produce a histamine-like substance, which causes the characteristic skin reaction. This is rendered more likely since we have been able to demonstrate antibody in saline extracts of the skin (Kerr *et al.* 1949).

Table 5. *Effect of phenergan and sphingosine when added to test fluid (strain 'Belfast'), introduced intradermally into highly sensitized animal (K* 10)

Tube	Phenergan (mg.)	Sphingosine (mg.)	Hapten (mg.)	Saline (ml.)	Mixture injected	Reading after 30 min. (mm.)	Remarks
1	0·125	—	—	0·1	Phenergan saline	1·0	Phenergan and saline had no effect
2	0·125	—	0·01	—	Hapten phenergan	0·5	Phenergan nullified action of the testing fluid (hapten)
3	—	0·2	—	0·1	Sphingosine saline	1·0	Sphingosine and saline had no effect
4	—	0·2	0·01	—	Sphingosine hapten	11·0	Sphingosine did not reduce the action of the hapten
5	—	—	0·01	0·1	Hapten saline	11·5	Testing fluid produced strong reaction in the sensitized animal

Quantities shown were injected in 0·1 ml.

The injection of phenergan intravenously inhibits the skin reaction, and it seems to do so directly by antagonizing the substance resulting from the combination *in vivo* of fixed antibody and antigen which excites the swelling. ACTH, cortisone and sphingosine do not seem to act in this way. In the first place there is no local inhibition when cortisone or sphingosine is injected along with the specific testing fluid (antigen or hapten) into the skin of sensitized animals. Nor can cortisone when placed in contact *in vitro* with the antiserum-antigen complex prevent the formation of the swelling when the mixture is injected intradermally into a normal animal. It would appear that ACTH, cortisone, sphingomyelin and sphingosine have some physiological action which prevents the substance released or produced by the junction of antibody and antigen from injuring the dermal tissues and structures, such as, for example, the capillaries.

The chain of events which occupy the hours between the injection of these agents and the manifestation of the inhibition remains obscure, but there does not seem

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to be any direct action of the hormones or of sphingosine as such at the local site. We have no evidence in this work as to whether the intervening processes, before the inhibition is in operation, are the same with the hormones and with sphingosine.

In sensitized guinea-pigs the action of ACTH and cortisone as shown by Long, Miles & Perry (1951) is dependent on the presence of an adequate supply of ascorbic acid and also of a certain degree of thyroid activity, while that of *N*-acetylsphingosine is not (Fisher *et al.* 1951). The conditions of the experiments in cattle are such that this difference is not apparent.

In cattle the reactions of the W.B.C. to the injection of ACTH, cortisone, sphingomyelin and sphingosine are very striking. The high percentage of lymphocytes and the generally much lower number of neutrophils in normal animals makes the induced lymphopoenia stand out with great clarity. It seems established that stimulation of the anterior pituitary or the injection of ACTH or cortisone produces a temporary lymphopoenia (White & Dougherty, 1946; Valentine, Craddock & Lawrence, 1948; Hills, Forsham & Finch, 1948; De Groot & Harris, 1950).

In our experiments we found that the injection of ACTH, cortisone and sphingosine in adequate doses provoked a definite lymphopoenia usually apparent by 5–7 hr. but sometimes not appearing till later. These doses also effectively inhibited the skin reaction in animals sensitized with *Trichomonas* antigen. The connexion between the lymphopoenia and the desensitization is difficult to assess. In a first injection of any of these substances the connexion appears to be very close, but we are still of the opinion that the lymphopoenia is an accompanying phenomenon. Our reason for this conclusion is that the inhibition of the skin reaction may last considerably beyond the period of true lymphopoenia, and a second injection following at an interval of a few days may have an excellent desensitization effect without a very pronounced cell response.

SUMMARY

1. ACTH, cortisone, sphingomyelin and sphingosine produce a delayed inhibition of the skin reaction in cattle sensitized to *Trichomonas foetus* antigen when they are injected intramuscularly. The inhibition is greatest 6–9 hr. after injection of the inhibitor, but may sometimes be further delayed. This reaction is associated with an absolute lymphopoenia of slightly variable duration.

N-acetylsphingosine retains the desensitizing effect of crude sphingomyelin and is of much the same potency as cortisone acetate of Merck.

2. The lymphopoenia associated with the injection of ACTH and cortisone occurs also with sphingosine, and these three substances are also alike in not producing the desensitizing effect until some hours after the injection.

3. Phenergan, a synthetic anti-histamine, has a different action, and its desensitizing effect which appears to depend on a direct antagonizing of the oedema-provoking substance resulting from the junction of antibody and antigen (hapten) takes effect apparently as soon as the injected drug itself is present at the site of the injection of the specific testing fluid.

4. Methods of testing the capacity of the hormones and drugs to prevent the skin reaction locally and to nullify the action of the *in vitro* antigen-antibody mixtures which can produce a reaction in normal animals are described.

Phenergan alone of the substances dealt with has any power to act locally. Phenergan mixed with the antigen-antiserum complex *in vitro* prevents the formation of the characteristic swelling in a normal animal.

Phenergan mixed with the specific testing fluid prevents the development of the skin reaction when the mixture is injected intradermally into a sensitive animal.

Cortisone and sphingosine do not have this action.

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