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SUPPRESSION OF ADJUVANT-INDUCED ARTHRITIS IN RATS WITH 2-BUTOXYCARBONYLMETHYLENE-4-OXOTHIAZOLIDINE

BY

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The intradermal injection of Freund's adjuvant (a fine suspension of dead tubercle bacilli in liquid paraffin) into the foot pad of rats produces an inflamed " primary lesion " at the site of injection and then, after a delay of approximately 10 days, inflamed " secondary " lesions in areas of the body remote from the injection site (Stoerk, Bielinski & Budzilovich, 1954; Pearson, 1956, 1959; Houssay & Frangione, 1961; Ward & Jones, 1962; Newbould, 1963).

The inflammation associated with both primary and secondary lesions in arthritic rats is reduced by treatment with most of the compounds currently used in the chemotherapy of rheumatoid arthritis in man (Newbould, 1963). However, in rats injected with adjuvant none of these chemotherapeutic agents interfere permanently with the disease process involved in the formation of secondary lesions and relapses occur when treatment is terminated. The object of this paper is to describe some of the biological properties of a new compound, 2-butoxycarbonylmethylene-4-oxothiazolidine (I.C.I. 43,823) (Clarkson, Hull & Newbould, 1961), one of a series studied by Clarkson, Hepworth, Hull, Maisey & Slatcher (unpublished). Unlike conventional antirheumatic drugs, this compound prevents completely the development of secondary lesions in rats injected with adjuvant into the foot pad.

METHODS

Male, specific pathogen-free albino rats, Alderley Park strain I, were used. They belonged to a colony-bred strain of rats of Wistar origin and weighed approximately 200 g.

The arthritic syndrome was induced by an intradermal injection of 0.05 ml. of a fine suspension of dead tubercle bacilli in liquid paraffin B.P. (concentration 5 mg/ml.) through a No. 20 needle into the plantar surface of the right hind-foot. The tubercle bacilli were derived from human strains PN, DT and C which were grown for 8 weeks, killed by steam and dried in a vacuum oven. In routine tests for chemotherapeutic activity groups of three rats were weighed and dosed by mouth with the compounds to be investigated, one untreated control group being included for every five groups treated. One day later, the thickness of the right hind-foot was measured with a micrometer and injected with adjuvant. Daily treatment was continued until the 13th day when the weight of each rat was again recorded, the severity of secondary lesions assessed as nil, mild, moderate, moderately severe or severe, and the thickness of the injected foot measured to enable the percentage inhibition of the increase in thickness of the injected foot to be calculated (Newbould, 1963). For more detailed evaluation the swellings in the hind-feet were measured with a micrometer daily (week-ends excepted) until the 30th day.

To see whether continuous daily dosing with I.C.I. 43,823 was necessary for successful chemotherapy, doses were given only around the time of injection in one group of rats, whilst in other groups dosing was started at different times before and after injection.

Additional tests for anti-inflammatory activity were conducted in the rat using the inflammation induced by injecting kaolin into the ankle, formalin into the foot pad and carrageenin into the flank. Carrageenin-induced oedema in the foot pad of rats was studied by the method of Winter, Risley & Nuss (1962).

For white-cell studies, samples of blood were taken from the tail. After haemolysis of the red cells with saponin, the white cells were counted using a " Coulter " counter. Blood smears were stained with Giemsa and one hundred cells counted to obtain the differential count.

Tuberculin tests were performed by injecting 0.1 ml. of a 1 : 10 dilution of Old Tuberculin intradermally into the flank. The diameter of the reaction was measured 24 and 48 hr after injection.

RESULTS

In a series of tests, when chemotherapeutic activity was assessed only on the 30th day after injection, daily oral doses of 50, 100 and 200 mg/kg of I.C.I. 43,823 inhibited the increase in thickness of the injected foot by 21.5, 30.7 and 37.2% respectively (Table 1). At each of these dose levels there was a mean weight gain during the period of the test whilst in the untreated controls there was a mean weight loss. More importantly, daily doses of 100 and 200 mg/kg of I.C.I. 43,823 prevented the development of secondary lesions in 63.5 and 87.4% of rats respectively. Phenylbutazone (100 mg/kg by mouth daily), which was used as a reference compound, inhibited primary lesions by 42% and resulted in a mean weight gain of 11.8 g. However, phenylbutazone had little effect on secondary lesions which were nearly always present and were classified as moderate in 56.4% of rats (Table 1).

TABLE 1

RESULTS OF A SERIES OF TESTS IN WHICH I.C.I. 43,823 AND PHENYLBUTAZONE WERE GIVEN TO RATS DAILY FOR 14 DAYS (SATURDAYS EXCEPTED) DURING THE DEVELOPMENT OF THE ARTHRITIC SYNDROME

Figures in parentheses are standard errors. Doses were oral per day

			Assessment 13 days after injection						
				Rats (%) with secondary lesions					
Compound	Dose (mg/kg)	No. of groups of rats	Inhibition of increase in foot thickness (%)	Nil	Mild	Mod.	Mod. sev.	Sev.	Mean weight change (g)
43,823	50	10	21·5 (±4·7)	26·7	30·0	33·3	10	0	+ 3·8 (±3·5)
43,823	100	37	30·7 (±2·7)	63·5	20·6	9·4	5·6	0·9	+ 7·3 (±1·8)
43,823	200	43	37·2 (±2·0)	87·4	12·6	0	0	0	+ 6·9 (±1·7)
Phenyl-butazone	100	52	42 (±1·3)	2·5	19·9	56·4	19·9	1·3	+11·8 (±1·5)
Controls	Nil	52	0	0	0	0	0	100	− 6·5 (±0·8)

Foot-thickness measurements recorded at frequent intervals after injection showed that daily treatment with I.C.I. 43,823 (200 mg/kg) had little effect on the swelling of the injected foot up to the 8th day (Fig. 1). In contrast, daily treatment with phenylbutazone (100 mg/kg) partially controlled the swelling of the injected foot during this period. From the 8th to the 14th day a secondary swelling developed in the injected foot of untreated rats, but no further swelling appeared in the injected foot of rats treated with I.C.I. 43,823 or phenylbutazone. Secondary lesions, which first appeared on the 10th day in untreated rats and rats treated with phenylbutazone, resulted in an increase in thickness of the hind-foot which

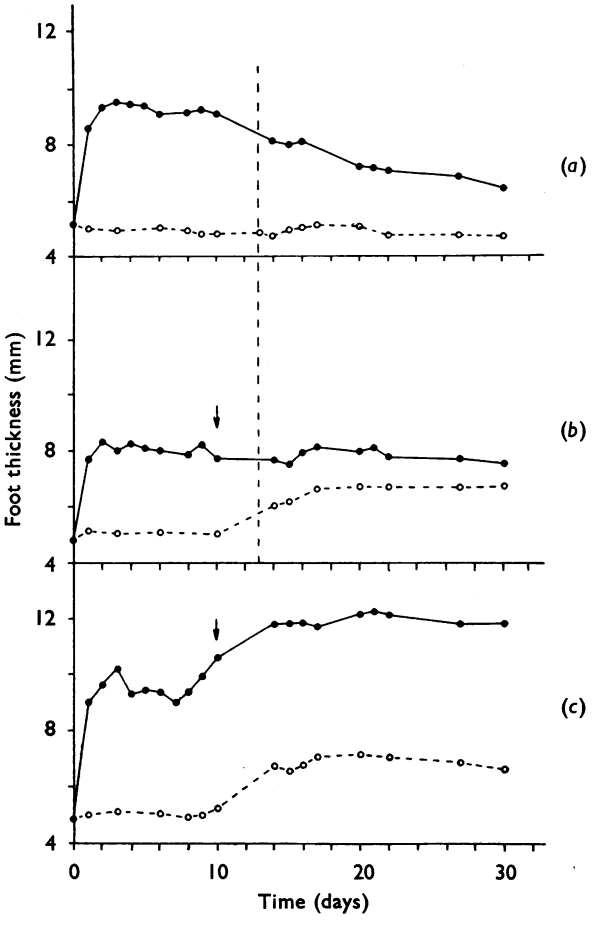

Fig. 1. Changes in the thickness of both hind-feet of treated and control rats during the development of the arthritic syndrome. Each point represents the mean thickness for three rats. Rats were given daily doses by mouth for 14 days (starting 1 day before injection into the foot pad) of: (*a*) I.C.I. 43,823 (200 mg/kg); (*b*) phenylbutazone (100 mg/kg); (*c*) untreated controls. ● —— ●, Thickness of the injected foot; ○ - - - - ○, thickness of the other hind-foot. The arrows indicate the day on which secondary lesions were first detected. The vertical dotted line indicates the time of the last dose administered.

had not been injected. Secondary lesions did not develop in the rats treated with I.C.I. 43,823 (200 mg/kg), hence the absence of swelling in the uninjected hind-foot. The appearance of a rat treated with I.C.I. 43,823 for 14 days is shown alongside that of an untreated control in Fig. 2. After withdrawing daily treatment on day 13, the swelling of the injected foot of rats previously treated with I.C.I. 43,823 continued to regress and secondary lesions did not develop.

During the development of the rat arthritis syndrome a biphasic increase in leucocyte counts was observed (Fig. 3). The first increase was associated with the development of the primary swelling in the injected foot whilst the second was associated with the secondary

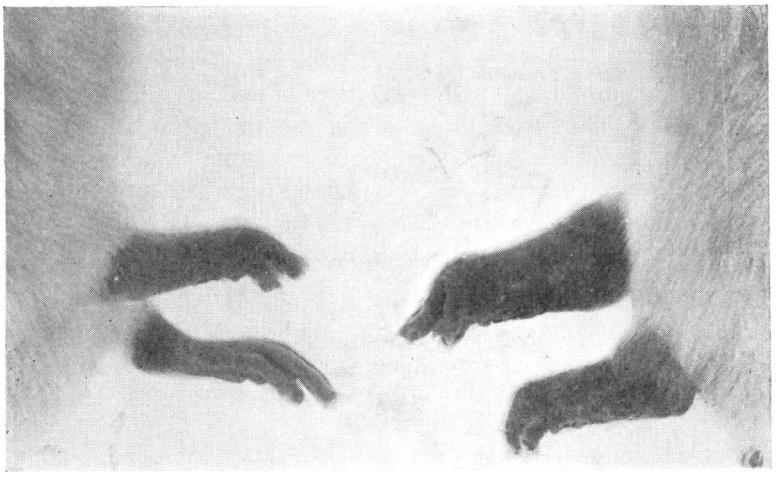

Fig. 2. Appearance, 13 days after the injection of adjuvant into the right hind-foot pad, of: left, a rat treated with I.C.I. 43,823 (200 mg/kg, orally daily for 14 days); right, untreated control.

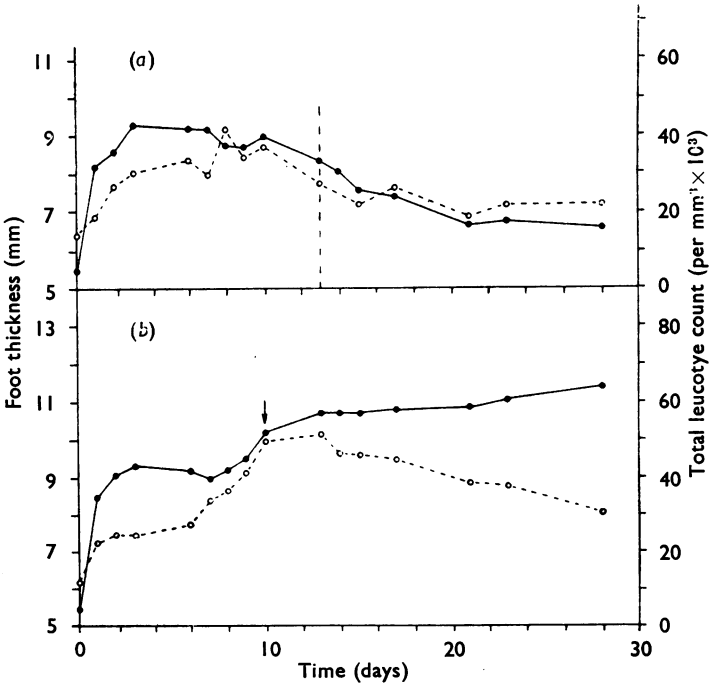

Fig. 3. Changes in the thickness of the injected foot and the blood leucocyte count during the development of the arthritic syndrome for: (*a*) rats treated daily for 14 days with I.C.I. 43,823 (200 mg/kg, orally daily, starting 1 day before injection into the foot pad); (*b*) untreated controls. Each point is the mean from six rats. ●——●, Thickness of the injected foot; ○ - - - - ○, total blood leucocyte count. The arrow indicates the day on which secondary lesions were first detected. The vertical dotted line indicates the time of the last dose administered.

increase in thickness of this foot and the development of secondary lesions. The leucocyte counts of rats treated daily with I.C.I. 43,823 (200 mg/kg) increased during the first 3 days after injecting adjuvant into the foot pad. Thereafter, the counts remained almost constant until day 10 when they started to decrease. Thus, rats treated with I.C.I. 43,823 did not exhibit the secondary increase in leucocyte counts seen in untreated controls. In control and treated rats the increases in total leucocyte counts were due primarily to increases in the number of polymorphonuclear leucocytes (Fig. 4).

Daily doses of I.C.I. 43,823 (500 mg/kg) given by mouth for 6 months to normal rats did not depress the number of circulating leucocytes.

A positive tuberculin reaction developed 24 hr after injecting 0.1 ml. of a 1 : 10 dilution of Old Tuberculin into the flank of rats which had severe secondary lesions as a result of injecting adjuvant into the foot pad 13 days previously (Table 2). Rats injected with

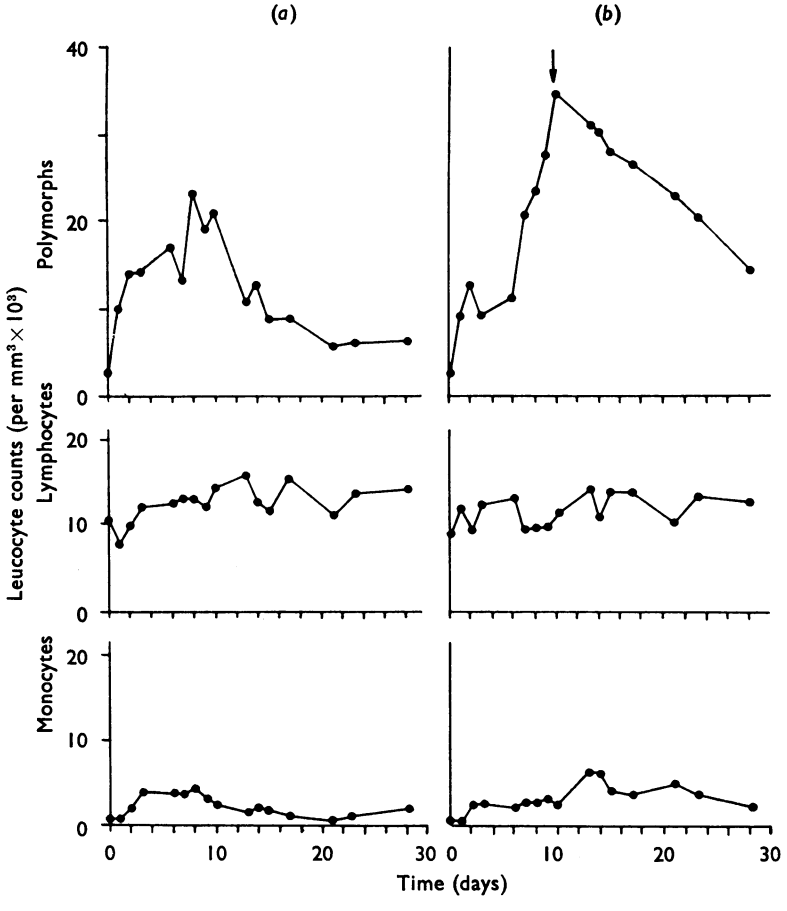

Fig. 4. Counts of polymorphonuclear leucocytes, lymphocytes and monocytes in the blood of rats during the development of the arthritic syndrome. (*a*) Rats treated with I.C.I. 43,823 (200 mg/kg, orally daily for 14 days, starting 1 day before injection into the foot pad); (*b*) untreated controls. Each point is the mean from six rats. The arrow indicates the day on which secondary lesions were first detected.

TABLE 2

DIAMETER OF THE LESION 24 AND 48 HR AFTER THE INTRADERMAL INJECTION OF 0·1 ML. OF A 1 : 10 DILUTION OF OLD TUBERCULIN INTO THE FLANK

The test was performed 13 days after injecting adjuvant intradermally into the food pad; I.C.I. 43,823 was administered for 14 days starting 1 day before the injection of adjuvant into the foot pad

Condition	Diameter of tuberculin reaction (mm) after 24 hr	Diameter of tuberculin reaction (mm) after 48 hr
I.C.I. 43,823 (200 mg/kg by mouth daily)	13	0
	11·5	9·5
	14	0
	10	0
	14	7·5
	Mean 12·5	Mean 3·4
Untreated, arthritic controls	13·5	13
	12	13·5
	15	17·3
	12·5	12·5
	11	0
	Mean 12·8	Mean 11·3
Normal	0	
	0	
	0	

adjuvant into the foot pad and treated with I.C.I. 43,823 daily did not develop secondary lesions yet the tuberculin reaction, assessed 24 hr after injecting Old Tuberculin into the flank on day 13, was indistinguishable in diameter and appearance from that seen in untreated controls (Table 2). The injection of Old Tuberculin into the flank of normal rats did not elicit any response. When the tuberculin reactions were assessed again 48 hr after injection, the erythema had disappeared in three of the five rats previously treated with I.C.I. 43,823 and had diminished in the two remaining rats. In four of the untreated controls, the diameter and appearance of the reaction after 48 hr was no different from that observed 24 hr previously.

When I.C.I. 43,823 (200 mg/kg) was administered daily by mouth for 3 days only, starting 1 day before the injection of adjuvant into the foot pad, the results of the assessment on the 14th day were similar to those obtained after continuous daily dosing (Table 3). If the delay from the time of injection to the administration of the first of a series of daily oral doses was longer than 3 days, the compound was ineffective (Table 3).

TABLE 3

THE EFFECT ON THE DEVELOPMENT OF SECONDARY LESIONS OF ADMINISTERING ORALLY I.C.I. 43,823 (200 MG/KG) AT DIFFERENT TIMES BEFORE AND AFTER THE INJECTION OF ADJUVANT INTO THE FOOT PAD

Each group contained five rats. Day 0=day of injection. Controls were untreated

Period of dosing (days)	Assessment 13 days after injection: Inhibition of increase in foot thickness (%)	Assessment 13 days after injection: No. of rats with secondary lesions
−1 to 13	39	Nil
1 to 13	35	1 mild
3 to 13	9	2 mild, 1 moderate
5 to 13	0	5 moderately severe
−1 to 1	40	Nil
Controls	—	5 severe

Doses of 200 mg/kg of I.C.I. 43,823 did not reduce the inflammation which followed the injection of formalin into the foot pad or kaolin into the ankle, nor was the granulation tissue which formed around a subcutaneous injection of carrageenin reduced by treatment with I.C.I. 43,823 (200 mg/kg daily for 5 days). Doses of 200 and 100 mg/kg of I.C.I. 43,823 partially reduced the oedema which followed the injection of carrageenin into the foot pad of rats.

DISCUSSION

Chemotherapeutic studies previously reported (Newbould, 1963) showed that the inflammation associated with both primary and secondary lesions in rats injected with adjuvant into the foot pad could be reduced by treatment with nonsteroidal compounds of known value in the treatment of rheumatoid arthritis in man. However, none of these compounds prevented the development of secondary lesions. During treatment with the anti-inflammatory steroid paramethasone, primary and secondary lesions did not develop, but both types of lesion subsequently appeared after treatment had been withdrawn. Thus, whilst all these compounds exhibited anti-inflammatory activity in this laboratory model they were unable to interfere permanently with the disease process involved in the establishment of secondary lesions.

I.C.I. 43,823 has biological properties which differ from those of known anti-inflammatory compounds. The compound is devoid of activity against most models of acute inflammation. It partially inhibited oedema due to carrageenin in the rat but this finding is probably irrelevant to its mode of action in the rat arthritis syndrome, since the secondary phase of this syndrome was suppressed by doses given only during the first 3 days. By far the most striking feature of I.C.I. 43,823 is its ability to prevent permanently the development of the secondary swelling in the injected foot and the development of secondary lesions in rats injected with adjuvant into the foot pad. These beneficial effects are reflected in the leucocyte counts of peripheral blood for, whilst untreated control rats exhibited a biphasic increase in leucocytes, the first phase associated with the development of the primary swelling and the secondary phase associated with the secondary increase in thickness of the injected foot and the development of secondary lesions, no secondary leucocytosis occurred in rats treated with I.C.I. 43,823. Since there was no evidence of general cytotoxic activity when high doses of I.C.I. 43,823 were administered for long periods to normal rats, the absence of a secondary leucocytosis during treatment with I.C.I. 43,823 is considered to be a consequence of the absence of secondary lesions and not the cause.

The mode of action of I.C.I. 43,823 is obscure and currently it is difficult to predict whether it is capable of being resolved on the basis of existing theories of the aetiology and pathogenesis of secondary lesions. The most acceptable theory of the origin of secondary lesions suggests that they are the result of a generalized immunological response to constituents of the tubercle bacillus which have become disseminated after injection (Pearson & Wood, 1959; Waksman & Sharp, 1960; Waksman, Pearson & Sharp, 1960; Waksman & Wennersten, 1963). These authors have presented evidence which strongly suggests that the development of secondary lesions involves an immunological response. However, whilst tubercle bacilli or their constituents have been implicated because arthritic rats exhibit a delayed reaction to intradermal injections of tuberculin and rats inoculated with adjuvant early in life fail to develop secondary lesions when challenged again with adjuvant

in later life, tubercle bacilli or their constituents have not been demonstrated specifically at sites where secondary lesions arise. Evidence is accumulating (Newbould & Snow, unpublished) which suggests that treatment with I.C.I. 43,823 does not interfere with the dissemination of tubercle bacilli from the injection site. If this is so, then I.C.I. 43,823 may be interfering with the immune response. However, rats which do not develop secondary lesions as a result of treatment with I.C.I. 43,823 still exhibit a delayed, 24-hr, reaction to tuberculin when this is injected on the 13th day after the injection of adjuvant. The 48-hr tuberculin reaction is much less striking in treated rats, but we do not yet know if this observation is relevant to the mode of action of the compound. The excellent chemotherapeutic effects obtained after administering I.C.I. 43,823 for 3 days only starting 1 day before the injection of adjuvant into the foot pad suggest that the compound is interfering specifically with an early phase in the development of the syndrome. Since from day 0 to day 5 lymph nodes have been shown to play an important role in the development of secondary lesions (Newbould, 1964a, b), the effects of I.C.I. 43,823 on the cellular changes within these nodes are now being studied.

We do not know, as yet, if I.C.I. 43,823 will be of use in the treatment of rheumatoid arthritis in man because a toxicological problem has been encountered which must be further evaluated before the compound can be administered to human beings.

SUMMARY

1. The intradermal injection of Freund's adjuvant into the foot pad of rats produces an inflamed "primary" lesion at the site of injection and then, after a delay of approximately 10 days, inflamed " secondary " lesions in areas of the body remote from the injection site.

2. The inflammation associated with the development of primary and secondary lesions is reduced by daily treatment with compounds of known value in the chemotherapy of rheumatoid arthritis in man but none of these compounds interferes permanently with the disease process involved in the establishment of secondary lesions in rats.

3. Daily treatment with I.C.I. 43,823 does not suppress the acute inflammation which develops at the site of the primary lesion. However, secondary lesions do not develop even if I.C.I. 43,823 is administered for 3 days only, starting 1 day before the injection of adjuvant into the foot pad.

4. The mode of action of I.C.I. 43,823 is unknown. High doses given for long periods to normal rats cause no reduction in the number of circulating leucocytes and rats in which the development of secondary lesions has been suppressed still exhibit a 24-hr tuberculin reaction.

I wish to thank my colleague, Mr G. E. Davies, for studying the effects of I.C.I. 43,823 on various models of acute inflammation. I also wish to thank Mrs R. Bradbury, Miss C. Dale, Miss S. Warrington and Mr R. Siddall for their expert technical assistance.

REFERENCES

CLARKSON, R., HULL, R. & NEWBOULD, B. B. (1961). United Kingdom Patent No. 971,176.

HOUSSAY, R. H. & FRANGIONE, B. (1961). On the pathogenesis of the arthropathy induced with Freund's adjuvant. In *Atti Del X Congresso della Lega Internazionale Contro il Reumatism*, vol. 2, p. 950. Turin: Tipografia Edizioni Minerva Medica.

NEWBOULD, B. B. (1963). Chemotherapy of arthritis induced in rats by mycobacterial adjuvant. *Brit. J. Pharmacol.*, **21**, 127–136.

NEWBOULD, B. B. (1964a). Lymphatic drainage and adjuvant induced arthritis in rats. *Brit. J. exp. Path.*, **45**, 375-383.

NEWBOULD, B. B. (1964b). Role of lymph nodes in adjuvant-induced arthritis in rats. *Ann. rheum. Dis.*, **23**, 392–396.

PEARSON, C. M. (1956). Development of arthritis, periarthritis and periostitis in rats given adjuvants. *Proc. Soc. exp. Biol. (N.Y.)*, **91**, 95–101.

PEARSON, C. M. (1959). Development of arthritis in the rat following injection with adjuvant. In *Mechanisms of Hypersensitivity*, ed. SHAFFER, J. H., LOGRIPPO, G. A. & CHASE, M. W., pp. 647–671. Boston: Little Brown.

PEARSON, C. M. & WOOD, F. D. (1959). Studies of polyarthritis and other lesions induced in rats by injection of mycobacterial adjuvant. 1. General clinical and pathologic characteristics and some modifying factors. *Arthr. and Rheum.*, **2**, 440–459.

STOERK, H. C., BIELINSKI, T. C. & BUDZILOVICH, T. (1954). Chronic polyarthritis in rats injected with spleen in adjuvant. *Amer. J. Path.*, **30**, 616.

WAKSMAN, B. H., PEARSON, C. M. & SHARP, J. T. (1960). Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. *J. Immunol.*, **85**, 403–417.

WAKSMAN, B. H. & SHARP, J. T. (1960). Immunologic nature of arthritis induced in rats by mycobacterial adjuvant. *Fed. Proc.*, **19**, 210.

WAKSMAN, B. H. & WENNERSTEN, C. (1963). Passive transfer of adjuvant arthritis in rats with living lymphoid cells of sensitized donors. *Int. Arch. Allergy*, **23**, 129–139.

WARD, J. R. & JONES, R. S. (1962). The role of adjuvant composition and its route of administration in the production of rat polyarthritis. *Arthr. and Rheum.*, **5**, 125–126.

WINTER, C. A., RISLEY, E. A. & NUSS, G. W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. exp. Biol. (N.Y.)*, **111**, 544–547.