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STUDIES ON THE INDUCTION OF IMMUNOLOGICAL TOLERANCE BY ANTIGEN IN GUINEA-PIGS ALREADY SENSITIZED TO DINITROCHLOROBENZENE

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SUMMARY

When guinea-pigs showing a high degree of contact sensitivity to dinitrochlorobenzene (DNCB) were injected intravenously with a related hapten, dinitrobenzene sulphonic acid sodium salt ($DNBSO_3$), the animals became totally unresponsive for 1 to 4 days and remained thereafter in a state of diminished sensitivity, lasting for 8 weeks. No lasting total unresponsiveness was achieved by an additional intradermal injection of DNCB (or various variations thereof) performed 6 hr after the intravenous injection ('double shot' procedure), in contrast to what had been observed in the Neoarsphenamine and potassium dichromate system. The effect of the intravenous injection of $DNBSO_3$ is attributed to a temporary disappearance of the sensitized lymphoid cells from the circulation, whereas the induction of a permanent unresponsiveness certainly requires some additional mechanism.

INTRODUCTION

The induction of a long lasting specific unresponsiveness in already sensitized individuals is of theoretical interest and could be of considerable importance in human medicine. In guinea-pigs already presenting a delayed-type hypersensitivity to Neoarsphenamine (NEO) we succeeded in inducing a specific unresponsiveness lasting up to 2 years merely by the intravenous injection of a high dose of NEO, followed within 6–24 hr by the intradermal injection of a much lower dose (Frey, Geleick & de Weck, 1964; Frey, de Weck & Geleick, 1966). Animals treated by this procedure ('double shot') no longer showed delayed type responses to NEO and could not be resensitized, even with NEO injected in Freund's complete adjuvant (FCA). The unresponsiveness obtained was clearly dependent upon the dose administered and the time interval between both injections.

These observations were confirmed by Polak & Turk (1968) in guinea-pigs sensitized to

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potassium dichromate and a comparable state of unresponsiveness may have been obtained in the patients previously sensitized to Mechlorethamine as described by Waldorf, Haynes & Van Scott (1967).

In previous experiments we have shown that in dinitrochlorobenzene (DNCB) sensitized animals an intravenous injection of DNCB (Frey & Geleick, 1962) as well as other immunologically related and crossreactive compounds like dinitrobenzenesulphonic acid sodium salt ($DNBSO_3$) and some dinitrophenyl (DNP)–amino acids or DNP–protein conjugates (Frey, de Weck & Geleick, 1964a; de Weck, Frey & Geleick, 1964) caused a specific but only *temporary* inhibition of the contact hypersensitivity.

The purpose of this study was to ascertain whether with the 'double shot' procedure a *permanent* tolerance could be induced in animals already contact sensitive to DNCB. It was of special interest to establish if this phenomenon also occurs with DNCB a non-metallic, low molecular and well known chemical sensitizer.

MATERIAL AND METHODS

Dinitrophenyl compounds. 2,4-dinitrochlorobenzene (DNCB) was obtained from Merck, Darmstadt, Germany; 2,4-dinitrofluorobenzene (DNFB) from Roche, Basle, Switzerland and 2,4-dinitrobenzenesulphonic acid sodium salt ($DNBSO_3$) from Eastman Kodak, Rochester, N.Y.; 2,4-dinitrophenyl-S-glycine (DNP-S-Gly) was obtained from Roche, Basle, Switzerland. Dinitrophenyl-bovine γ-globulin (DNP–BGG) and dinitrophenyl-guinea-pig serum (DNP-GPS) were prepared as described elsewhere (de Weck & Frey, 1966). These conjugates had the following degrees of substitution: DNP_{13}-BGG, 13_M DNP/$1{\cdot}6 \times 10^5$ g BGG; DNP_{18}-GPS, 18_M DNP/10^5 g GPS protein.

Animals. White spotted Himalayan guinea-pigs of both sexes, weighing 300–500 g and originating from a closed colony, were used throughout. They were fed on a pellet diet, with additional greens and water ad libitum.

Epicutaneous sensitization to DNCB. 0·002 ml of a 50% solution in acetone containing 1 mg DNCB was applied with a pipette to the shaved skin of the guinea-pigs' necks. The site was then covered with adhesive tape, which was left in place for 7 days.

Epicutaneous tests with DNCB: 0·025 ml solutions of DNCB in acetone at 0·09, 0·05 and 0·03% were applied with a pipette to three skin areas each measuring 2 cm^2. Such concentrations are non-toxic for normal guinea-pigs. The contact reactions were evaluated after 24 hr according to an arbitrary scale from 0 to 3, described previously (Frey, de Weck & Geleick, 1964a). The average degree of contact sensitivity is given by the arithmetical mean of all reactions of an animal group.

Intravenous injection of $DNBSO_3$. Doses of 175–600 mg/kg contained in 2–3 ml of water were injected into the vein of the front or hind legs with a 22 gauge needle. After the hair had been plucked, the vein was exposed by an incision of the skin, the wound being closed afterwards with 2–3 Michelclamps.

Intradermal injection of DNCB. Doses of 10 μg were administered by injecting into the skin of the flank 0·05 ml of a 0·02% solution in a mixture of ethanol–water 1:25. To inject 1 mg, we administered 0·1 ml of a 1% solution in ethanol (94%). Given with Freund's complete adjuvant (FCA) equal parts of a 2% solution of DNCB in ethanol were mixed with FCA (Difco) and 0·1 ml of the mixture containing 1 mg DNCB was injected intradermally.

Statistical methods. To determine whether the sensibility in a group of animals increased or decreased significantly we used the Wilcoxon matched pairs signed rank test (Sachs, 1968). To evaluate whether the treated groups differed significantly from the control groups we used the χ-test by van de Waerden (1957) and the Smirnoff-Kolmogoroff-test (Sachs, 1968). 0·05 was chosen as the level of significance.

Determination of anti-dinitrophenyl antibodies by passive cutaneous anaphylaxis (PCA). Guinea-pigs were bled by cardiac puncture 1, 4 and 15 days after the intravenous injection of $DNBSO_3$ whether followed or not by a subsequent intradermal injection of DNCB. The sera obtained were diluted 1 : 5 in 0·01 M phosphate-saline, and 0·1 ml was injected intradermally in two or three normal guinea-pigs. The intravenous challenge was performed after 20–24 hr with 0·5 ml of DNP_{17}-HSA containing approximately 2 mg of conjugate and 0·5 ml 2% Evans blue. The diameter of blueing reactions was recorded after 20–30 min.

General design of experiments. To ascertain whether a permanent tolerance could be induced in animals contact sensitive to DNCB by the 'double shot' procedure described above, the following experiment and corresponding controls were performed.

Four groups of eight highly contact-sensitive animals were formed and treated as follows:

Animal Groups	Intravenous injection	Intradermal injection
(a)	saline 3 ml/kg	not given
(b)	saline 3 ml/kg	DNCB 1 mg
(c)	$DNBSO_3$ 600 mg/kg	not given
(d)	$DNBSO_3$ 600 mg/kg	DNCB 1 mg

The time interval between the two injections was 6 hr. All animals were tested epicutaneously with DNCB 6 hr after the intravenous injection and again 1, 2, 3, 4, 7, 14 and 56 days later.

RESULTS

Influence of the 'double shot' procedure on contact sensitivity

As indicated in Fig. 1, when tested 6 hr after the intravenous injection the animals of Groups (c) and (d) showed a strong decrease in contact sensitivity, most of them becoming totally unresponsive. After 24 hr, a gradual recovery of hypersensitivity was observed which continued from days 2–4. However, after 1 and 2 weeks, the animals had still not regained their initial hypersensitivity level, the difference between the initial and the level at 2 weeks being significant. As the depression observed in Group (d) was not stronger than in Group (c) this effect had to be attributed exclusively to the intravenously injected $DNBSO_3$, the additional intradermal injection of DNCB being irrevelant in this system.

The sensitivity level of Groups (a) and (b) remained unchanged up to the first week; later, a slight but significant increase was observed, which may possibly be due to the booster effect of repeated testing. When again testing all animals after 8 weeks, Groups (c) and (d) had almost regained their initial hypersensitivity, although their responses were still lower than the initial level. At that time, however, Groups (c) and (d) were significantly less hypersensitive than Groups (a) and (b).

Influence of the 'double shot' procedure on circulating antibodies.

Preliminary experiments were performed in order to evaluate the effect of the 'double shot' procedure on the circulating skin sensitizing antibodies of DNP specificity which are

frequently found in guinea-pigs sensitized epicutaneously with DNCB. Accordingly, sera of the four groups of animals described (a, b, c, d) were investigated by passive cutaneous anaphylaxis. From the results summarized in Table 1, it appears that the intravenous injection of $DNBSO_3$ in addition to depressing the contact sensitivity also reduces temporarily the presence of circulating anti-DNP antibodies. In fact, the PCA reactions became negative with sera of one half of the animals of Groups (c) and (d), which had

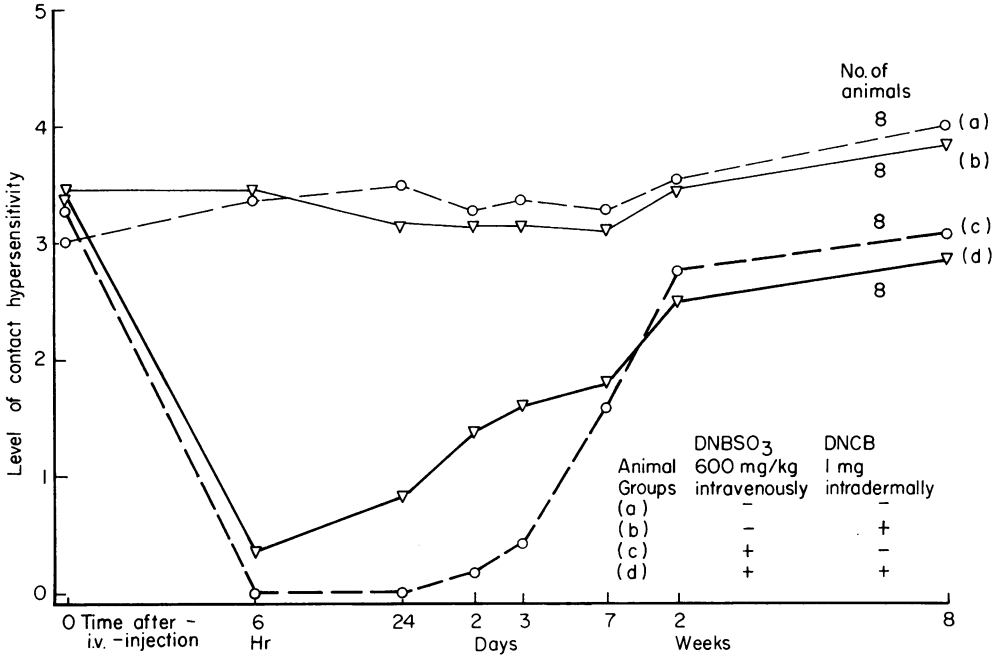

FIG. 1. Effect of an intravenous injection of 600 mg/kg $DNBSO_3$ and/or an intradermal injection of 1 mg DNCB on the level of contact hypersensitivity to DNCB.

Animal Groups	Intravenous injection	Intradermal injection
(a)	saline 3 ml/kg	not given
(b)	saline 3 ml/kg	DNCB 1 mg
(c)	$DNBSO_3$ 600 mg/kg	not given
(d)	$DNBSO_3$ 600 mg/kg	DNCB 1 mg

received $DNBSO_3$ intravenously 24 hr before and which had demonstrable PCA antibodies before the injection. As for contact sensitivity, this depressing effect was independent of the subsequent intradermal injection of DNCB. The result of PCA reactions performed on days 4 and 15 showed in some animals a parallelism to the evolution of contact sensitivity, i.e. both reactions became restored. However, there were also numerous cases of complete dissociation, i.e. animals showing complete abolition of contact sensitivity followed by restoration but with PCA antibodies remaining demonstrable throughout or, on the contrary, cases of complete abolition of the PCA reaction which was not restored on day 15 when contact sensitivity had partially reappeared.

Determination of antibody titres and dialysis of the serum samples (especially those of the first post-injection day) should permit us to ascertain that the intravenous injection really impairs antibody production and not merely 'masks' the presence of anti-DNP antibodies by hapten inhibition. This, however, is most unlikely for serum samples taken 15 days after the intravenous injection of $DNBSO_3$. In such cases, the negativation of the PCA reaction should actually reflect a central effect on the formation of skin sensitizing antibodies.

TABLE 1. Influence of the 'double shot' procedure on circulating anti-DNP-antibodies

Animal Groups	PCA	Contact-sensitivity level	Double shot		Contact-sensitivity level	PCA	Contact-sensitivity level	PCA	Contact-sensitivity level	PCA
Days	−2	−1	0		1	1	3	4	14	15
			i.v.	i.d.						
(a)	12/12	3·9	ϕ	ϕ	3·8	11/11	3·9	7/7	3·9	7/7
(b)	7/7	3·3	ϕ	DNCB (1 mg in FCA)	4·8	5/7	5·2	7/7	5·6	7/7
(c)	19/19	4·4	$DNBSO_3$ (600 mg/kg)	ϕ	0·4	9/19	0·6	6/13	2·2	9/13
(d)	7/7	5·2	$DNBSO_3$ (600 mg/kg)	DNCB (1 mg in FCA)	0·1	4/7	0·9	5/7	3·6	6/6

Passive cutaneous anaphylaxis (PCA). No. of animals with positive sera/total of animals tested. Contact sensitivity level: mean of contact sensitivity of the group (see 'Material and Methods').

Experimental variations. As in the first experiment, only an initial short lasting total unresponsiveness followed by a slight and partial unresponsiveness could be achieved after 2 and 8 weeks, further experiments were performed in which we varied the dose, number, frequence and substance used for the intravenous and for the intradermal injections.

As indicated in Table 2, the total dose of $DNBSO_3$ injected intravenously was fractionated in four doses, administered every 4 hr, or single daily doses were repeated at different intervals. In all schedules, the maximal tolerated dose of the compound was administered. On the other hand, DNCB was injected intradermally in very low doses (keeping in mind low-zone tolerance), given in FCA or administered in single or repeated daily doses at different intervals, following the intravenous injection. In some experiments, twenty intradermal doses of 5 μg DNCB were injected into different regions of the skin in order to reach all accessible regional lymph nodes, or two thirds of the skin surface of the animals were painted with a 0·5% DNCB solution in acetone, applying in this way a total dose of 15 mg. In other experiments, DNCB was given orally in doses of 6 mg daily some days before and after the intravenous injection of $DNBSO_3$. Finally, DNCB was replaced in some experiments by DNP-BGG, DNP-GPS, DNPS-Glycine and $DNBSO_3$.

In all these experiments, groups of 4–12 'experimental animals' receiving intravenous plus intradermal injections Group (d), were compared to control groups being tested only Group (a), receiving only intradermal injections Group (b), or only intravenous injections Group (c).

When more than one intravenous or intradermal injection was given, the series of epicutaneous tests were started 6 hr after the last pair of injections. The time interval between the intravenous and the intradermal injection was 6 hr in all experiments. The results of all the experimental variations mentioned in Table 2 were essentially similar to those of the first

TABLE 2. Experimental variations of the 'double shot' procedure in attempt to induce long lasting unresponsiveness in guinea-pigs contact sensitive to DNCB

Intravenous injection of $DNBSO_3$ (mg/kg)	Intradermal injection of DNCB or other DNP-compounds
1 × 600	1 × 1 mg
1 × 600	$1{\cdot}3_M$ DNP/$_M$BGG
1 × 600	$1{\cdot}79_M$ DNP/$_M$GPS
4 × 175 every 4 hr	4 × 0·01 mg every 4 hr
4 × 300 on consecutive days	4 × 0·01 on consecutive days
8 × 300 on days 0 to 10	8 × $1{\cdot}75_M$ DNP/$_M$GPS; days 0 to 10
4 × 400 on alternate days	4 × 1 mg in FCA
4 × 400 on alternate days	4 × 1 mg $DNBSO_3$
4 × 400 on alternate days	4 × 1 mg DNP-S-glycine
4 × 500 every 4 day	no i.d. injection
4 × 500 every 7 day	no i.d. injection
2 × 500 on day −14 and day 0	1 × 1 mg on day 0
1 × 600	1 × 1·0 μg
1 × 600	1 × 0·1 μg
1 × 600	1 × 0·01 μg
1 × 600	20 × 5 μg on different skin sites
1 × 600	Skin painting with DNCB in aceton
1 × 600 on day 0	6 mg DNCB orally on days −21 to 4
4 × 300 on days 0 to 3	6 mg DNCB orally on days −21 to 4
1 × 600	6 mg DNCB orally on days 0 to 7
4 × 300 on days 0 to 3	6 mg DNCB orally on days 0 to 7

experiment. In all cases, the animals of Groups (c) and (d) (ninety-one and forty-seven animals respectively) showed a total unresponsiveness after 6–24 hr, a gradual recovery of hypersensitivity from days 7–14 and a significantly lower level of hypersensitivity after 8 weeks, which at this time was also significantly lower than the levels of Groups (a) and (b) (forty-six and twenty-six animals respectively).

DISCUSSION

As the problem of the induction of unresponsiveness in already sensitized individuals has theoretical and practical implications, it may be worthwhile to discuss briefly the possible causes of the different results obtained in the DNP, NEO and Cr systems.

One may safely postulate that in the case of delayed hypersensitivity, already sensitized individuals possess a considerable population of circulating 'sensitized lymphoid cells' consecutively to the stimulation of stem cells located in the central lymphoid organs, e.g. the lymph nodes and the spleen. We have already shown that in the DNP, NEO and Cr

systems the intravenous injection alone induced only temporary unresponsiveness, probably by destroying or inactivating all the circulating sensitive cells. However, the capacity of the central lymphoid organs to produce circulating sensitized lymphoid cells does not appear to have been seriously impaired, as hypersensitivity was partially restored within a few days. As stated already, a permanent and total unresponsiveness was only achieved with neoarsphenamine or potassium dichromate when a second intradermal or epicutaneous application of the same hapten was administered no later than 6 hr to 5 days after the intravenous one. We therefore have to assume that the second application either influences in some decisive and permanent way the stem cells in the central lymphoid organs or 'masks' the hypersensitivity by preventing sensitized lymphoid cells from accumulating in the circulation.

A possible reason for achieving only a limited degree of unresponsiveness in the DNP system and for the ineffectiveness of the second (intradermal) injection could be that the doses of $DNBSO_3$ injected intravenously were too low. Because of systemic toxicity, no higher daily doses could be reached, even when injecting the total dose in fractions administered every 4 hr, and no better results were achieved by injecting $DNBSO_3$ four times on consecutive or alternate days or even at longer intervals (Table 2). The relationship between the intravenous dose of $DNBSO_3$ and the population of immunocompetent cells to be reached in order to achieve complete immunological tolerance, is stressed by the fact that normal adult guinea-pigs require doses of 250 mg/kg (Frey, de Weck & Geleick, 1964b) and newborn animals doses of 50 mg/kg (unpublished). Accordingly, doses of 600 mg/kg may be too low to achieve long-lasting unresponsiveness in already sensitized adult animals possessing a much larger number of specifically sensitized and/or immunocompetent cells.

This reasoning is supported by the results of Polak & Turk (1968), who obtained a permanent unresponsiveness in the Cr system, injecting intravenously 20 mg/kg of potassium dichromate followed by topical application, whereas lower intravenous doses induced only temporary desensitization. However, if such low doses were supplemented by antilymphocytic sera and immunosuppressive drugs, which may diminish the population of sensitized cells, a permanent unresponsiveness was again achieved.

This is also in agreement with our earlier experiments with NEO (unpublished), where a clear dose dependence of the intravenous injection to induce long-lasting unresponsiveness was demonstrated. It seems, therefore, that the intravenous dose must be high enough to enable the intradermal injection to work. In our present experiments with DNCB, despite all our efforts to enhance the activity of the intradermal injection, no long-lasting total unresponsiveness was achieved, possibly because intravenous doses of $DNBSO_3$ were too low.

However, this apparently simple explanation may not be so readily acceptable. The intravenous doses of $DNBSO_3$ (not followed by an intradermal one) were sufficient to achieve an early, complete but only temporary unresponsiveness, the situation a few hours after the intravenous injection (presumably a disappearance of sensitized lymphoid cells from the circulation) appearing very similar in the DNP, the neoarsphenamine and the Cr systems. Therefore, one is led to postulate the existence of two dose levels for the intravenous injection, namely, one sufficient to achieve early and temporary unresponsiveness and a higher one capable of achieving long-lasting tolerance, provided that an additional intradermal injection is administered. It should not be overlooked that in the NEO and Cr systems this second intradermal or epicutaneous application is essential in order to achieve

long-lasting unresponsiveness and may not be replaced by higher and/or repeated intravenous injections.

What is the role of the topical deposit of antigen in a sensitized animal temporarily deprived of circulating sensitized lymphoid cells by the previous intravenous injection? One interpretation (I. Lefkowits, personal communication) could be that the topical application 'calls out' from the central lymphoid organs, immunocompetent cells which would otherwise not be accessible to the intravenously injected hapten. Hapten, still present in the circulation and/or in the organism, then exerts its tolerogenic effect and true tolerance would ensue. The difference observed between the DNP and the other systems would then be essentially attributed to a quantitative difference in the elimination rate within the critical period of 1–2 days following the intravenous injection.

Another possibility is that the topical administration of hapten creates a permanent depot of antigen, able to continuously activate (exhaustive stimulation) and 'filter out' the sparsely-arising, sensitized lymphoid cells developing during the recovery phase. In such a case, we are dealing not with a true tolerance but with a masking of hypersensitivity by some 'treadmill' mechanism, as demonstrated for unresponsiveness towards non metabolizable polysaccharides (Felton *et al.*, 1955; Howard, 1969). The difference observed between the DNP and the other systems would reside not in their short but in their long range elimination rates. In favour of the last possibility there is the observation (Polak, Frey & Turk, unpublished) that peritoneal exudate cells from Cr desensitized animals still demonstrate immunological reactivity. Consequently, such 'desensitized' animals still possess a specifically reacting cell clone. However, it could also be envisaged that the 'double shot' procedure induces in the Cr-system a split tolerance, namely a permanent desensitization (clone abolished) for delayed hypersensitivity but no impairment of the antibody-producing clone. Apparently against the concept of continuous inactivation of specific cells by antigen deposit is the fact that the local site of topical application needs only to remain in place for 24 hr to permit achievement of long-lasting unresponsiveness (Polak & Turk, 1969). Although in already sensitized animals no long-lasting unresponsiveness could be achieved in the DNP system, our observations point to some of the factors probably required. Further investigations on the half-life of haptens, whether effective or not in the double shot procedure, and experiments on the immunological reactivity of lymphoid cells from desensitized animals should throw more light on this interesting and potentially important biological phenomenon.

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REFERENCES

FELTON, L.D., KAUFMANN, GLADYS, PRESCOTT, B. & OTTINGER, BARBARA (1955) Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides. *J. Immunol.* **74,** 17.

FREY, J.R. & GELEICK, H. (1962) Desensibilisierung durch intravenöse Injektion von Dinitrochlorbenzol beim Kontaktekzem des Meerschweinchens. *Dermatologica,* **125,** 132.

FREY, J.R., GELEICK, H. & DE WECK, A.L. (1964) Immunological tolerance induced in animals previously sensitized to simple chemical compounds. *Science,* **144,** 853.

FREY, J.R., DE WECK, A.L. & GELEICK, H. (1964a) Inhibition of the contact reaction to Dinitrochlorobenzene by intravenous injection of Dinitrobenzene sulfonate in guinea pigs sensitized to Dinitrochlorobenzene. *J. invest. Derm.* **42,** 189.

FREY, J.R., DE WECK, A.L. & GELEICK, H. (1964b) Immunological unresponsiveness in allergic contact dermatitis to Dinitrochlorobenzene in guinea-pigs. *J. invest. Derm.* **42,** 41.

FREY, J.R., DE WECK, A.L. & GELEICK, H. (1966) Sensitization, immunological tolerance and desensitization of guinea-pigs to Neoarsphenamine. IV. Desensitization to NEO. *Int. Arch. Allergy*, **30,** 521.

HOWARD, J. (1969) *Properties of Antigen. Immunological Tolerance* (Ed. by M. Landy and W. Braun), p. 28. Academic Press, New York and London.

POLAK, L. & TURK, J.L. (1968) Studies on the effect of systemic administration of sensitizers in guinea-pigs with contact sensitivity to inorganic metal compounds. I. The induction of immunological unresponsiveness in already sensitized animals. *Clin. exp. Immunol.* **3,** 207.

POLAK, L. & TURK, J.L. (1969) Studies on the effect of systemic administration of sensitizers in guinea-pigs with contact sensitivity to inorganic metal compounds. III. The effect of immunosuppressive agents on enhancing the unresponsiveness of already sensitized animals and an investigation of the action of the epicutaneous test within 24 hr. *Clin. exp. Immunol.* **4,** 423.

SACHS, L. (1968) *Statistische Auswertungsmethoden*, pp. 291 and 312. Springer, Berlin.

VAN DE WAERDEN, B.L. (1957) *Mathematische Statistik*, p. 285. Springer, Berlin.

WALDORF, D.S., HAYNES, H.A. & VAN SCOTT, E.J. (1967) Cutaneous hypersensitivity and desensitization to Mechlorethamine in patients with mycosis fungoides lymphoma. *Ann. Intern. Med.* **67,** 282.

WECK DE, A.L., FREY, J.R. & GELEICK, H. (1964) Specific inhibition of contact dermatitis to Dinitrochlorobenzene in guinea pigs by injection of haptens and protein conjugates. *Int. Arch. Allergy* **24,** 63.

WECK DE, A.L. & FREY, J.R. (1966) Immunotolerance to simple chemicals. *Monogr. Allergy*, **1,** 137.