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Presence of a Substance in the Third International Standard of Old Tuberculin that Interacts negatively with the Biological Potency of the Preparation

OTTO GALTUNG,[1] FINN FODSTAD,[2] KÅRE MORDAL[3] & HANS WAALER[4]

In the course of experiments designed as part of a biological assay of the Third International Standard of Old Tuberculin (OT) it was noticed that this preparation at the usual concentrations failed to elicit the expected concentration-response in persons on whom the von Pirquet was performed.

Further specially designed experiments have shown that this phenomenon is due to the presence in the Third OT Standard of a substance that interferes with, or blocks, the biological response to the tuberculin. However, the substance has not yet been identified nor has a model yet been constructed to explain in detail the nature of the interaction between that substance and the tuberculin.

Tuberculin preparations—whether PPD or OT—are composed of many different specific agents with widely different biological properties. In addition to these specific tuberculin agents, the preparations generally used also contain a series of non-specific substances, some intrinsic as a result of the production process and some deliberately added for a given purpose. Some of these non-specific substances may actively interfere with the biological response to the preparation administered.

Among these are: *Tween-80*, which is sometimes added to PPD products in order to prevent the adsorption of specific tuberculin molecules to the glass of the vials in which the PPD is dispensed, but which also affects the intensity of the tuberculin reactions (Guld & Roelsgaard, 1965; Wijsmuller et al., 1962); *adrenaline* (epinephrine), which is often added to OT products intended for von Pirquet tests with the expressed objective of increasing the potency of the product (by changing or enhancing the effective contact between the active substance and the biological tissue) (Galtung Hansen, 1937); and *glycerol*, which is inherent in all OT products although in varying concentrations (unpublished experiments have shown that adding glycerol will enhance the biological response when the tuberculin is used in high concentrations, but that it will only do so up to a certain concentration, after which the response is reduced again).

In the present paper evidence will be given suggesting that the Third International Standard of Old Tuberculin contains a substance (apart from glycerol) that strongly interferes with the biological potency of the product or at least the biological response when it is used in high concentrations in cutaneous tests. No attempt is made to investigate the actual mechanism (on the micro level); only the phenomenon itself is presented.

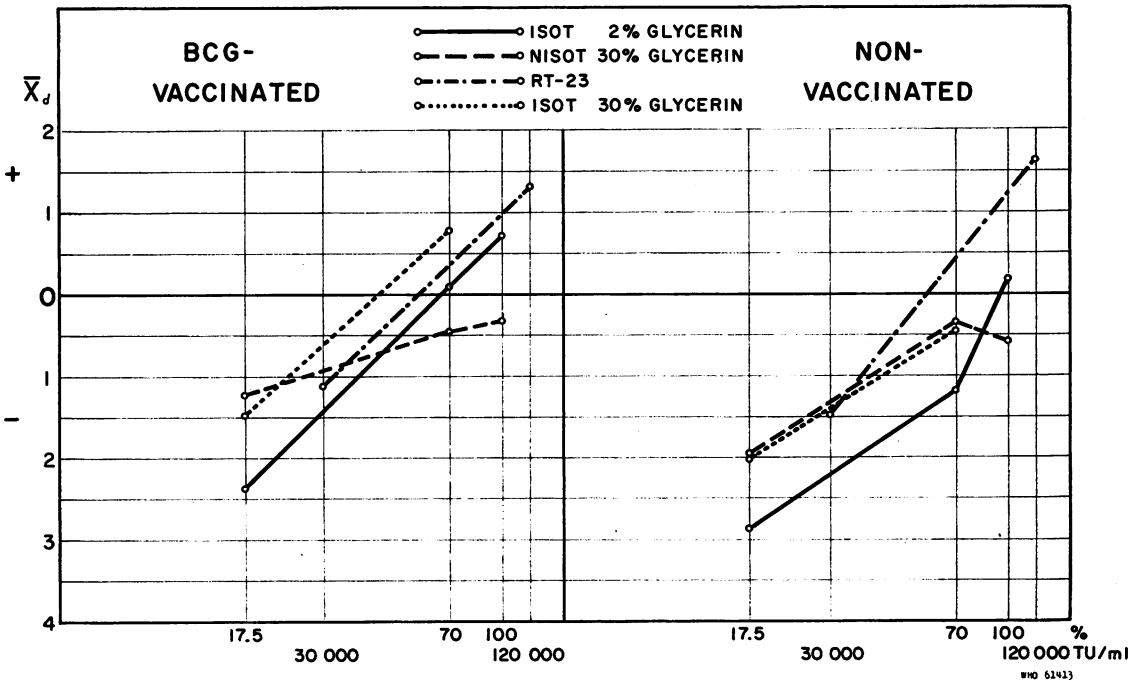
METHODS AND TEST TECHNIQUE

The test technique applied was a modified von Pirquet test. A drop of the concentrated tuberculin was placed on the volar aspect of the left forearm by means of a special nib-shaped scarificator. A scarification, 5 mm long, was then made in the superficial layer of the skin by means of the same instrument through the drop of tuberculin, and the site was left to dry for 5 minutes. The test reaction was read after 2–3 days. The present analysis is based on the width of the infiltrated area, measured

[1] Chief Officer for Tuberculosis, Directorate of Health, Oslo, Norway.
[2] Head of Department, Veterinary Institute, Oslo, Norway.
[3] Consultant, National Mass Radiography Service, Oslo, Norway.
[4] Senior Statistician, National Tuberculosis Register, Oslo, Norway.

FIG. 1

DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 1[a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

in millimetres at right angles to the scar. The tuberculin reactions were read by trained readers who had no knowledge of the kind or the concentrations of the tuberculin products used.

The standard von Pirquet test consists of 2 parallel scarifications on the upper two-thirds of the forearm. In the studies reported here, one scarification was made for 1 of 10 test preparations, the other for a reference tuberculin which was the same for all persons tested throughout each study. The two preparations were alternately assigned to the upper and lower scarification. The analysis presented here has been based on the difference between the two reactions.[1] Reactions smaller than 3 mm to both preparations have been excluded from the present analysis. The experiments mentioned in this report were all carried out in human populations. Tuberculin testing was carried out in conjunction with the current mass X-ray survey of the general population of Norway and a specially trained tuberculin-testing team was attached to one of the mobile X-ray units.

RESULTS

The Third International Standard of Old Tuberculin (NISOT) was included in a series of experiments at the request of the WHO International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, as a part of an international assay of this product. It was also included in experiments in connexion with the assay of Norwegian-made OT products.

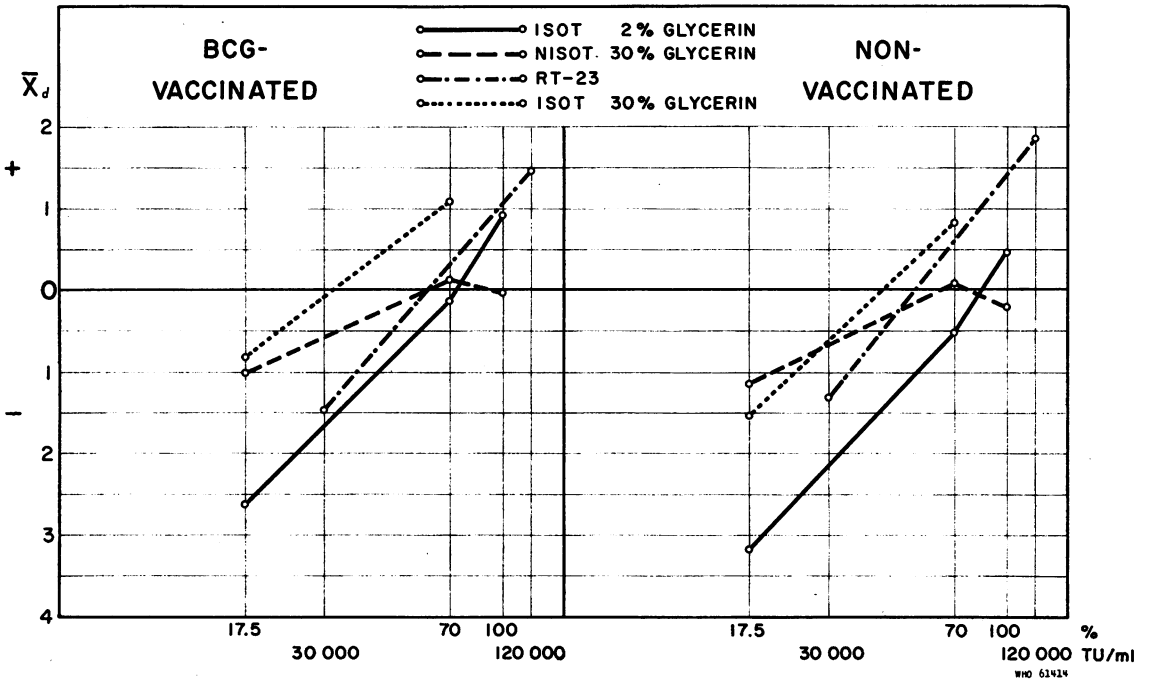
Studies 1 and 2

The products included in the first experiment are shown at the top of Fig. 1.[2] Because of the wide difference in glycerol content between the Second and Third Standards of Old Tuberculin (2% and 30%

[1] The corresponding correlation tables are not given here but are available to interested readers upon request to the authors.

[2] Tables showing the basic data used in plotting the graphs in Fig. 1, 2, 3, 4 and 5 are available to interested readers upon request to the authors.

FIG. 2
DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 2[a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

respectively), the Second Standard was also tested in dilutions in which the glycerol content was adjusted to 30%. Fig. 1 shows that increasing the glycerol content from 2% to 30% increased the potency by about 40%, but the dose-response (i.e., slope) remained the same. The crucial observation in the present context is, however, the deviating concentration-response for the Third OT Standard, which was almost non-existent for the highest concentrations.

This experiment was then repeated (Fig. 2) in order to exclude trivial coding-errors and the like.

Study 3

In spite of these results the Third Standard of Old Tuberculin was included in study 3 in the routine assay of a new batch of Norwegian OT (SMT-8) in relation to the previous batch (SMT-7) and to RT-23. The results (Fig. 3) are essentially the same as in studies 1 and 2. The Third OT Standard at this level of concentration and with this technique differed from the other products by its lack of any concentration-response. The effect of adrenaline added to the concentrated preparations of the Third OT Standard and of SMT-8 on the size of the tuberculin reaction was not significantly different for the two products.

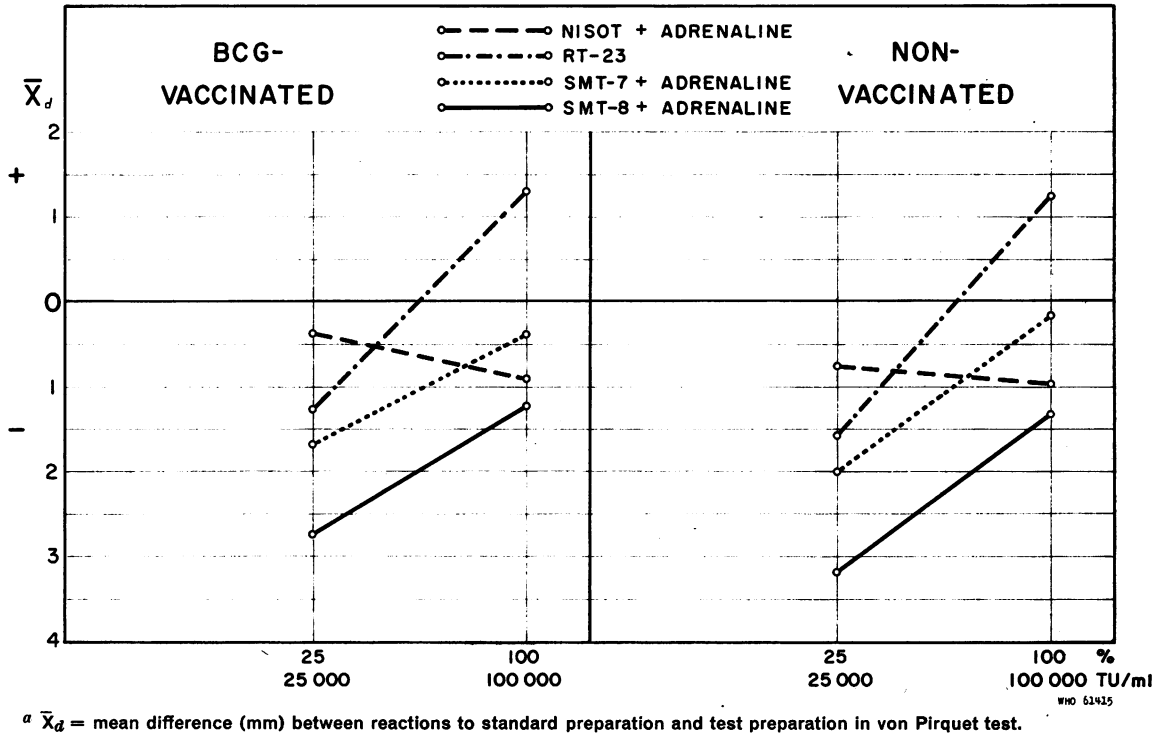
Study 4

When a tuberculin preparation is diluted, the relative content of various substances will change if the diluent is not identical with the original solution.

In order to examine whether such a change could elucidate the findings, a fourth study was undertaken in which the 25% concentrations were produced in 3 different ways, i.e., by dilution in (*a*) saline, (*b*) glycerinum purissimum and (*c*) glycerol pro analysis. The glycerinum purissimum was the kind of glycerol that was originally used in the production of the tuberculin preparation.

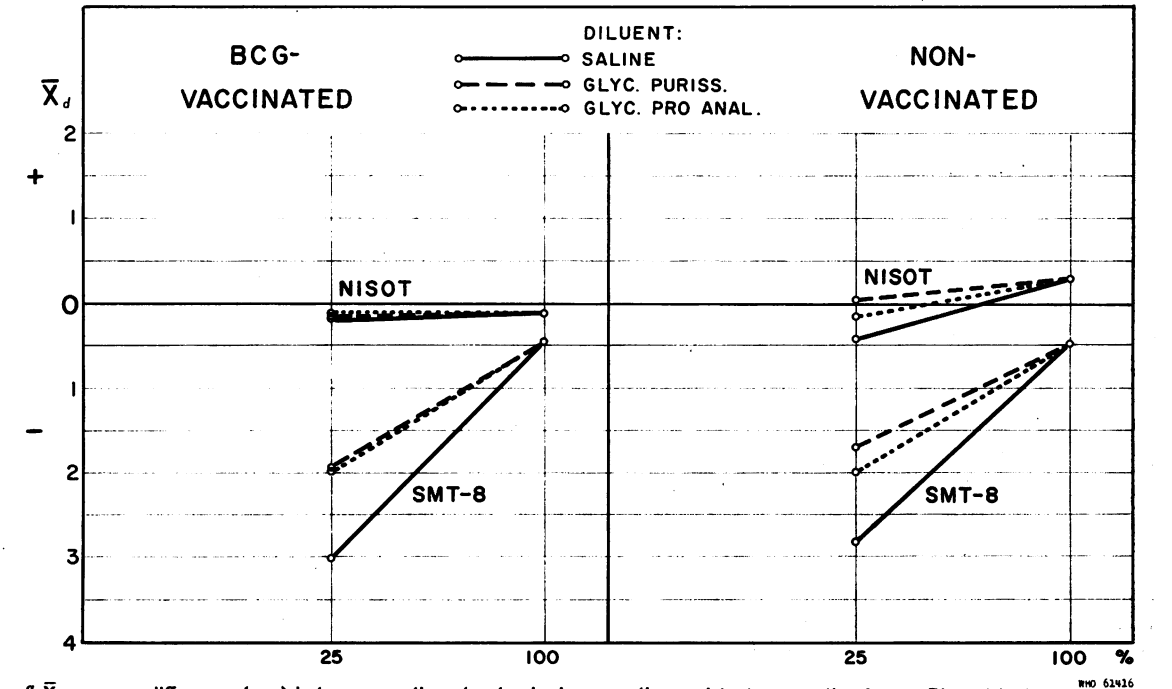
The results of this study (Fig. 4) showed that the factor under investigation could not be responsible for the anomaly noted as the lack of concentration-response remained almost unchanged.

FIG. 3
DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 3 [a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

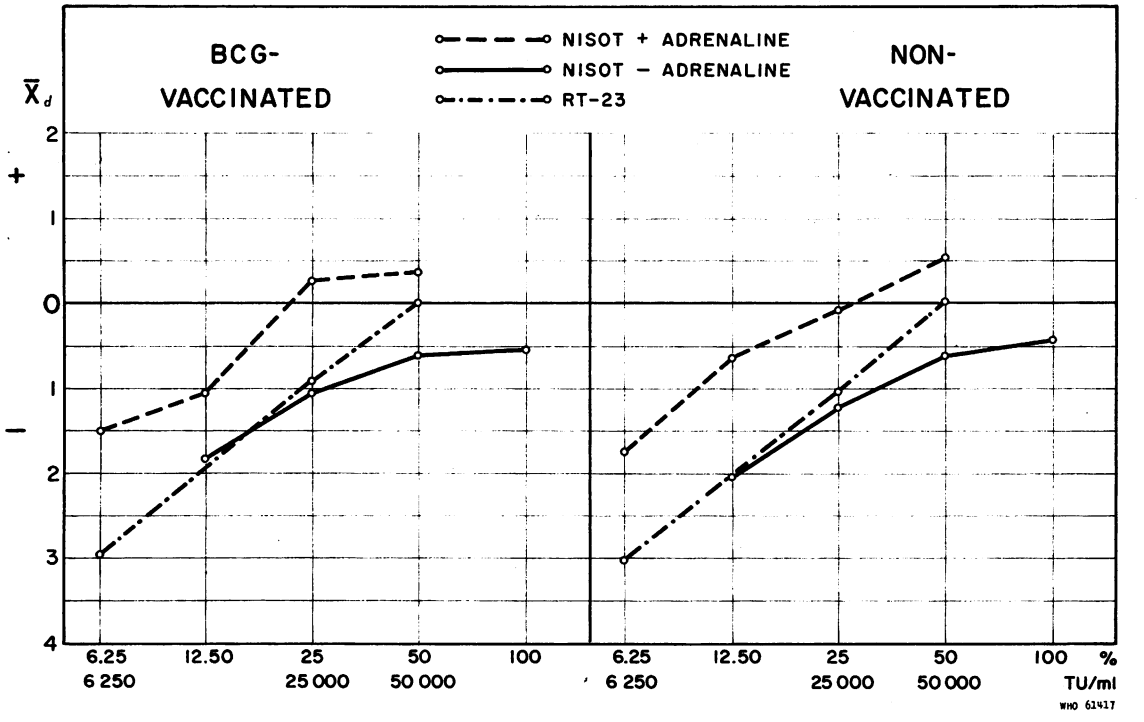
FIG. 4
CONCENTRATION-RESPONSE CURVES IN STUDY 4 [a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

FIG. 5

DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 5[a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and tests preparation in von Pirquet test.

Study 5

In a fifth study (Fig. 5) the lower range of dilutions was examined; 2 dilution-series were tested both with 20% glycerol and one with and the other without adrenaline. It was assumed that, if the preparation was sufficiently diluted, a concentration-response must sooner or later be observed. Concentrations as low as 1/16th of the original were examined. At this level a positive concentration-response was observed. The result, however, shows the function not to be log-linear at the level usually applied for von Pirquet tests. The function indicates an increasing slope for decreasing concentrations approaching parallelism with RT-23. This is true for dilutions both with and without adrenaline. The effect of adrenaline consequently cannot be measured as usual, but seems to be of the same magnitude as that usually observed.

These experiments indicate that the Third Standard of Old Tuberculin contains a substance that somehow depresses or interferes with the biological potency of the preparation. Making certain assumptions, it might be possible to estimate the strength of this effect from this experiment.

It is reasonable (see Fig. 5) to assume that the slopes for RT-23 and the Third OT Standard are parallel below the 12.5%-level of the Third OT Standard and that, at this level, p% of the Third OT Standard corresponds to $p \times 1000$ TU/ml of RT-23.

In other words, but for the effect of interference, the curves in Fig. 5 for RT-23 and the Third OT Standard would coincide.

This means that the ratio between the observed and the expected potency values of the Third OT Standard at various concentrations can be calculated. The vertical differences in mm between expected and observed values for the Third Standard have been expressed in corresponding potency ratios based on the estimated regression line for RT-23.

TABLE 1

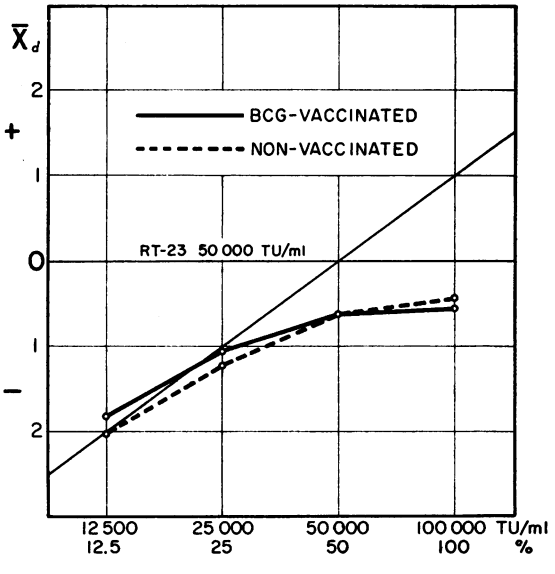
PERCENTAGE REDUCTION OF THE THIRD INTERNATIONAL STANDARD OF OLD TUBERCULIN RELATIVE TO RT-23

	BCG-vaccinated			Non-vaccinated		
	25% concn	50% concn	100% concn	25% concn	50% concn	100% concn
Regression line values for RT-23	−1.0	0	1.0	−1.0	0	1.0
Observed values for the Third OT Standard	−1.05	−0.61	−0.56	−1.24	−0.63	−0.45
Difference (d)	−0.05	−0.61	−1.56	−0.24	−0.63	−1.45
$\frac{d}{b\ (=3.32)}$	−0.015	−0.1837	−0.4699	−0.0723	−0.1898	−0.4367
Percentage reduction	3.4	34.5	66.1	15.3	35.4	63.4

The results are given in Table 1 and indicated in Fig. 6.

FIG. 6

OBSERVED AND EXPECTED CONCENTRATION-RESPONSE CURVES FOR NISOT WITHOUT ADRENALINE IN STUDY 5[a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

It is clear that there is a two-thirds reduction in the inherent potency of the 100% concentration of the Third OT Standard as a result of the action of an interfering substance.

In the non-vaccinated the reduction in potency is seen to be close to proportional to the concentration of dilution: 50% Third OT Standard contains half as much of the substance and consequently is reduced in potency by 38% only; at the 25%-level the potency reduction is more than halved, i.e., to 3%–15%; at the 12.5%-level there will still be a reduction but it is negligible and has been set at zero as an assumption in these calculations. For the vaccinated group the reduction seems to be even more pronounced.

Study 6

However, this still does not constitute proof that a substance with the property described really does exist. A final experiment was therefore designed to test the hypothesis.

The RT-23 and the Third OT Standard were mixed together in various concentrations. Any substance present in the Third OT Standard that interfered with potency should thereby be demonstrated to act upon the RT-23 preparation as well.

If 2 tuberculin products, T_1 and T_2 (TU/ml), are mixed with c of T_1 and $(1-c)$ of T_2, the combined strength would be:

$$\text{Exp} = cT_1 + (1-c) \times T_2$$

The objective was to demonstrate whether this also holds true when RT-23 is mixed with the Third OT Standard or whether RT-23 is reduced in potency by the fact of mixing. In the latter case, the observed strength of the mixture would be:

$$\text{Obs} = cT_1 + (1-c) \times T_2\ \theta$$

from which θ (the potency reduction of RT-23)

TABLE 2

CONCENTRATIONS, RELATIVE COMPOSITION, NUMBER TESTED (N), MEAN SIZE OF INDURATION BETWEEN STANDARD PREPARATION (RT-23 50 000 TU/ml) AND TEST PREPARATION ($\bar{X}_d$) AND VARIANCE OF DISTRIBUTION OF DIFFERENCES (s^2) IN STUDY 6

Composition		BCG-vaccinated			Non-vaccinated		
Third OT Standard	RT-23	N	$\bar{X}_d$	s^2	N	$\bar{X}_d$	s^2
0.9 · 100 %	0.1 · 150 000 TU/ml	161	−1.22	5.80	128	−1.10	3.67
0.9 · 100 %	0.1 · 600 000 TU/ml	164	−0.39	3.82	137	−0.05	4.99
0.8 · 100 %	0.2 · 85 000 TU/ml	167	−1.33	5.29	115	−0.51	4.85
0.8 · 100 %	0.2 · 310 000 TU/ml	173	−0.24	5.88	139	0.08	6.29
0.5 · 100 %	0.5 · 55 000 TU/ml	153	−1.08	4.24	142	−0.39	6.14
0.5 · 100 %	0.5 · 145 000 TU/ml	160	−0.11	5.03	142	0.54	5.56
RT-23 45 000TU/ml		161	0.62	6.65	149	0.46	5.88
RT-23 90 000 TU/ml		179	1.85	5.50	154	1.92	5.98
Third OT Standard 50 %		145	−2.46	7.24	138	−1.82	6.25
Third OT Standard 100 %		161	−1.85	5.99	122	−1.45	6.00

could be estimated. It would further be expected that this reduction in itself would be a function of c (proportion of the Third OT Standard in the mixture).

The concentrations and relative composition of the products applied as well as the results are given in Table 2.[1]

Fig. 7, in which the results are given with the content of RT-23 along the horizontal axis, confirms the hypothesis. The dilutions containing 60 000 TU/ml to 70 000 TU/ml RT-23 with various amounts of the Third OT Standard are shown to be considerably weaker than expected from estimates of the pure RT-23 product. The difference of about 1.5 mm corresponds to a reduction of 50%. In other words, it is at least clear that the interfering effect on RT-23 is more pronounced than would be expected from the inherent potency of the Third OT Standard.

Fig. 8 gives the results, showing along the horizontal axis the *expected* strength of the combined dilutions calculated on the basis of the observed relative values in this experiment.

Again, the formula is:

$$\text{Exp} = cT_1 + (1-c) \times T_2$$

where T_1 values are those of the Third OT Standard from this experiment. The proportions are 90%, 80% and 50%, and the corresponding values in terms of TU/ml can be found in Table 2 from interpolation of the Third OT Standard curves. (A linear interpolation has been made although it is assumed, and has been observed in the previous experiment, that this is not strictly permissible. The deviations are negligible, however.)

Finally, the actual reduction in potency of RT-23 in the mixed dilution can be estimated from:

$$\text{Obs} = cT_1 + (1-c) \times T_2\,\theta$$

$$\theta = \frac{\text{Obs} - cT_1}{(1-c) \times T_2}$$

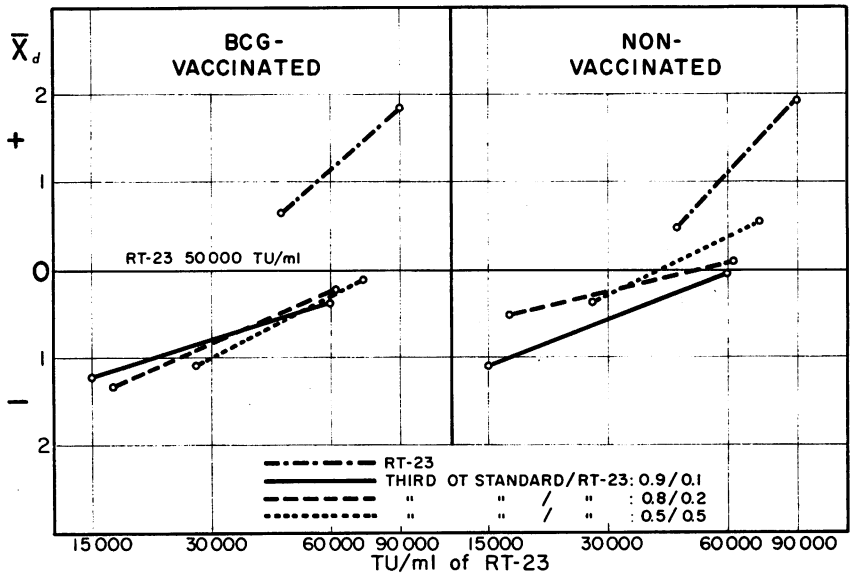
where θ expresses the potency reduction of RT-23, Obs is the observed potency in terms of RT-23 units, T_1 is the estimated strength of the Third OT Standard, and T_2 is the strength of RT-23.

The θ values, given in Table 3 separately for the vaccinated and the non-vaccinated, vary from 0.28 to 0.83. However, it is clear that, as expected, the reduction is greater for the dilutions with higher proportions of the Third OT Standard. It also seems to be greater for dilutions containing high concentrations of RT-23.

[1] The strength of 100% of the Third OT Standard in terms of RT-23 units was estimated on the basis of the previous experiment and was planned to produce dilutions of 45 000 TU/ml and 90 000 TU/ml. For unknown reasons, however, the RT-23 used in the present experiment turned out to be considerably stronger (later animal experiments confirmed this difference).

FIG. 7

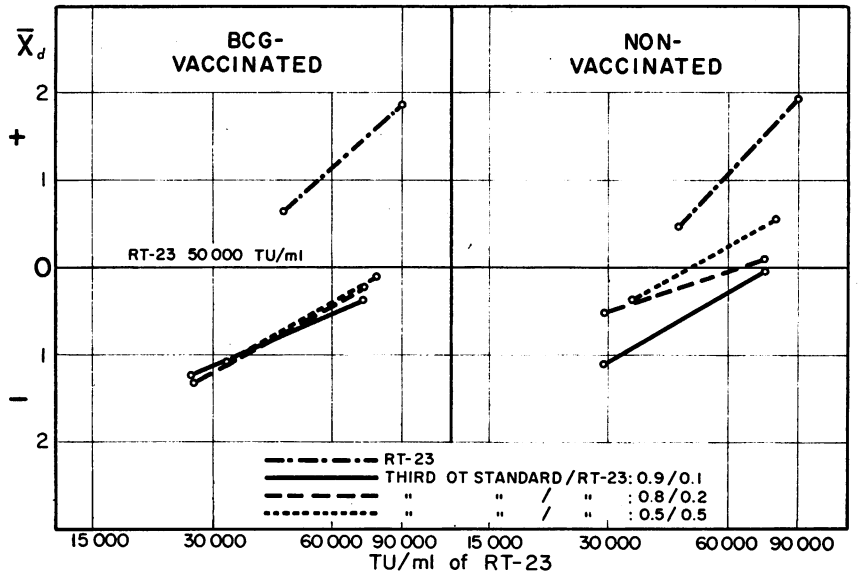
DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 6, WITH ACTUAL CONTENT OF RT-23 ALONG THE HORIZONTAL AXIS [a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

FIG. 8

DOSE-RESPONSE AND CONCENTRATION-RESPONSE CURVES IN STUDY 6, WITH EXPECTED STRENGTH ALONG THE HORIZONTAL AXIS [a]



[a] $\bar{X}_d$ = mean difference (mm) between reactions to standard preparation and test preparation in von Pirquet test.

TABLE 3
EXPECTED AND OBSERVED STRENGTH OF DILUTIONS COMPOSED OF THE THIRD STANDARD OF OLD TUBERCULIN AND OF RT-23

Composition		Concentration (TU/ml)					Reduction of RT-23 [a] (Θ)	
Third OT Standard (T_1)	RT-23 (T_2)	RT-23 only	Expected [b]		Observed [c]			
			BCG-vacc.	Non-vacc.	BCG-vacc.	Non-vacc.	BCG-vacc.	Non-vacc.
0.9 · 100 %	0.1 · 150 000 TU/ml	15 000	26 304	29 157	18 220	19 385	0.46	0.35
0.9 · 100 %	0.1 · 600 000 TU/ml	60 000	71 304	74 157	27 910	33 250	0.28	0.32
0.8 · 100 %	0.2 · 85 000 TU/ml	17 000	26 532	29 184	17 220	26 250	0.45	0.83
0.8 · 100 %	0.2 · 310 000 TU/ml	62 000	71 532	74 184	30 160	35 550	0.33	0.38
0.5 · 100 %	0.5 · 55 000 TU/ml	27 500	32 316	34 190	19 585	27 920	0.54	0.77
0.5 · 100 %	0.5 · 145 000 TU/ml	72 500	77 316	79 190	32 240	45 030	0.38	0.53

[a] $\Theta = \frac{\text{Obs} - cT_1}{(1-c \times T_2)}$
[b] $\text{Exp} = cT_1 + (1-c) \times T_2$, where T_1 values are estimated from the figures in Table 2 (see text).
[c] The observed values are estimated on the basis of the common regression line for RT-23: $y = -20.3 + 4.48 \times \log x$.

CONCLUSIONS

The 67% potency reduction of 100% Third OT Standard observed earlier (Fig. 5) is thus in agreement with the results of study 6, and the presence of an interfering or blocking substance is proved. However, no simple and clear-cut model has been constructed to explain in detail the behaviour of the interaction between this substance and the tuberculin.

It is believed that the presence of similar substances in various tuberculin products might provide a simple explanation of other differences observed in dose-responses.

ACKNOWLEDGEMENTS

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RÉSUMÉ

Au cours d'une série d'essais effectués dans le cadre de l'évaluation biologique du troisième étalon international de vieille tuberculine, on a constaté que cette préparation, aux concentrations habituelles, ne suscitait pas les réactions attendues, en fonction de la dose, chez les personnes subissant le test de von Pirquet.

Les auteurs montrent que ce phénomène est dû à la présence dans le troisième étalon international d'une substance qui inhibe la réponse biologique normale à la tuberculine. Si l'épreuve est pratiquée à l'aide de préparations contenant un mélange, à concentrations variables, de PPD RT23 et du troisième étalon international, les réactions sont beaucoup plus faibles que si l'on utilise le PPD RT23 seul, bien que le troisième étalon international, lorsqu'il est utilisé seul, provoque également des réactions tuberculiniques.

La substance en cause n'a pas encore été identifiée. Il n'est pas impossible que les différences notées dans les courbes dose-réponse obtenues avec diverses préparations de tuberculine soient dues à la présence, dans ces préparations, de substances inhibitrices similaires.

REFERENCES

Galtung Hansen, O. (1937) *Acta tuberc. scand.*, **11**, 130–161
Guld, J. & Roelsgaard, E. (1965) *Bull. Wld Hlth Org.*, **33**, 345–363
Wijsmuller, G., Magnusson, M. & Larsen, S. O. (1962) *Acta tuberc. scand.*, **42**, 79–108