

Effects of homœopathic preparations of organic acids and minerals on the oxidative metabolism of human neutrophils

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Abstract

A number of different potencies of commercially available homœopathic preparations in saline solution were tested for their ability to regulate the oxidative metabolism (superoxide production) and adhesion function of human neutrophils *in vitro*. 15% to 30% inhibition of oxidative metabolism was caused by *Sulphur* 6x, *Manganum phosphoricum* 6x and 8x, and *Magnesium phosphoricum* 6x and 8x. *Phosphorus* slightly reduced superoxide production, with varying results in a series of experiments. Using *Magnesium phosphoricum* and *Phosphorus*, small inhibitory effects (8-11%) were noted even at high potencies. Among the organic acids, a group (*Acidum malicum* 4x and *Acidum fumaricum* 4x) enhanced superoxide production, while others either inhibited the response (*Acidum citricum* and *Acidum succinicum*, 3x and 4x) or had no effect (*Acidum α -ketoglutaricum* and *Acidum cis-aconitum*). Attempts to reproduce these effects using solutions prepared in the laboratory confirmed the inhibitory effects of *Manganum phosphoricum* 6x and of organic acids in the 3x, while other data indicated that critical factors in the methodology of preparation may affect the results.

KEY WORDS: Leukocyte function; Manganum phosphoricum; Magnesium phosphoricum; Phosphorus; Homotoxicology; Free radicals; Inflammation.

Introduction

Homœopathy is a global, integrated clinical and pharmacological approach, which raises a series of problems regarding both its efficacy and its action mechanism(s). Homœopathic issues may therefore be investigated from multiple standpoints, and biochemistry and cell biology may help to provide answers to specific questions.

In vitro testing of homœopathic potencies on leukocyte functions may provide important clues by examining possible effects on cell function directly. Homœopaths have claimed from the beginning that homœopathically prescribed medicines act by regulating endogenous defence mechanisms, and studies of leukocytes would therefore be highly significant due to the widespread involvement of

these cells in inflammatory reactions. To the best of our knowledge, only a few studies of the effects of homœopathic drugs on neutrophil functions have been reported.¹⁻⁴

In view of the above we have undertaken a series of experiments to test various homœopathic drugs for their possible effect on human neutrophils. Two relevant functions were considered—superoxide production, which is involved in bactericidal and cytotoxic activity of these cells, and adhesion to serum-coated plastic surfaces, a parameter reflecting the first steps of cell migration from bloodstream to connective tissue.

Tests were done on compounds such as *Sulphur* and *Phosphorus*, which are supposed to interact with neutrophils, since phosphorylation processes and sulphydryl groups have an

important role in the regulation of many biochemical mechanisms in these cells.^{5,6} Another series of compounds were chosen because on the basis of clinical homœopathic and homotoxicological experience they are presumed to have regulatory effect on the inflammatory reactions.^{7,8} These include *Manganum phosphoricum* and *Magnesium phosphoricum*. Finally, other compounds were included because of possible actions at the level of the cell bioenergetic metabolism: a selection of organic acids, intermediates of the energy-generating tricarboxylic acid (Krebs) cycle. All available potencies of the selected drugs were tested, starting with low potencies which are supposed to act on the basis of molecular mechanisms, and going on to very high potencies where no molecule of the original solute is present.

Materials and methods

Reagents

Fe³⁺ cytochrome c was purchased from Boehringer, Mannheim, Germany, formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, a bacterial tripeptide) from Sigma, St Louis, Mo., USA, foetal bovine serum (FBS) and 96-well microtitre plates with flat base from ICN-Flow, Costa Mesa Ca, USA, Percoll from Pharmacia, Uppsala, Sweden, lactose from Farmitalia Carlo Erba, Milano, Italy. The drugs were purchased from Staufien Pharma, Goeppingen, Germany. Manufactured according to the *German Homœopathic Pharmacopoeia (GHP)*⁹ they were supplied in vials (1 or 2 ml) as decimal (x) potencies in 0.9% NaCl (physiological saline.) Other materials and reagents were of the highest purity available. In order to avoid any contamination that could cause artifactual activation of the cells, sterile solutions and disposable plastic ware were used throughout, when possible under a laminar flow hood. Reagents were made up using sterile pyrogen-free water or 0.9% NaCl solution.

Preparation of diluted and succussed solutions

Where indicated, 6x and 8x dilutions of magnesium phosphate ($\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$, here referred to as *Magnesium phosphoricum*) and of manganese phosphate ($\text{MnHPO}_4 \cdot \text{H}_2\text{O}$, here referred to as *Manganum phosphoricum*) were prepared in the laboratory according to the *GHP*.⁹ Briefly, the 1x trituration was

prepared by mixing 0.2 g of the magnesium or manganese phosphate salt with 2.7 g of lactose in a stoneware mortar (70 × 40 mm) and triturating for 1 hour, using a clockwise rotating movement. The 2x trituration was prepared by mixing 0.3 g of the 1x trituration with 2.7 g of lactose and triturating as above. Manual trituration in lactose was carried out up to the 6x.

The 6x solution for the assays was prepared from the 4x trituration, according to Method 11 in the *GHP*: 1 g of the 4x trituration was combined with 9 ml of 0.9% NaCl solution in a sterile 50 ml polypropylene tube provided with a cap and succussed, using 100 vertical strokes. 1 ml of the resulting solution (5x) was combined with 9 ml of 0.9% NaCl solution and succussed as above, thus obtaining the 6x liquid diluted and succussed dilution. The 8x liquid solution for use in the assays was prepared from the 6x trituration by the same method.

Parallel to the 6x and 8x dilutions, control solutions were made as follows: a) 6x and 8x dilutions of 'unsuccussed' *Magnesium phosphoricum* and *Manganum phosphoricum*, using the same mixtures of metal salt and lactose but mixing them briefly and gently rather than triturating and succussing; b) diluted and succussed solutions of lactose only, using the same method of trituration and dilution and succussion; c) diluted but not succussed solutions of untrituated lactose only.

Isolation of neutrophils

Human neutrophils were prepared from EDTA-anticoagulated blood from healthy donors. Cells from a single donor were used for each experiment, with different donors for different experiments. The blood was fractionated by centrifugation over Percoll gradients.¹⁰ The cells (> 95% neutrophils) were suspended in Hank's balanced salt solution (137 mmol/l NaCl, 5.4 mmol/l KCl, 0.4 mmol/l KH_2PO_4 , 4.2 mmol/l NaHCO_3 , 0.4 mmol/l Na_2HPO_4 , pH 7.4), containing 0.2% of human serum albumin, 5 mmol/l glucose, 0.5 mmol/l CaCl_2 and 1 mmol/l MgSO_4 (HCGMA solution), at a concentration of $2.8 \cdot 10^6$ neutrophils/ml.

Assay of superoxide production and adhesion

The method of simultaneous evaluation of oxidative metabolism and adhesion on micro-

TABLE 1. List of homœopathic medicines tested. For details of dilutions with significant inhibitory or stimulatory effect (shown in parentheses) see Table 2.

Compound	Decimal dilutions	Effect on O ₂ ⁻ production	Effect on adhesion
1 Sulphur	6, 8, 10, 12, 15, 30, 60, 100, 200, 300, 400, 500, 600	inhibition (6x)	none
2 Phosphorus	6, 8, 12, 15, 30, 60, 100, 200, 300, 400, 500, 600	inhibition (6x, 8x, 30x)	none
3 Manganum phosphoricum	6, 8, 10, 12, 200, 500	inhibition (6x, 8x)	none
4 Magnesium phosphoricum	6, 8, 12, 15, 30, 60, 100, 200, 400	inhibition (6x, 8x, 10x, 12x, 30x, 60x)	none
5 Acidum malicum	4, 5, 6, 8, 10, 12, 15, 30, 60, 100, 200	potentiation (4x)	potentiation (4x?)
6 Acidum fumaricum	4, 5, 6, 8, 10, 12, 15, 30, 60, 100, 200	potentiation (4x)	potentiation (4x?)
7 Acidum citricum	3, 4, 5, 6, 8, 10, 12, 15, 30, 60, 100, 200	inhibition (3x, 4x)	none
8 Acidum succinicum	2, 3, 4, 5, 6, 8, 10, 12, 15, 30, 60, 100	inhibition (2x, 3x, 4x, 6x)	inhibition (2x)
9 Acidum cis-aconitum	4, 5, 6, 8, 10, 12, 30, 60, 100, 200	none	none
10 Acidum α-ketoglutaricum	4, 5, 6, 8, 10, 12, 15, 30, 60, 100, 200	none	none

plates was used.^{11,12} Briefly, the 96-well microtitre plates were coated with 50% FBS for 2 hours and then washed twice with physiological saline. 25 µl of homœopathic solution was dispensed into multiple microwells (3–6 replicates for every experimental point). As controls, 25 µl of 0.9% NaCl solution or, where indicated, 25 µl of lactose in 0.9% NaCl solution were put in replicates of microwells. The plates were heated to 37°C and after addition of 75 µl of neutrophil suspension incubated at 37°C for 10 minutes. 50 µl of the stimulant fMLP at the final concentration of 10⁻¹ mmol in HCGMA was then added to each well. The stimulant solution contained 450 µmol/l of Fe³⁺ cytochrome c, a compound whose chemical reduction is a marker of superoxide production. The plates were incubated at 37°C for 30 minutes. During this time, superoxide production of activated cells was measured with a microplate optical reader recording the increase in absorbance at 550 nm relative to that at 540 nm. Soon after the end of the incubation period, the plates were washed twice with phosphate-buffered saline and adherent cells quantified by measuring the membrane enzyme acid phosphatase relative to the enzyme activity of a known number of cells.

Concentration of test solutions

The doses of test solutions are given in decimal homœopathic potencies (x). The actual

molar concentrations of the highest doses of compound used, calculated taking into consideration the molecular weight of the compound, the dilution procedures and the final dilution into the assay system, are as follows:

<i>Manganum phosphoricum</i> 6x	1.1 · 10 ⁻⁶ mol/l
<i>Magnesium phosphoricum</i> 6x	9.5 · 10 ⁻⁶ mol/l
<i>Phosphorus</i> 6x	1.3 · 10 ⁻⁶ mol/l
<i>Sulphur</i> 6	6.5 · 10 ⁻⁷ mol/l
<i>Acidum succinicum</i> 2x	1.4 · 10 ⁻² mol/l
<i>Acidum citricum</i> 3x	8.7 · 10 ⁻⁴ mol/l
<i>Acidum malicum</i> 4x	1.25 · 10 ⁻⁴ mol/l
<i>Acidum fumaricum</i> 4x	1.4 · 10 ⁻⁴ mol/l
<i>Acidum cis-aconitum</i> 4x	9.5 · 10 ⁻⁵ mol/l
<i>Acidum α-ketoglutaricum</i> 4x	9.5 · 10 ⁻⁵ mol/l

Results

Superoxide production and adhesion of human neutrophils were measured in the absence and in the presence of the compounds shown in Table 1. Various effects were noted. Only 3 of the tested homœopathic preparations had an effect on neutrophil adhesion. *Acidum succinicum* 2x strongly inhibited cellular adherence, probably due to nonspecific toxic effects of such a high dose. *Acidum malicum* 4x and *Acidum fumaricum* 4x potentiated adhesion, but only in 1 of the 4 experiments performed.

As regards O₂⁻ production, *Acidum cis-aconitum* and *Acidum α-ketoglutaricum*

TABLE 2. Potentized drugs showing an effect on superoxide production by fMLP-activated human neutrophils

	n	Control ¹	Test ¹	Effect (%)	t-test ²
<i>Magnesium phos.</i>					
6x	11	10.2 ± 3.9	8.6 ± 4.1	- 15.7	p < 0.001
8x	11	10.8 ± 4.5	8.3 ± 3.7	- 23.1	p < 0.001
10x	5	12.4 ± 4.4	10.9 ± 3.9	- 12.1	p < 0.05
12x	5	12.7 ± 4.6	10.7 ± 3.9	- 15.7	p < 0.05
30x	4	11.3 ± 4.7	10.4 ± 4.8	- 8.0	p < 0.05
60x	4	14.1 ± 2.5	12.5 ± 2.5	- 11.3	p < 0.05
<i>Manganum phos.</i>					
6x	10	9.1 ± 4.9	6.9 ± 4.3	- 24.2	p < 0.001
8x	10	9.4 ± 4.5	7.3 ± 4.3	- 22.3	p < 0.001
<i>Sulphur</i>					
6x	4	9 ± 6.5	6.4 ± 5	- 29.0	p < 0.05
<i>Phosphorus</i>					
6x	6	14.2 ± 4.6	12.2 ± 3.7	- 14.1	p < 0.05
8x	6	14.8 ± 4.6	13.2 ± 4.9	- 10.8	p < 0.05
30x	5	15.9 ± 5.7	14.4 ± 5.2	- 9.4	p < 0.05
<i>Ac. succinicum</i>					
3x	6	14.8 ± 5.4	4.3 ± 1.7	- 70.1	p < 0.01
4x	7	12.2 ± 5.0	10.5 ± 4.0	- 12.5	p < 0.05
6x	4	17.6 ± 5.4	16.4 ± 4.8	- 6.8	p < 0.05
<i>Ac. citricum</i>					
3x	5	10.6 ± 3.2	2.4 ± 2.2	- 77.3	p < 0.01
4x	5	13.0 ± 6.4	12.3 ± 6.2	- 5.4	p < 0.05
<i>Ac. malicum</i>					
4x	5	11.1 ± 7.9	14.0 ± 8.0	± 26.1	p < 0.05
<i>Ac. fumaricum</i>					
4x	4	7.8 ± 5.6	10.7 ± 6.6	± 37.2	p < 0.05

¹Units are nmoles O₂⁻/10⁶ cells/10 minutes. The table shows mean ± standard deviation for the indicated number of experiments (n). Due to the high variability of responses of fMLP in different individuals, replicates of test assays were run parallel to control assays in every experiment.

²Significance was calculated comparing test and control values of the indicated number of experiments (n) using Student's t-test for paired data. Control solutions in this series were 0.9% NaCl.

showed neither stimulatory nor inhibitory effects. *Manganum phosphoricum*, *Magnesium phosphoricum*, *Phosphorus*, *Acidum succinicum*, *Acidum citricum* and *Sulphur* inhibited neutrophil fMLP-stimulated superoxide production. Table 2 shows details of these inhibitory effects. Most of these homœopathic preparations were active only at low dilutions, the effect on O₂⁻ production ranging from 10 to 30%, except for *Acidum citricum* 3x and *Acidum succinicum* 3x, which inhibited over 70%. A representative experiment showing the effect of several dilutions of *Manganum phosphoricum* is shown in Fig. 1.

A small inhibitory effect of *Phosphorus* potencies was also noted. We performed several experiments with this, as the phosphorylation and dephosphorylation reactions are fundamental in regulating a number of biochemical processes, including neutrophil oxidative metabolism.⁵ In a series of 5 separate

experiments, using leukocytes from different subjects, we found inhibitory effects of *Phosphorus* in all cases, but at different dilutions (Fig. 2a-e). As a consequence, when all the experiments were averaged, very small inhibitory effects resulted at almost all dilutions (Fig. 2f). The 6x, 8x and 30x dilutions showed statistically significant effects (Table 2).

On the other hand, *Acidum malicum* and *Acidum fumaricum* had a small but significant potentizing effect in the 4x; the 3x was not available.

We attempted to clarify whether the observed effects were due to homœopathic dynamization or to more conventional biochemical interaction with cell metabolism. A series of 76 experiments was carried out using *Magnesium phosphoricum* and *Manganum phosphoricum*, the two preparations showing most consistent effect in the 6x and 8x potencies. The purchased solutions were compared

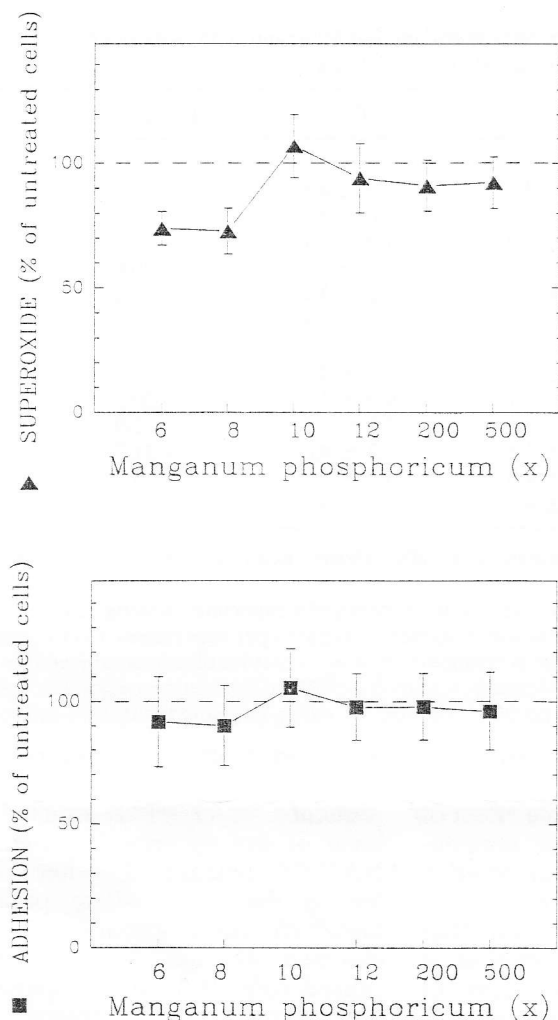


Fig. 1. Effect of Manganum phosphoricum in decimal dilutions on superoxide production (above) and adhesion (below) of human neutrophils. Values indicate mean activity relative to control, \pm SD of 6 replicates from an experiment representative of 4 performed.

with corresponding preparations made in the laboratory either by the homœopathic method⁹ or by simple dilution, without trituration and succussion (Table 3). The purchased solution decreased neutrophil superoxide production, as expected. It is worth noting that in this series of experiments, the control solutions were dilutions of lactose in 0.9% NaCl solution, containing exactly the same lactose concentration as the 6x and 8x homœopathic solutions (0.17% in the final assay). The inhibition observed is therefore not due to interference by lactose.

On the other hand, the data in Table 3 also

show that among the solutions made in our laboratory, only diluted and succussed *Manganum phosphoricum* 6x had the same inhibitory effect as the purchased solution. Other dilutions prepared in our laboratory also had small inhibitory effects on superoxide production, but the results were not statistically significant.

Acidum succinicum and *Acidum citricum* 3x dilutions made with and without succussion (100 vertical strokes) in our laboratory had the same inhibitory effect as the purchased homœopathic preparations. On the other hand, *Acidum malicum* and *Acidum fumaricum* 4x dilutions made with and without succussion in our laboratory did not have a stimulating effect but a small, though not significant, inhibitory effect (data not shown).

Discussion

Since little is known about the putative targets of homœopathic drugs in the human body and

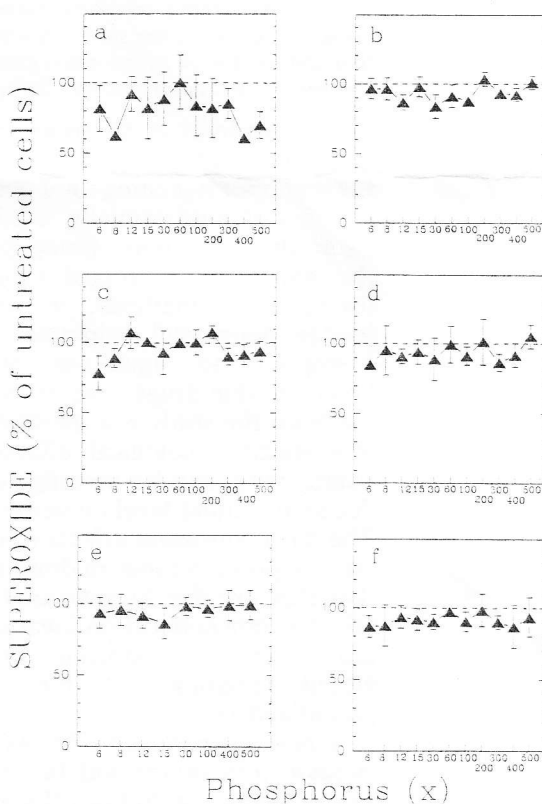


Fig. 2. Effect of Phosphorus in decimal dilutions on superoxide production of human neutrophils. Values in a, b, c, d and e are mean \pm SD of 3 (a-d) and 10 (e) replicates from experiments carried out using cells from different donors. Values in f are mean \pm SD for values in experiments a-e.

TABLE 3. Effect of different Mangesium phosphoricum and Magnanum phosphoricum preparations on in vitro human neutrophil superoxide production (see Method for details)

	Control ¹	Test ¹	Effect (%)	t-test ²
<i>Magnesium phosphoricum</i>				
6x purchased	8.9 ± 3.9	7.6 ± 4.3	- 14.6	p < 0.05
6x diluted & succussed	8.6 ± 4.0	8.0 ± 4.1	- 7.0	p = ns
6x diluted, not succussed	8.5 ± 3.9	7.9 ± 3.1	- 7.0	p = ns
8x purchased	9.2 ± 4.2	7.3 ± 3.5	- 20.6	p < 0.01
8x diluted & succussed	9.1 ± 4.2	8.5 ± 3.7	- 6.6	p = ns
8x diluted, not succussed	8.7 ± 3.8	8.6 ± 3.8	- 1.1	p = ns
<i>Manganum phosphoricum</i>				
6x purchased	8.6 ± 4.1	6.7 ± 4.1	- 22.1	p < 0.001
6x diluted & succussed	8.7 ± 4.1	6.9 ± 3.6	- 20.7	p < 0.01
6x diluted, not succussed	8.3 ± 4.0	7.3 ± 3.7	- 12.0	p = ns
8x purchased	8.5 ± 3.5	6.7 ± 4.0	- 21.2	p < 0.001
8x diluted & succussed	8.7 ± 4.0	8.2 ± 3.8	- 5.7	p = ns
8x diluted, not succussed	8.5 ± 4.0	8.5 ± 3.6	0	-

¹Units are nmoles O₂⁻/10⁹ cells/10 minutes. The table shows mean ± standard deviation for 6 separate experiments.

²Significance was calculated comparing test and control values of 6 experiments using Student's t-test for paired data. At least 4 replicates of test and control solutions were compared per experiment. Controls of purchased and diluted & succussed test solutions were the corresponding 6x or 8x lactose dilutions subjected to trituration, dilution and succussion as described under Methods. Controls of diluted but not succussed test solutions were the corresponding 6x or 8x lactose dilutions prepared by simple mixing, omitting trituration and succussion.

of the sensitivity of different cell types to different potencies, this work was designed as the first pilot screening for a possible effect of a series of commercially available homœopathic drugs on human blood cells involved in the inflammatory process. Using well-established assay methods, we observed that human neutrophil oxidative metabolism is susceptible to regulation by a series of homœopathic drugs. The main point emerging from the study is a direct demonstration that specific biological effects of homœopathic drugs can be investigated and reproduced at cellular level on neutrophils *in vitro*. The most consistent effects were observed in the low-potency range of dilutions, suggesting that they are due to molecular regulation of selected biochemical pathways; some results did however also indicate the existence of effects relating to the homœopathic potentization.

Acidum succinicum (3x, 4x and 6x) and *Acidum citricum* (3x and 4x) inhibited superoxide production but not the adhesion function, indicating that these compounds are specific for oxidative metabolism, not affecting the receptor systems of the cell nor the mechanical apparatus necessary for adhesion. The inhibition of superoxide production by succinate and butyrate has been described

by others,¹³ suggesting fine regulation of the intracellular pH by these acids and, as a consequence, of O₂⁻/H₂O₂ stoichiometry at the level of the superoxide-generating enzyme NADPH oxidase. 2 other organic acids among the intermediate products of the metabolic energy-producing cycles, *Acidum malicum* 4x and *Acidum fumaricum* 4x, caused enhancement of superoxide production. With 2 groups of organic acids showing opposite effects on cell metabolism, it is conceivable that these findings reflect the existence of some specific transport or regulatory mechanism of organic acids which remains to be identified.

Phosphorus, *Sulphur*, *Manganum phosphoricum* and *Magnesium phosphoricum* in different potencies inhibited superoxide production by neutrophils. The results seen with mineral homœopathic potencies are interesting for the following reasons:

- In several instances, the extent of the inhibition was almost the same at different potencies. *Manganum* and *Magnesium* potencies (Fig. 1, Table 2 and Table 3) caused similar inhibition at potencies differing, in terms of molecular concentration, by a factor of 100 (6x and 8x).
- Phosphorus* and *Magnesium phosphoricum* showed small but significant effects even in

the high potency range (Fig. 2, Table 2). In our experience with the test and in statistics, these effects cannot be due to intra-assay experimental errors.

- The inhibition of superoxide production by these homœopathic mineral preparations has been obtained using an incubation medium containing 1 mmol/l of magnesium and 0.7 mmol/l of phosphate, concentrations several orders of magnitude higher than the doses of *Phosphorus* and *Magnesium phosphoricum* used in the test.

These observations suggest that the effects may be accounted for by mechanisms which are not directly dose-dependent and differ from those considered in conventional biochemistry. It is tempting to speculate that homœopathic preparations of magnesium, manganese and phosphorus may interfere with the complex biochemical reactions leading to superoxide generation, in which magnesium, manganese and phosphorylation processes may have a number of regulatory functions.^{5,14-18}

Control experiments showed that not all the effects obtained with commercially available homœopathic solutions could be reproduced with the corresponding solutions made in our laboratory. At least one important correspondence between purchased and 'home-made' solutions was found: *Manganum phosphoricum* 6x prepared in the laboratory by trituration, dilution and succussion inhibited superoxide production to the same extent as the purchased solution, while a smaller and non-significant inhibition was caused by *Manganum phosphoricum* 6x prepared without dynamization. Taken together, these results suggest that trituration and succussion may actually increase the potency of serially produced solutions, but also point to the existence of other critical factors, possibly related to materials or to methodological procedures, or even to the unpredictability of cellular behaviour,¹⁹ which cannot be identified on the basis of present experience.

The classical homœopathic indications for phosphorus and for manganese and magnesium salts are conditions like acute inflammation, hepatitis, pancreatitis, hypersensitivity, haemorrhage, etc. Among the various molecular and cellular systems involved in these pathologies, the activation of blood neutrophils and production of oxidative free radicals play a primary role.^{20,21}

Modulation of leukocytes by low doses of Phosphorus, Manganum phosphoricum and Magnesium phosphoricum diluted according to the homœopathic method could be beneficial in these inflammatory conditions. However, considerable caution is needed in applying the molecular paradigm directly to homœopathic therapeutics, which developed mainly as a 'holistic' approach to health and disease. The question as to whether the *in vivo* effects of homœopathic drugs are due to regulation of peripheral cells or to other complex and integrated homeostatic regulatory systems has yet to be investigated. Moreover, from the homœopathic standpoint one should also consider the effect of specific medicines on cells isolated from patients presenting symptoms specific to these medicines. This problem represents a further degree of complexity in this type of studies.

In conclusion, this work strongly suggests that 'homœopathic' effects of specific drugs, in particular magnesium and manganese phosphates, may be demonstrated at cellular level. A number of problems remain to be clarified, however. They include reproducibility of the effects in different subjects (see the results obtained with *Phosphorus*), the differences between purchased and 'home-made' potencies and, finally, how to integrate the results obtained with laboratory models into traditional homœopathic principles.

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