Effect of loop diuretics on cholinergic neurotransmission in human airways in vitro

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Abstract

Background - Frusemide can inhibit various indirectly acting bronchoconstrictor stimuli in asthmatic patients. Both frusemide and bumetanide also modulate airway neurotransmission in some species but there are no data on the effect of loop diuretics on neurotransmission in man. An in vitro study was performed in human airways to investigate the possible neuromodulatory action of two loop diuretics, frusemide and bumetanide, and to elucidate whether a cyclooxygenase inhibitor such as indomethacin could modulate the effect of frusemide. The effect of acetazolamide, a carbonic anhydrase inhibitor, was also investigated.

Methods - Electrical field stimulation (EFS; 40 V, 0.5 ms, 0.5-32 Hz for 15 seconds) in human airways with or without epithelium was used to induce a cholinergic contraction (n=5 in all experiments). Indomethacin was present throughout. After obtaining a control frequency-response curve, different concentrations of diuretic were added to the organ bath and another frequency-response curve was constructed. To determine whether the effect of the diuretic was prejunctional or postjunctional a cumulative concentration-response curve to exogenous acetylcholine (Ach, 0.3 µmol/l to 10 mmol/l) was constructed in the presence of a diuretic (frusemide 1 mmol/l or bumetanide 0.1 mmol/l) or its vehicle. In some experiments indomethacin was omitted from the organ bath to investigate the possible involvement of cyclooxygenase products.

Results - Both frusemide (10 µmol/l to 1 mmol/l) and bumetanide (1 µmol/l to 0.1 mmol/l) produced a concentration-dependent inhibition of the EFS-induced cholinergic contraction in human airways in vitro but only in epithelium denuded tissues. Frusemide (1 mmol/l) produced a maximum inhibition of 46.3% (SE 9.9%) at 0.5 Hz and bumetanide (0.1 mmol/l) 39.6 (6.2)% at 0.5 Hz. Without indomethacin in the organ bath the frusemide-induced inhibition was enhanced at 4, 8, and 16 Hz, but bumetanide-induced inhibition was not enhanced at any frequency when indomethacin was omitted. Frusemide (1 mmol/l) and bumetanide (0.1 mmol/l) had no effect on the cumulative concentration-response curve to exogenous Ach (0.3 µmol/l to 10 mmol/l). Acetazolamide (100 µmol/l) had no effect on the EFS-

induced cholinergic contraction in tissues with or without epithelium.

Conclusions - In human airways in vitro both frusemide and bumetanide produced a concentration-dependent inhibition of the EFS-induced cholinergic contraction. This inhibition is mediated through a prejunctional mechanism. Epithelium removal was necessary to achieve this effect. The mechanism of action of frusemide and bumetanide on airway nerves remains unclear: inhibition of the Na-K-Cl cotransporter is a possibility and, for frusemide, release of endogenous cyclooxygenase products may be involved. Carbonic anhydrase inhibition, on the other hand, is unlikely to be a factor.

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It has recently been shown that frusemide, a loop diuretic, can inhibit various indirectly acting bronchoconstrictor challenges in asthmatic patients, including exercise,¹ inhaled allergen,² sodium metabisulphite,³ distilled water,⁴ adenosine,⁵ and hypertonic saline.⁶ However, frusemide had no inhibitory effect on methacholine or prostaglandin F_{2α} induced bronchoconstriction.⁷⁻⁹ Although this drug seems to prevent effects of indirect challenges in asthmatics, it is curious that bumetanide, a more potent loop diuretic, causes little or no protection against indirect bronchoconstrictor challenges. Speculation continues, therefore, about the potential mechanisms of action, which may not be related to the diuretic effect of these drugs.¹⁰

In vitro results point to different sites of action and these have been reviewed recently.¹⁰⁻¹² The in vitro data have come from tissues of different species including guinea pig,¹³ equine,¹⁴ and bovine.¹⁵ Only a few experiments have been performed on human tissue, and no data are available on the possible effects of loop diuretics on neurotransmission in human airways in vitro.

We performed this study to assess the effects of frusemide and bumetanide on the cholinergic neurotransmission in human airways in vitro. We also investigated the effect of acetazolamide, a carbonic anhydrase inhibitor, on cholinergic neurotransmission as frusemide, but not bumetanide, possesses an inhibitory effect on carbonic anhydrase.¹⁶ Electrical field stimulation (EFS) was used to release acetylcholine (Ach) from postjunctional nerves in human airways and the contractile response to exogenous Ach was measured to evaluate a possible neuromodulatory effect.

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Methods

TISSUE PREPARATION

Human airways were obtained from 35 patients (33 men, two women) of mean age 63.6 (range 43–76) years undergoing pulmonary resection, mostly for bronchial carcinoma. Immediately after surgery the airways were macroscopically dissected in the operating theatre, placed in cold (4°C) Krebs-Henseleit (KH) solution (NaCl 118, KCl 5.9, MgSO₄ 1.2, CaCl₂ 2.5, NaH₂PO₄ 1.2, NaHCO₃ 25.5, and glucose 5.5 mmol/l) and transported to the laboratory. The airways were carefully stripped from surrounding lung tissue and cut into strips (main bronchi) or ring segments (segmental and subsegmental bronchi) which were mounted between two platinum wire electrodes in 10 ml organ baths containing KH solution at 37°C and continuously bubbled with 95% oxygen and 5% carbon dioxide. Indomethacin (10 µmol/l) was present in the organ bath throughout the experiment to block the formation of prostaglandins which may interfere with neurotransmission.¹⁷ In a different series of experiments indomethacin was omitted from the organ bath to investigate the possible involvement of cyclooxygenase products.

In most experiments the epithelium was removed by gently rubbing the luminal surface of the bronchi for two minutes with a cotton wool applicator. Complete removal of the epithelium was checked by light microscopy by an experienced pathologist in a blind fashion.

Bronchial strips or rings were connected via silk threads to Grass FT-03 force displacement transducers (Grass Instruments, Quincy, Massachusetts, USA) to measure isometric changes in tension. The tissues were allowed to equilibrate for one hour whilst being washed with fresh KH solution every 20 minutes under a resting tension of 2 g, which was found to be optimal for measuring changes in tension.¹⁸ EFS was produced by a Harvard student stimulator (Harvard, Kent, UK). Biphasic square wave pulses of a supramaximal voltage of 40 V at source and a pulse duration of 0.5 ms were applied for 15 seconds every four minutes at frequencies ranging from 0.5 to 32 Hz. Isometric contractile responses were visualised on a computer screen after digitalisation of the signal (Codal, Dataq Instruments, Ohio, USA) and recorded on a personal computer.

PROTOCOL

Electrical field stimulation

In human airways EFS causes a rapid contraction that can be abolished by pretreatment of the tissue with atropine, confirming that the contractile response is due to Ach release. After a one hour period of equilibration a frequency-response curve was constructed and discarded. After washing the tissues a control frequency-response curve was performed after which a diuretic or its vehicle (in a separate tissue) was added to the organ bath. The diuretics were incubated for 10 minutes

which gives a maximal inhibitory effect. Only one concentration of diuretic was added per tissue. After 10 minutes incubation a third frequency-response curve was performed. The responses to EFS in vehicle-treated tissues were stable throughout the period of the experiment. Six tissues were simultaneously studied with at least one time control tissue per experiment. The responses to EFS were completely blocked by tetrodotoxin (1 µmol/l), confirming their neuronal origin.

Acetylcholine concentration-response curve

The effect of frusemide (1 mmol/l) or bumetanide (100 µmol/l) on a cumulative concentration-response curve to exogenous Ach (0.3 µmol/l to 10 mmol/l) was studied in human bronchi after a 10 minute incubation period. The results were expressed as a percentage of the maximum contractile response to Ach (10 mmol/l) which was determined at the beginning of the experiment.

DRUGS

The drugs used in these experiments were obtained from the Sigma Chemical Co (Eupen, Belgium). Stock solutions of frusemide were made up in 100 µmol/l sodium hydroxide and further diluted with distilled water. Stock solutions of bumetanide were made up in 100% ethanol and further diluted with ethanol to the appropriate concentration. Acetazolamide was dissolved in 100 µmol/l sodium hydroxide and further diluted with distilled water. All control responses were obtained in the presence of an equivalent amount of solvent. Indomethacin was made up in alkaline phosphate buffer (KH₂PO₄ 20 mmol/l, Na₂HPO₄ 120 mmol/l; pH 7.8). Fresh drug solutions were made up daily. Drug additions did not exceed 1% of the bath volume. All concentrations refer to the final organ bath concentrations.

ANALYSIS OF RESULTS

All contractile responses were measured as the difference between peak tension and the resting tension that developed. The effect of the diuretic was expressed as percentage inhibition, comparing the contractile responses at each frequency after pretreatment with the diuretic with the contraction at the same frequency in the control response. Since each tissue acted as its own control analysis of data was possible using a Student's *t* test for paired data. Contractile responses to exogenous Ach were compared between diuretic-treated and vehicle-treated tissues by the Student's *t* test for unpaired data. Results are expressed as mean (SE), *p* values of <0.05 being considered significant.

Results

EFS in human airways (40 V, 0.5 ms, 0.5–32 Hz for 15 seconds) resulted in a rapid cholinergic contraction which increased with increasing frequencies of stimulation. A typical trace, which also demonstrated the inhibitory effect of frusemide, is shown in fig 1. The

Figure 1 Effect of electrical field stimulation (EFS) (40 V, 0.5 ms, 0.5–32 Hz for 15 seconds) in human airways (■). Frusemide (1 mmol/l) clearly inhibited the cholinergic contraction at all frequencies.

Mean (SE) inhibitory effect of frusemide (100 µmol/l) in absolute measurements (mg)

Frequency (Hz)	Control	Frusemide	% inhibition
0.5	302(78)	211(58)	30.2(5)
1	327(69)	223(51)	31.9(4)
2	463(71)	299(63)	35.5(4.6)
4	618(91)	516(73)	16.6(2)
8	809(72)	717(89)	12.5(2.9)
16	1064(99)	986(108)	7.4(1.1)
32	1358(109)	1238(73)	8.8(2.1)

Figure 2 Inhibition of EFS-induced cholinergic contraction by different concentrations of frusemide in human epithelium-denuded airways in the presence of indomethacin (10 µmol/l): ●, 10 µmol/l frusemide; ▲, 100 µmol/l frusemide; ■, 1 mmol/l frusemide. The frusemide vehicle (○) had no effect on the cholinergic contractions. Means (SE) values of five tissues of different patients are shown. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

inhibitory effect of frusemide (100 µmol/l) in absolute measurements is shown in the table.

EFFECT OF FRUSEMIDE ON CHOLINERGIC RESPONSES

In tissues with epithelium frusemide (1 mmol/l) produced no inhibition of the cholinergic contractions to EFS, with or without indomethacin (10 µmol/l). When the epithelium was removed and indomethacin (10 µmol/l) was added to the organ bath, frusemide (100 µmol/l and 1 mmol/l) inhibited the cholinergic contractile responses at all frequencies, which was, however, not significant at 32 Hz. Frusemide (10 µmol/l) had no more significant inhibitory effect on the cholinergic responses. Frusemide (1 mmol/l) produced a maximum inhibition of 46.3 (9.9)% ($n = 5$, $p < 0.05$) at 0.5 Hz and 45 (4.6)% at 1 Hz stimulation ($n = 5$, $p < 0.001$). There was no significant effect of the vehicle on the cholinergic responses (fig 2).

Without indomethacin in the organ bath frusemide (10 µmol/l) produced no inhibition of the cholinergic responses. The inhibition produced by 100 µmol/l frusemide, however, was significantly enhanced at 4, 8, and 16 Hz stimulation; at 8 Hz, for example, frusemide (100 µmol/l) produced an inhibition of 12.5 (2.9)% with indomethacin (10 µmol/l) and 27.4 (2.3)% without ($n = 5$, $p < 0.01$) (fig 3).

The responses to exogenous ACh (0.3 µmol/l to 10 mmol/l), with or without indomethacin, were not significantly altered by frusemide (1 mmol/l) (fig 4).

EFFECT OF BUMETANIDE ON CHOLINERGIC RESPONSES

In tissues with epithelium bumetanide (100 µmol/l) had no inhibitory effect on the EFS-induced cholinergic contractions. When the epithelium was removed bumetanide (10 and 100 µmol/l) inhibited the cholinergic responses at all frequencies, but not significantly at 16 Hz and 32 Hz for 100 µmol/l bumetanide. Bumetanide (100 µmol/l) produced a maximum inhibition of 39.6 (6.2)% at 0.5 Hz ($n = 5$, $p < 0.01$) and 38.4 (6.6)% at 1 Hz stimulation ($n = 5$, $p < 0.05$). At 1 µmol/l bumetanide had no significant inhibitory effect on the cholinergic responses, and no significant effect was seen with the vehicle for bumetanide (fig 5).

When indomethacin (10 µmol/l) was omitted the inhibition produced by bumetanide (10 µmol/l) was similar to that produced by the same concentration of bumetanide in the presence of indomethacin (10 µmol/l) (fig 6).

The responses to exogenous ACh (0.3 µmol/l to 10 mmol/l) were not affected by bumetanide (100 µmol/l) (fig 7).

EFFECT OF ACETAZOLAMIDE ON CHOLINERGIC RESPONSES

In tissues with or without epithelium acetazolamide (100 µmol/l) produced no significant inhibitory effect (2.2(1.5)%, $n = 5$) on the cholinergic responses to EFS in human bronchi (data not shown).

Figure 3 Inhibition of EFS-induced cholinergic contraction in epithelium-denuded tissues by frusemide ($100 \mu\text{mol/l}$) in the presence (▲) and absence (□) of indomethacin ($10 \mu\text{mol/l}$). Indomethacin attenuated the inhibitory effect of frusemide. Mean (SE) values of five tissues are shown. ** $p < 0.01$, * $p < 0.05$.

Figure 4 Cumulative concentration-response curve to exogenous acetylcholine ($0.3 \mu\text{mol/l}$ to 10 mmol/l) in the presence (●) and absence (○) of frusemide (1 mmol/l). Mean (SE) values of five different epithelium-denuded tissues are shown.

Discussion

These results show that the loop diuretics frusemide and bumetanide inhibit the cholinergic contractions in response to EFS in human airways in vitro in a concentration dependent manner. Removal of the epithelium was necessary to achieve this effect. Neither diuretic, however, affected the cumulative concentration-response relationship to exogenous Ach, suggesting that they may act through a prejunctional mechanism. When indomethacin was omitted from the organ bath inhibition of the cholinergic contraction induced by frusemide, but not by bumetanide,

was enhanced, although not significantly at all frequencies. This suggests that cyclooxygenase products may be involved in the mechanism of action of frusemide. Acetazolamide, a carbonic anhydrase inhibitor, produced no inhibition of the cholinergic contraction.

The mechanism of action of frusemide is not fully understood. In our experiments both frusemide and bumetanide inhibited the cholinergic contraction to EFS, so inhibition of the Na-K-Cl cotransporter, which is one of the mechanisms of action of loop diuretics in the kidney,¹⁹ cannot be excluded. Moreover, bumetanide was found to be about 10 times more potent than frusemide, as has been shown in previous in vitro studies.^{13,20} In in vivo challenges bumetanide has little or no effect.³ The same is true for piretanide and toresamide which are more potent diuretics but have less protective effect than frusemide in bronchoconstrictor challenges in asthmatic patients.²¹ Some investigators therefore question whether the inhibition of the Na-K-Cl cotransporter is a possible explanation for the anti-asthmatic effects of frusemide.¹⁰ However, our results in human airways in vitro indicate that this effect certainly can not be dismissed, and the discrepancy between in vivo and in vitro results remains difficult to interpret. It has been postulated that local pharmacokinetic effects in the airways in vivo might explain this discrepancy and, in fact, frusemide, but not bumetanide, has been shown to produce an acute elevation of plasma renin, and an increased peripheral blood flow,²² which could account for an increased washout of exogenously administered bronchoconstrictor agents. Moreover, bumetanide, in contrast to frusemide, is highly lipid soluble, suggesting that bumetanide is not maintained in a high enough concentration at its site of action in the airways in vivo.²³

Another explanation for the different effects of frusemide and bumetanide in vivo could be the fact that frusemide also possesses an inhibitory effect on carbonic anhydrase activity which bumetanide does not share, and that frusemide exerts its effect through inhibition of this enzyme.³ Our experiments, however, failed to demonstrate an inhibitory effect of acetazolamide on the EFS-induced cholinergic contraction, which makes this possibility unlikely. Moreover, carbonic anhydrase inhibition is unimportant in vitro as bumetanide, which has no such activity, was more effective than frusemide in our experiments.

Loop diuretics have an effect on airway nerves seen in guinea pig bronchi^{13,24} and trachea²⁰ in vitro. Frusemide also has an inhibitory effect on various inflammatory cells, and the latter mechanism is probably important in some challenges in asthmatic patients. Frusemide inhibits the release of histamine and leukotrienes in passively sensitised human lung in vitro²⁵ and in actively sensitised guinea pig lung.²⁶ It also inhibits mediator release from eosinophils in vitro²⁷ by inhibition of chloride transport. From these findings it has been suggested that frusemide exerts its effects through inhibition of a specific chloride channel.¹⁰ This seems an attractive hypothesis as

Figure 5 Inhibitory effect of different concentrations of bumetanide on the EFS-induced cholinergic contractions in human epithelium-denuded airways in the presence of indomethacin ($10 \mu\text{mol/l}$): $\blacksquare$, $1 \mu\text{mol/l}$ bumetanide; $\blacktriangle$, $10 \mu\text{mol/l}$ bumetanide; $\blacksquare$, $100 \mu\text{mol/l}$ bumetanide. The bumetanide vehicle ($\triangle$) had no effect on the cholinergic contractions. Mean (SE) values of five different tissues are shown. ** $p < 0.01$, * $p < 0.05$.

Figure 6 Inhibition of the EFS-induced cholinergic contraction in epithelium-denuded tissues by bumetanide ($10 \mu\text{mol/l}$) in the presence ($\circ$) and absence ($\bullet$) of indomethacin ($10 \mu\text{mol/l}$). Indomethacin withdrawal did not result in an increased inhibitory effect of bumetanide. Mean (SE) of five tissues are shown.

nedocromil sodium possesses an inhibitory effect on chloride transport,^{28,29} but whether chloride channel blockers can inhibit cholinergic contraction in human airways in vitro remains to be investigated.

Frusemide enhances the synthesis of prostaglandin E_2 in the kidney and it has therefore been postulated that prostaglandin release may be involved in the protective effects of frusemide. Indeed, prostaglandins E_1 and E_2 have

been shown to modulate airway neurotransmission.^{17,30} Recent data provide evidence that inhibitory prostaglandins are involved in the prevention of exercise-induced asthma by frusemide. Indomethacin (50 mg three times daily) attenuated the protective effect of frusemide on exercise-induced asthma,³¹ and flurbiprofen, another cyclooxygenase inhibitor, did not modulate the protective effect of frusemide on metabisulphite-induced bronchoconstriction. This is either evidence against the release of inhibitory prostaglandins by frusemide,³² or for the release of prostaglandins by frusemide under certain conditions such as exercise-induced asthma or other mast cell degranulating stimuli. Others have also failed to show increased production of prostaglandin E_2 in human nasal mucosa in vivo after frusemide instillation.³³ In our experiments the frusemide-induced inhibition of the cholinergic contraction was enhanced when indomethacin was omitted from the organ bath, although this enhancement was only seen with a high dose ($100 \mu\text{mol/l}$). The bumetanide-induced inhibition was, however, not enhanced when indomethacin was omitted from the organ bath, perhaps because it has no inhibitory effect on 15-hydroxyprostaglandin dehydrogenase, an enzyme involved in the inactivation of prostaglandins.³⁴ Indomethacin in a concentration of $10 \mu\text{mol/l}$ augments the responsiveness of unrubbed canine bronchial rings to acetylcholine, which may be due to inhibition of the generation of prostaglandins.³⁵ These results suggest that the release of prostaglandins may be involved in the inhibition induced by frusemide, but not by bumetanide, although this effect may not be important. Other in vitro experiments also suggest an influence of inhibitory prostaglandins.³⁶

In our experiments loop diuretics only produced inhibition of the cholinergic contractions in tissues without epithelium and it seems unlikely that these inhibitory prostanooids are released by the epithelium. The site of prostaglandin release therefore remains speculative.

Frusemide failed to produce an inhibitory effect on cholinergic contractions in equine tracheal tissue without epithelium, suggesting that it produces its effects in this species through release of an epithelium-dependent relaxant factor.¹⁴ This is in agreement with the effect of frusemide on vascular smooth muscle where the vasoconstrictor response to periarterial electrical stimulation in rat tail artery with intact endothelium was inhibited, but this effect was absent in endothelium-denuded preparations.³⁷ This contrasts with the findings of the previous studies – for example, Elwood *et al*¹³ showed that frusemide modulated the cholinergic and non-cholinergic contractions in guinea pig airways by a prejunctional mechanism which was independent of the epithelium. In the present study, however, epithelium removal was necessary to achieve an inhibition of the cholinergic contraction by frusemide and bumetanide.

Mechanical removal of the epithelium enhances the responsiveness and sensitivity to

Figure 7 Cumulative concentration-response curve to exogenous acetylcholine (0.3 $\mu\text{mol/l}$ to 10 mmol/l) in epithelium-denuded tissues in the presence (●) and absence (○) of bumetanide (100 $\mu\text{mol/l}$). Mean (SE) values of eight tissues are shown.

several spasmogens in airway preparations from several animal species including man.³⁸ This increased reactivity may be due to the existence of an inhibitory signal generated by the epithelium.³⁵ The loss of an inhibitory factor cannot explain the inhibitory effect of loop diuretics in epithelium-denuded human airways in vitro.

Epithelium removal results in loss of a barrier function.³⁹ A direct inhibitory effect of frusemide on the smooth muscle is unlikely, however, as it has no effect on smooth muscle contractions induced by various constrictor agents.¹⁵ This was also seen in the present study as frusemide did not affect the dose-response curve to exogenous Ach.

Epithelium removal also exposes airway nerves, a state similar to the airways of asthmatic subjects, where chronic inflammation causes damage to the epithelium, exposing sensory nerve endings.^{40,41} By removing the epithelium and exposing airway nerves frusemide could readily reach the nerves to exert its inhibitory effect. As high concentrations of frusemide are needed to inhibit the cholinergic contraction, however, a possible toxic effect of the loop diuretics on the airway nerves cannot be excluded.

This study has shown that loop diuretics, in a concentration-dependent manner, inhibit the cholinergic contraction in human airways in vitro by a prejunctional mechanism, but only in epithelium-denuded tissues. Cyclooxygenase products enhance this inhibitory effect with frusemide, whilst a carbonic anhydrase inhibitor has no inhibitory effect at all. Our experiments suggest that inhibition of Na-K-Cl cotransporter may explain the inhibitory effect of loop diuretics on cholinergic contraction. However, there is no explanation as to why epithelium removal is necessary to achieve this effect in human airways in vitro.

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Adventitia

A Philadelphia summer

"The August heat was merciless. From the seventh to the twenty-seventh there were only two cool days. Twice there was rain and afterwards the sun shone through a steamy mist. Those who have lived through Philadelphia summers know these afternoons and would view with compassion the moist faces of the delegates". Thus wrote William Johnson, a Connecticut delegate to the Constitutional Convention which followed the Declaration of Independence two centuries ago.

Things were much the same in August 1976. The sweltering city of the founding fathers was again host to a great variety of groups celebrating the bicentenary of independence. There were conventions of magicians and candlemakers, a Eucharistic congress, national archery and rowing championships – and the fall meeting of the American Physiological Society to which I was to present a paper.

We had one great advantage over the perspiring delegates of 200 years ago – air conditioning – or did we? The first hint that something was amiss was when they switched off the air conditioning at my hotel. I was assured that this was a routine servicing procedure – but why in the middle of an August heat wave and who were those heavies snooping around the lobby and corridors? And then the story broke with screaming headlines in the *Philadelphia Daily News*: dozens of delegates to the convention of the Pennsylvania branch of the American Legion, held in the city two weeks earlier, were being smitten by a mysterious disease from which 19 had already died. Hotel bookings were cancelled and the city, which had invested billions of dollars in the bicentennial celebrations, was facing bankruptcy. Rumours abounded. The veterans of foreign wars accused drug companies of conspiring to release a fatal flu virus in order to promote their vaccine sales. High levels of nickel had

been found in the bodies of fatal cases and a left wing group (perhaps communist sailors from a Polish ship moored in the Delaware river) were thought to have injected nickel carbonyl gas into the air conditioning systems of hotels where the legionnaires were staying. Or was there some noxious element in the droppings of those rabbits and doves conjured out of hats at the magicians' convention?

These rumours were not confined to the tabloids and taverns of Philadelphia. Even the *Lancet*, as late as June 1977, was still promoting the nickel theory and contemplating the possibility that "careless chemists," "mad scientists," "saboteurs," or "criminals" were responsible; or perhaps the nickel carbonyl gas was coming from the garbage cans in which Philadelphians were burning their rubbish because of a garbage collectors' strike? And so the theories proliferated until Joseph McDate noticed a curious red patch on a culture from a guinea pig inoculated with lung tissue from a dead legionnaire. From this he grew *Legionella pneumophila*, the Gram negative bacillus now known to be the cause of the outbreak.

Of 182 patients with legionella pneumonia, 29 died. The victims included 149 legionnaires, nine Eucharists, two candlemakers, and one magician – but no physiologists. The majority of these had – as indeed had I – entered the Bellevue-Stratford hotel or strolled along the adjacent sidewalk on Broad Street. Raised serum titres among the hotel employees suggested long term exposure to the bacillus and a degree of immunity, for only one of the 400 employees contracted the disease. This solitary case was the air conditioner man. Perhaps, like our famous predecessors two centuries ago, we too should have sweated out that Philadelphia summer and eschewed the comforts of modern technology.

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