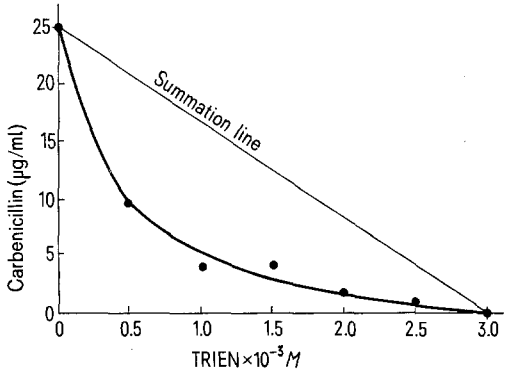
Triethylene Tetramine. A New Potentiator of Antibiotic Activity

There is a large and shameful pool of resistance among bacteria to classical antibiotic and chemotherapy. It is well established that ethylenediamine tetra-acetate (EDTA) increases the sensitivity in vitro of Gram negative bacteria to antibiotics[1,2], but NEU and WINSHELL[3] suggest that this effect is not appreciable and unlikely to be useful clinically. Moreover, even if antibiotic activity were considerably enhanced by EDTA, this compound is too toxic for routine systemic use[4]. I have found that another chelating agent, triethylene tetramine (TRIEN), significantly enhances antibiotic activity both in vitro and in vivo. TRIEN, like EDTA, selectively binds divalent cations but is neither toxic nor teratogenic; it has found limited clinical use in the management of Wilson's disease[5].

Experiments and results. In the following study TRIEN specially purified as the dihydrochloride was used because commercial preparations of the free base contain toxic impurities. *Pseudomonas aeruginosa* was chosen as the test organism because it shows extensive resistance to antibiotics and is a serious hospital pathogen; the strain used was isolated post-mortem from a baby which died of septicaemia.

The minimum inhibitory concentration (MIC) of carbenicillin was determined by a tube dilution technique. The tubes, containing 2 ml growth medium inoculated with 10^5 pseudomonads, were incubated for 18 h at 37 °C; the MIC was taken as the lowest concentration of antibiotic which prevented visible growth. The effect of TRIEN on the MIC was investigated in a parallel test in which the growth medium contained a subinhibitory concentration of TRIEN. With brain heart infusion + 25% serum, pH 7.4, as the growth medium the presence of 5×10^{-3} *M* TRIEN consistently reduced the MIC of carbenicillin 8-fold (from 62.5 to 7.8 µg/ml).

The interaction between TRIEN and carbenicillin was analyzed by plotting the isobologram. Using tube dilutions in brain heart infusion + 25% serum, the OD_{50} concentration (that which reduces growth by 50% compared with growth in a control tube) was measured by comparing optical densities in an 'EEL' colorimeter. The OD_{50} concentrations of carbenicillin were determined also in the presence of different TRIEN concentrations which were below the previously estimated OD_{50} concentration of TRIEN. The result is given in the Figure. The differences between the observed OD_{50} concentrations and those predicted for a summation of the individual antibacterial actions of carbenicillin and TRIEN are significant ($0.05 > p > 0.02$), indicating that their action is synergistic.



Isobologram showing OD_{50} concentration of carbenicillin for *Ps. aeruginosa* in the presence of different concentrations of triethylene tetramine (TRIEN).

The methods of OLITZKI[6] and GORRILL[7,8] were used to produce acute and chronic infections in male Balb/c mice aged 14–15 weeks.

In one experiment mice were injected i.p. with the minimum lethal dose of pseudomonads (3.5×10^6 organisms in 0.4 ml 5% mucin). Pretreatment of the animals with a single i.v. dose of 75 mg carbenicillin, the maximum that could be given, did not offset endotoxaemic death. But pretreatment with only 50 mg carbenicillin plus 10 mg TRIEN did protect against acute death and the mice survived indefinitely.

In another experiment 33 mice were injected i.v. with 9.0×10^7 pseudomonads in 0.4 ml broth; with this dose renal abcesses develop within 48 h. 11 of the mice remained untreated. Another 11 received 2 doses of 50 mg carbenicillin i.v., the first 48 h and the second 72 h after injection of the organisms. The third group of 11 similarly received 2 doses of 50 mg carbenicillin but each time with 10 mg TRIEN in addition. 96 h after the start of the experiment the surving mice were killed and their kidneys examined for visible abcesses; assessment of the condition of the kidneys was performed by an assistant lacking knowledge of the specific treatments received by the animals. $^7/_{10}$ of the animals in the untreated group and $^5/_{11}$ of those given carbenicillin alone had renal lesions: there is no statistical difference between these results ($p < 0.30$). $^1/_{10}$ of the mice treated with carbenicillin plus TRIEN had renal abcesses and compared with the untreated group this is significant ($p < 0.01$).

Discussion. In the experiments described, TRIEN definitely increased the efficacy of carbenicillin therapy both in vitro and in vivo. This is in contrast to the findings of WEISER (personal communication) who, using EDTA plus tetracycline, managed to sterilize the surface of experimental burns infected with *Ps. aeruginosa* but could not prevent death from septicaemia.

Like EDTA[9], TRIEN probably increases the permeability of the bacteria to the antibiotic so that this can reach its site of action. Possibly TRIEN acts by binding divalent cations in the cell envelope lipopolysaccharide. (It is unlikely that TRIEN enhances the activity of carbenicillin by chelating ions in the growth medium because the addition of Mg^{++} or Ca^{++} ions as the chlorides did not affect the MIC of carbenicillin.)

From the results with TRIEN it would seem that the unfavourable conclusions of NEU and WINSHELL[3] do not apply generally and that there is a real prospect of

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overcoming bacterial permeability barriers in clinical practice.

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[11] Present address: The Middlesex Hospital Medical School, London W1P 7PN, England.

Résumé. In vivo aussi bien qu'in vitro, la triéthylène tétramine (TRIEN), agent qui forme des chélats avec les cations bivalents, renforce significativement l'activité antibactérienne de l'antibiotique carbénicilline envers la résistante *Pseudomonas aeruginosa.*

G. Smith[10,11]

St Catharine's College, University of Cambridge, Cambridge (Great Britain), 7 August 1974.

Phlorizin Binding to Bilayer Vesicles of Phospholipids and Phospholipid-Cholesterol

Phlorizin inhibits sugar transport in kidney, intestine, and erythrocytes[1,2]. In erythrocytes, it also inhibits anion exchange and increases anion conductance[3,4]. However, the mechanism of its interaction with cell membranes is unclear. In renal cell membranes, phlorizin was found to bind not only to a specific, high-affinity protein receptor[5], but also to low-affinity sites of unknown nature[5,6], possibly including lipid. In this context, it seemed useful to study the binding of phlorizin to sonicated bilayer vesicles of phospholipids and phospholipid-cholesterol.

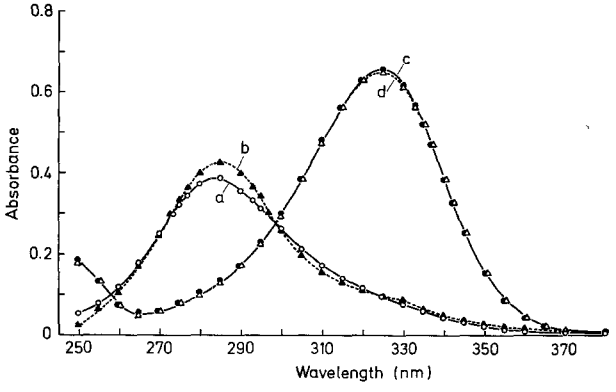

Fig. 1. Phlorizin absorbance spectra at pH 4.5 and 9.5 and the effect of PC vesicles. Phlorizin, 25 μM; phospholipid, 2 mM. At pH 4.5: a) keto-phlorizin; a_M, 1.62×10^4 M^{-1} cm^{-1} (285 nm); b) phlorizin and PC. At pH 9.5: c) enol-phlorizin; a_M, 2.73×10^4 M^1 cm^{-1} (325 nm); d) phlorizin and PC. To amplify changes in spectra, lipid concentrations were increased to 4 times that used in the binding studies.

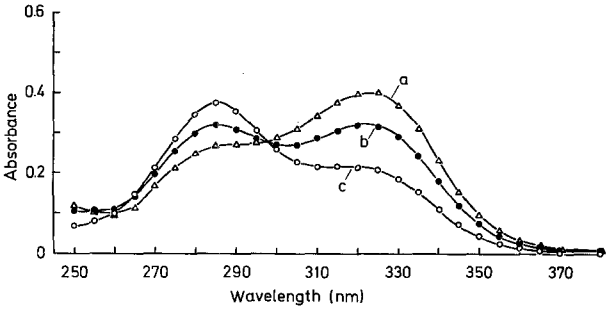

Fig. 2. Effect of PC and PS vesicles on the phlorizin absorbance spectrum at pH 7.2. Phlorizin, 25 μM; phospholipid, 2 mM. a) phlorizin; b) phlorizin and PS; c) phlorizin and PC.

Methods. Chromatographically pure egg yolk phosphatidylcholine (PC) and ox-brain phosphatidylserine (PS) were obtained from Lipid Products (South Nutfield, U.K.). Cholesterol (99%) (Merck) was recrystallized from methanol. Phlorizin (Roth) was purified to remove all traces of phloretin[4].

PC and PS dispersions in 50 mM KCl and 30 mM Glycylglycine buffer at pH 7.2 were sonicated at 5 °C under N_2 to minimum optical density[7,8]. Phlorizin binding to lipid vesicles was measured spectrophotometrically in double-chamber mixing cuvettes (Hellma) at 25 °C. The 2 compartments of the reference cuvette were filled with equal volumes (1 ml) of lipid vesicles and buffer. The sample cuvette contained vesicles in one chamber and phlorizin (20–75 μM) in the other. The absorbances of keto-phlorizin at 285 nm and of enol-phlorizin at 325 nm were measured before mixing and within 2 min afterwards. On the assumption that the enolic form of phlorizin is not bound to the lipid vesicles, the concentrations of free and bound keto-phlorizin were calculated from these absorbances[9].

Results and discussion. The absorption spectra of the keto and enol tautomers[10] of phlorizin are shown in Figure 1 (Curves a and c). Also shown is the effect of PC vesicles on the spectra. The spectrum of phlorizin plus lipid at pH 9.5 is identical to that of the enolic species alone. The spectrum of phlorizin plus lipid at pH 4.5 differs slightly from that of the ketonic species alone. These observations suggest that an association of the ketonic species with the phospholipid occurs, accompanied by a change in the molar absorbancy index (a_M). The effect of PS on a_M was qualitatively similar, but smaller. In the calculation of free and bound phlorizin concentrations, corrections were made for this small change in a_M.

The addition of liposomes to phlorizin at pH 7.2, close to the pK_a of phlorizin, lowers the absorbance at 325 nm and increases that at 285 nm (Figure 2). Together with

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