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The biochemical basis of synergy between the antibacterial agents, chlorocresol and 2-phenylethanol

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Summary

The combined antimicrobial action of chlorocresol and 2-phenylethanol has been investigated. Synergy in growth inhibition experiments was reflected by increased membrane permeability to hydrogen, potassium and phosphate ions and an inability to accumulate glutamate actively. No gross disruptive effects on the membrane were observed. ATP synthesis and glucose uptake were largely unaffected by combinations of these two agents despite a marked inhibition of respiratory activity. The biochemical basis of synergy is believed to result from a combined action on the generation and coupling of a proton gradient to active solute transport and retention processes.

Introduction

The phenomenon of synergy between non-antibiotic antimicrobial substances has recently been reviewed (Denyer et al., 1985). Among the synergistic pairs of preservatives identified, that of chlorocresol (CC) and 2-phenylethanol (PEA) was noteworthy (Richards, 1971; Richards and McBride, 1971, 1972). Using this combination, an attempt has been made in this paper to establish whether synergy demonstrated by microbiological evaluation is paralleled by synergy in biochemical studies.

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Materials and Methods

Chemicals and reagents

L-α-Phosphatidyl-DL-glycerol (PG), Hepes buffer and Fiske and SubbaRow (1925) inorganic phosphate microassay kit were obtained from Sigma Chemicals, Poole, glucose/fructose assay kit was from Boehringer Mannheim, F.R.G., ATP monitoring kit from LKB-Wallace, Finland, and L-[U-^{14}C]glutamic acid was from Amersham International plc, Bucks. Fentichlor [bis(2-hydroxy-5-chlorophenyl)sulphide] was obtained from BTP Cocker Chemicals, Lancs. Chlorocresol (CC), 2-phenylethanol (PEA) and all other chemicals were from BDH, Poole.

Culture media

Nutrient broth contained (g/l): peptone, 7.5;

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Lab Lemco, 4; sodium chloride, 5 and was solidified when required with Agar No. 1, 15. The semi-synthetic medium used was a modification of that of Davis and Mingioli (1950) and contained (g/l): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1; glucose, 5 and peptone, 20. All media were adjusted to pH 7.4 and sterilized at 121°C for 20 min. Media constituents were from Oxoid, Basingstoke.

Organism

Staphylococcus aureus NCTC 6571 was used throughout this work. Washed cell suspensions were prepared by the method of Mlynarcik et al. (1981).

Determination of minimum growth inhibitory concentrations (MIC's) at low inoculum size

An MIC determination in nutrient broth was performed for both CC and PEA (using concentration increments of 2.5 mg% w/v and 25 mg% v/v, respectively) against an inoculum of 1×10^6 cells/ml in stationary culture at 37°C. Combinations of the two antibacterial agents were tested, up to and including the MIC of each individual compound, using the chessboard method and the results plotted as an isobologram (Denyer et al., 1985).

Determination of growth inhibition at high inoculum levels

This was examined for five concentrations of CC and PEA and selected combinations (Table 1)

TABLE 1

CONCENTRATIONS OF CHLOROCRESOL (CC) AND 2-PHENYLETHANOL (PEA) STUDIED AND THE COMBINATIONS INVESTIGATED

PEA conc. (% v/v)	CC conc. (% w/v)	Combination
0.095	0.007	0.007% w/v CC + 0.38% v/v PEA(B)
0.19	0.014	0.014% w/v CC + 0.285% v/v PEA(C)
0.285	0.021	0.021% w/v CC + 0.19% v/v PEA(D)
0.38	0.028	0.028% w/v CC + 0.095% v/v PEA(E)
0.475(A)	0.035(F)	

Letters in parentheses refer to preservative systems in Figs. 2 and 3.

in the modified semisynthetic medium using an inoculum of 4×10^9 cells/ml. Incubation was at 37°C in a shaking water bath (100 oscillations/min). Evidence of growth at intervals over a 24 h period was obtained by optical density measurements at 420 nm on diluted 0.1 ml samples. The diluent was quarter-strength Ringer's solution.

Physical interaction of preservatives and phospholipid monolayers

Surface pressures of PG monolayers spread on an aqueous subphase were measured by a modification of the static (immersion) technique using an automated Wilhelmy plate apparatus and Langmuir trough (Proudfoot, 1971). 30 μl of phospholipid was introduced at the liquid–air interface in a hexane : ethanol (19 : 1) spreading solution (0.35 mg/ml) and allowed to form a monolayer. This monolayer was compressed by means of a moveable waxed barrier. The subphase consisted of CC, PEA or a combination of the two agents (Table 1) dissolved in triple-distilled water and maintained at 37°C. From the surface pressure/area curves generated, the area per mg of PG at a surface pressure of 10 mN/m was determined and expressed as a percentage of a control monolayer spread on triple-distilled water alone. Results are the mean of a minimum of three determinations.

Measurement of proton translocation

A modification of the method of Gilby and Few (1958) was used. A two-way pH-stat automatic titrator (Mettler Instrumente, Switzerland) connected to an E-PH converter (DK12) and linear chart recorder was employed to maintain the pH of a cell suspension (4×10^9 cells/ml) in 20 ml distilled water at pH 5.5 for 15 min. The titrant was 0.02 M HCl and the suspension was held at 37°C with stirring. 0.2 ml of drug or drug combination (Table 1) in ethanol was then added and the volume of acid required to maintain the pH recorded over a further 15 min period. Transmembrane proton flow was expressed as the volume of titrant added within the first 2 min of drug addition and was compared with control cells. 1% v/v ethanol had no effect on proton translocation.

Measurement of leakage of cellular components

Cell suspensions (4×10^9 cells/ml), prepared in

0.01 M Hepes buffer, pH 7.4 and containing either CC, PEA or their combinations (Table 1), were shaken (100 oscillations/min) at 37°C for 15 min (potassium and phosphate experiments) or 1 h (pentose experiments). At the end of this period cells were removed by filtration (0.2 μm cellulose nitrate Whatman membrane filter) and the filtrate assayed for potassium (Fuller et al., 1985), inorganic phosphate (Fiske and SubbaRow, 1925) or pentose (Meijbaum, 1939), as appropriate. The antibacterial agents did not affect the assays.

Measurement of glucose oxidation

Oxygen uptake was followed by Warburg manometry (Umbreit et al., 1972). Organisms were suspended at 4×10^9 cells/ml in 0.01 M Hepes buffer, pH 7.4, containing the appropriate antibacterial agent or combination of agents (Table 1). Glucose was supplied at a limiting concentration of 0.5 mM.

Measurement of glucose uptake

This was determined by following the disappearance of glucose from the medium, as measured with a glucose/fructose determination kit (Fuller et al., 1985). Freshly harvested organisms were suspended to 4×10^9 cells/ml in Hepes buffer (0.01 M, pH 7.4) containing appropriate concentrations of antibacterial agent(s) (Table 1), and maintained in a shaking water bath (100 oscillations/min) at 37°C. The experiment was initiated by adding glucose to a final concentration of 0.5 mM. After 60 min duplicate 5 ml samples were withdrawn, the preservative(s) extracted with chloroform, and the extract assayed for glucose content. Results were expressed as a percentage of control cell uptake.

Determination of cellular ATP levels

Organisms (4×10^9/ml) in 0.01 M Hepes buffer, pH 7.4, were incubated overnight in a shaking water bath (100 oscillations/min) at 37°C to deplete their ATP reserves (Mlynarcik et al., 1981). ATP synthesis in these organisms was initiated by the simultaneous addition of antibacterial agent(s) to the required concentrations(s) (Table 1) and glucose to a final non-limiting concentration of 5 mM. ATP was extracted from the cells at 60 min by the method of Sharpe et al. (1970). *n*-Butanol and residual antimicrobial agent(s) were removed by chloroform extraction (Mlynarcik et al., 1981). Dissolved chloroform was removed by vacuum evaporation (50°C for 10 min) and ATP determined by the luciferin/luciferase reaction (ATP monitoring kit). ATP synthesis in the presence of drug(s) was expressed as a percentage of control cells.

Measurement of L-glutamate uptake

Starved cells (4×10^9/ml) prepared by overnight incubation as described above, were suspended in 0.01 M Hepes buffer, pH 7.4, containing 5 mM glucose. To this was added an appropriate aliquot of an ethanolic solution of antibacterial agent(s) (Table 1; the alcohol did not exceed 1% of the final reaction volume), immediately followed by L-[U-^{14}C]glutamic acid to a final activity (concentration) of 0.1 μCi/ml (18 μM), to initiate the experiment. The mixture was incubated at 37°C with shaking (100 oscillations/min). 1 ml samples were removed after 60 min and passed through a 0.2 μm Millipore cellulose nitrate membrane filter which had been presoaked with ice-cold unlabelled glutamic acid solution. The bacteria were washed on this filter with 10 ml of the same solution. Counting was performed on a Kontron SL4000 Liquid Scintillation Counter after placing the filter in 5 ml of Filter-Count scintillation fluid (Packard, IL) in low-potassium glass vials. Passive uptake by cells was determined in the absence of glucose or following exposure to 5 μg/ml Fentichlor. The proportion of glutamate actively accumulated was estimated by subtraction of passive uptake from the total cellular ^{14}C-count. The results were expressed as a percentage of active glutamate uptake by control cells. Ethanol alone did not affect the uptake of the amino acid by bacteria.

Determination of Mg^{2+}-ATPase activity

A reaction mixture designed to contain finally (in 2 ml) 100 mM KCl, 5 mM $MgCl_2$, 0.1 units Mg^{2+}-ATPase, an appropriate concentration of antibacterial agent or combination of agents (Table 1), and 0.01 M Hepes buffer, pH 7.4, was prepared. The mixture was incubated at 37°C for

15 min and the reaction initiated by the addition of prewarmed disodium ATP in Hepes buffer to give a final concentration of 5 mM and a volume of 2 ml. The reaction was halted at 30 min by the addition of 1 ml of acid molybdate solution (Sigma Chemicals) and the inorganic phosphate produced was determined by the method of Fiske and SubbaRow (1925). Mg^{2+}-ATPase activity, calculated as an inorganic phosphate yield, was expressed as a percentage of enzyme activity in the absence of drug. The assay method was unaffected by the antimicrobial agents.

Calculation of the theoretical response to preservative combinations

Experimentally determined responses to individual agents, when expressed as a percentage of control experiments, can be used to calculate the theoretical additive effect of combined preservative treatment in the following manner.

If the response in the presence of preservative A is a% of control experiments and that in the presence of preservative B is b% of the control response, then the theoretical additive effect of preservatives A and B in combination is: ab/100 (calculated as a % of control).

Results

CC and PEA exhibit anti-staphylococcal activity, inhibiting growth in a concentration-dependent manner (Tables 2 and 3). An interaction with the cytoplasmic membrane may be central to this action since both agents produced an expansion of the monolayer of PG (a major phospholipid in the *S. aureus* membrane), presumably by their intrusion into the layer and the weakening of phospholipid–phospholipid interactions. In the intact cell it was found that leakage of intracellular material was restricted to small ions with PEA but extended to include pentoses in the case of CC. Active transport of glutamate and respiratory activity were inhibited by high concentrations of both compounds although respiratory stimulation was observed at low levels of CC. ATP synthesis and ATPase activity were markedly affected by CC but not PEA. Glucose uptake was largely unaffected by both compounds.

When used in combination CC and PEA demonstrate the capability for synergy against a low inoculum (10^6 cells/ml) of *S. aureus*, giving a summed fractional inhibitory concentration (ΣFIC) (Denyer et al., 1985) of 0.66 at the inflexion of the isobologram obtained from the MIC's of combinations of CC and PEA (Fig. 1). With a larger inoculum (4×10^9 cells/ml), the same organism was again challenged with CC and PEA individually and in combination. The results of growth inhibition studies are presented in Figs. 2a and 3a, where the actual response obtained is compared with the expected additive response calculated from the effects of the individual com-

TABLE 2

EFFECT OF CHLOROCRESOL (CC) ON THE GROWTH, METABOLISM AND MEMBRANE INTEGRITY OF *S. aureus*, PHOSPHATIDYLGLYCEROL (PG) MONOLAYER STRUCTURE AND Mg^{2+}-ATPase ACTIVITY

Parameter studied	Activity (% of control) in the presence of CC					
	CC concentration (% w/v): 0 (control)	0.007	0.014	0.021	0.028	0.035
Growth	100	90	75	63	50	38
Expansion of PG monolayer	0	−4	−6	3	19	46
Increase in proton permeability	0	0	4	53	96	118
Increase in potassium ion leakage	0	3	20	41	97	111
Increase in phosphate ion leakage	0	3	5	8	66	279
Increase in pentose leakage	0	23	118	158	162	221
Respiration	100	113	123	58	36	21
Glucose uptake	100	100	100	99	91	53
Active transport of glutamate	100	53	39	32	25	19
ATP synthesis	100	75	61	56	30	9
Isolated Mg^{2+}-ATPase activity	100	82	66	54	34	23

TABLE 3

EFFECT OF PHENYLETHANOL (PEA) ON THE GROWTH, METABOLISM AND MEMBRANE INTEGRITY OF *S. aureus*, PHOSPHATIDYLGLYCEROL (PG) MONOLAYER STRUCTURE AND Mg^{2+}-ATPase ACTIVITY

Parameter studied	Activity (% of control) in presence of PEA					
	PEA concentration (% v/v): 0 (control)	0.095	0.19	0.285	0.38	0.475
Growth	100	94	88	83	78	40
Expansion of PG monolayer	0	5	10	16	25	47
Increase in proton permeability	0	0	0	7	22	29
Increase in potassium ion leakage	0	23	38	46	55	64
Increase in phosphate ion leakage	0	0	0	0	10	380
Increase in pentose leakage	0	−18	0	0	0	21
Respiration	100	80	65	45	40	12
Glucose uptake	100	100	99	99	98	97
Active transport of glutamate	100	72	51	37	28	10
ATP synthesis	100	92	84	85	79	75
Isolated Mg^{2+}-ATPase activity	100	86	86	79	76	73

pounds at the concentrations employed in each combination. Combinations C, D and E (Table 1) of the two agents showed a greater growth inhibitory effect than anticipated from experiments using CC and PEA alone, the maximum synergistic effect being noted at combination D. With this combination growth inhibition was in excess of that achieved by 0.475% v/v PEA or 0.035% w/v

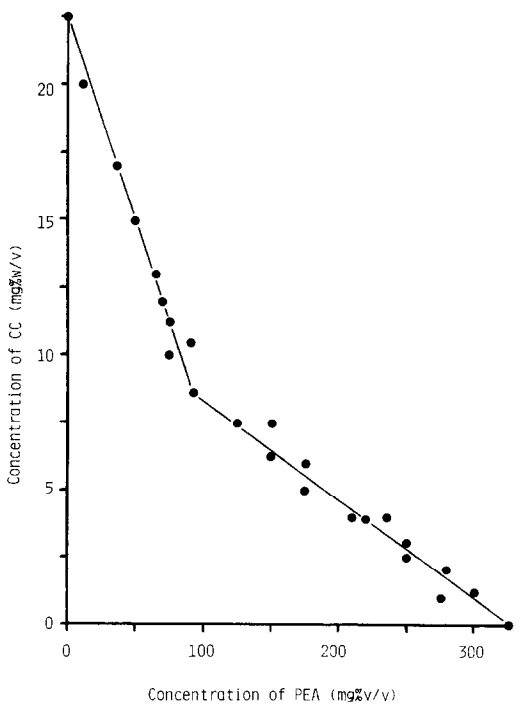

Fig. 1. Isobologram drawn from minimum growth inhibitory concentrations of chlorocresol (CC) and 2-phenylethanol (PEA) used alone and in combination against *Staphylococcus aureus*.

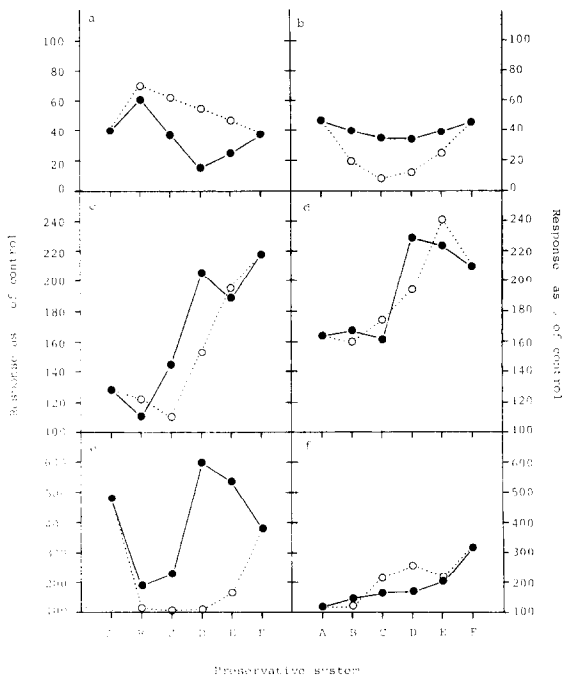

Fig. 2. Effect of chlorocresol (CC) and 2-phenylethanol (PEA) alone and in combination on: (a) *S. aureus* growth; (b) PG monolayer expansion; (c) proton permeability; (d) potassium ion leakage; (e) phosphate ion leakage; (f) pentose leakage. For details of preservative systems studied see Table 1. Experimentally determined response, ●———●; theoretical additive response, ○·····○.

CC alone. The pattern of growth inhibition was largely unaltered over an incubation period of 3–24 h and the results at 10 h are presented in Figs. 2a and 3a.

Results of biochemical and physical studies performed in the presence of CC/PEA combinations are presented in a similar manner to growth inhibition data and are similarly compared against a calculated theoretical additive response (Figs. 2b–f and 3b–f). Maximum enhancement of individual drug action was observed with combination D for hydrogen, potassium and phosphate ion permeability studies and glutamate active transport (Figs. 2c, 2d, 2e and 3d, respectively), while combination C showed greatest effect in PG monolayer (Fig. 2b) and respiration (Fig. 3b) experiments. Conversely, pentose leakage was reduced in the presence of these two combinations (Fig. 2f) and inhibition of ATP synthesis and ATPase activity was, in all cases, less than that calculated from the effects of CC and PEA alone (Figs. 3e and f). Glucose uptake followed closely the predicted pattern of inhibition (Fig. 3c).

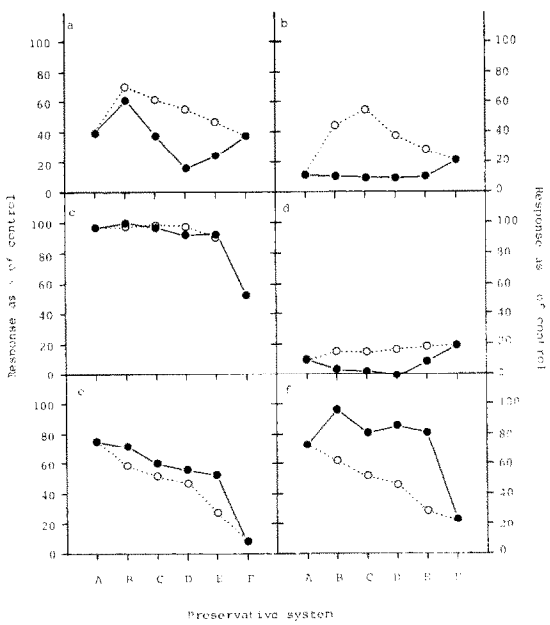

Fig. 3. Effect of chlorocresol (CC) and 2-phenylethanol (PEA) alone and in combination on: (a) *S. aureus* growth; (b) respiration; (c) glucose uptake; (d) active transport of glutamate; (e) ATP synthesis; (f) Mg^{2+}-ATPase activity. For details of preservative systems studied see Table 1. Experimentally determined response, ●———●; theoretical additive response, ○·····○.

Discussion

CC and PEA are known to act on the cytoplasmic membrane. At antibacterial concentrations CC acts as a membrane disruptor (Judis, 1962) while PEA affects membrane fluidity (Halegoua and Inouye, 1979) and also the transport of precursors for macromolecular synthesis (Richardson et al., 1969; Jones 1979; Duff and Wheller, 1981; Harding et al., 1984). In this work the membrane activity and resultant effects on metabolism of each compound are confirmed to be subtlely different (Tables 2 and 3). Indeed the compression of the drug–PG mixed monolayers to small areas/mg resulted in the collapse of the CC–PG monolayer at a lower surface pressure than the mixed PEA–PG or pure monolayers (data not shown). Thus, mixed monolayers of PEA–PG were more stable to compression than those of CC–PG indicating that PEA is capable of reducing intramembrane PG interactions without causing gross monolayer disruption (Kaye and Proudfoot, 1971).

CC exhibits many features of an uncoupling agent, i.e. conduction of protons, stimulation of respiration at low concentrations, and inhibition of ATP synthesis. Further, active transport of glutamate, an amino acid accumulated in response to a transmembrane proton gradient (Niven and Hamilton, 1974; Clement et al., 1984) was inhibited. No effect was demonstrated on glucose uptake which is a group translocation process except at concentrations where membrane disruption was apparent.

PEA does not appear to act in the same manner: proton conduction was weak, there was no stimulation of respiration and only slight inhibition of ATP synthesis, which, in the absence of respiration, is likely to be synthesized by substrate level phosphorylation. According to the chemi-osmotic hypothesis (Mitchell, 1972) hydrolysis of ATP should generate a proton gradient sufficient to permit the accumulation of glutamate in the absence of respiration-driven proton expulsion. This

did not appear to occur and lends support to the arguments of Duff and Wheller (1981) that PEA-induced inhibition of glutamate uptake is not the result of protonophoric action.

In combination these agents demonstrated enhanced growth inhibitory activity for the combinations C, D and E and this was reflected in increased membrane permeability to hydrogen, potassium and phosphate ions and monolayer expansion. CC-induced membrane disruption resulting in pentose release appeared to be inhibited by the presence of PEA, suggesting that the synergy noted in these combinations did not result from an increase in gross disruptive effects. Uptake and retention of potassium and phosphate ions is known to occur by charge (Harold and Papineau, 1972) and proton (Jeacocke et al., 1972; Rosenberg et al., 1979) driven processes, respectively, and their efflux may reflect an inhibitory effect on these concentration processes as much as a change in membrane permeability.

The areas of metabolic activity most substantially inhibited by combinations of CC and PEA (especially combinations C and D) were respiration and glutamate active transport. Respiratory inhibition was maintained at the level generated by 0.475% v/v PEA alone while active transport

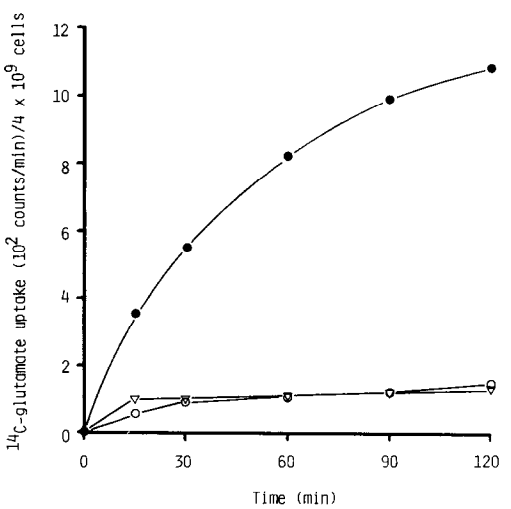

Fig. 4. Uptake of ^{14}C-labelled glutamate by *S. aureus*. ●, 5 mM glucose + [^{14}C]glutamate; ○, [^{14}C]glutamate alone; ▽, 5 mM glucose + 5 μg/ml Fentichlor + [^{14}C]glutamate.

of glutamate was totally inhibited. The reduction in uptake of even one amino acid is known to be sufficient to cause marked inhibition of growth in a nutritionally exacting organism such as *S. aureus* (Gale and Folkes, 1953; Hamilton, 1968). Some passive glutamate uptake still remained (Fig. 4) which was unaffected by Fentichlor, a known uncoupling agent (Bloomfield, 1974) and this may account for the residual growth observed with combinations C and D. Glucose transport by group translocation was unaffected by combinations of PEA and CC indicating a selective effect on transport mechanisms dependent upon effective coupling to a transmembrane pH gradient. No synergistic effect on ATP synthesis was demonstrated despite respiratory inhibition and proton conduction. This may be a consequence of successful substrate level phosphorylation and a greater resistance of the ATPase enzyme, as demonstrated by the isolated Mg^{2+}-ATPase, to the agents in combination rather than when used alone.

Only a few reports have appeared which attempt to explain the biochemical basis of synergy (Denyer et al., 1985) and of these the majority have invoked the principle of permeabilization where one compound decreases the cellular permeability barriers to the other (Hugbo, 1976; Quesnel et al., 1978; El-Nima, 1984). From the outset this mechanism was unlikely to account for the synergy observed between CC and PEA since they both act upon the cytoplasmic membrane. Instead our findings suggest a mechanism involving an inhibition of proton gradient-supported transport processes. Here, inhibition of respiration, possibly by disorganization of the structured respiratory chain, combined with a substantial proton-conducting effect leads to impaired production of a pH gradient and reduced coupling efficacy. Under these circumstances material accumulated and retained by pH-dependent processes would leak out and the cell would be unable to make good this loss. In addition, a direct action on the transport processes cannot be ruled out. It is likely that the basis of synergy in this combination lies with the ability to affect simultaneously several interrelated energy-creating and energy-dependent functions of the cell.

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