Inhibition of Bacterial Transport by Uncouplers of Oxidative Phosphorylation

EFFECTS OF PENTACHLOROPHENOL AND ANALOGUES IN *BACILLUS SUBTILIS*

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Analogues of the potent uncoupler of oxidative phosphorylation pentachlorophenol were tested as inhibitors of proline and glycine transport by *Bacillus subtilis*. These analogues included less highly substituted chlorophenols and pentachlorothiophenol. Like pentachlorophenol, they are non-competitive inhibitors of proline transport and uncompetitive inhibitors of glycine transport. However, the less highly substituted chlorophenols are weaker acids than pentachlorophenol and also weaker inhibitors. Analysis indicated that the anionic form of the uncouplers is the inhibiting species. Pentachlorothiophenol, a water-insoluble anion, is also a potent inhibitor. These results support previous studies that concluded that uncouplers of oxidative phosphorylation inhibit amino acid transport by binding at specific sites on proteins, the free energy of interaction stabilizing 'unproductive' conformations. Such specific interactions of uncoupler with protein are probably commonplace.

There is good reason to believe that uncouplers of oxidative phosphorylation can have a variety of effects on bacteria and mitochondria. Hopfer *et al.* (1968) found that the uncouplers 2,4-dinitrophenol and carbonyl cyanide trifluoromethoxyphenylhydrazine greatly increased conductance of protons across artificial phospholipid membranes, and Scholes & Mitchell (1970) found that carbonyl cyanide trifluoromethoxyphenylhydrazine likewise increased conductance of protons across the plasma membrane of *Micrococcus denitrificans*. Others, some of whose experiments are summarized by Brummett & Ordal (1977), have carried out experiments best interpreted by postulating that an uncoupler can interact at a specific site to promote its inhibiting effect. In one such series of experiments, Brummett & Ordal (1977) found that three chemically dissimilar uncouplers (pentachlorophenol, 3,3',4',5-tetrachlorosalicylanilide and carbonyl cyanide trifluoromethoxyphenylhydrazine) inhibited proline and glycine transport into *Bacillus subtilis* competitively in two instances, non-competitively in two instances and uncompetitively in two instances. These experiments were carried out in the presence of an exogenous energy source, so that, despite the presence of the uncoupler, the ATP concentrations were unaffected and thus, in all likelihood, the membranes were fully energized (see Ordal & Goldman, 1976). Thus these experiments were readily interpreted within the framework of

specific interaction of each uncoupler with a transport system as described in Brummett & Ordal (1977).

In the present paper we examine in detail the inhibition resulting from incubation of *B. subtilis* with pentachlorophenol and its analogues, including less highly substituted chlorophenols and pentachlorothiophenol. The object was to assess the effect of changing the pK_a , i.e. the propensity of the compound to be predominantly anionic or neutral under the conditions of the experiment. We find again that the motif of specific interaction of uncoupler with transport protein affords a straightforward interpretation of the data. In particular, we find that the anionic form of the uncoupler is the inhibiting species and give evidence that the sites of interaction are within the membrane.

Materials and Methods

Bacterial strain

B. subtilis strain OI 8 has been described previously (Ordal & Goldman, 1975).

Media

Minimal sorbitol medium (Ordal & Goldman, 1975) contained 50mM-potassium phosphate, pH 7, 1.2mM-MgCl₂, 1mM-(NH₄)₂SO₄, 0.14mM-CaCl₂, 10μM-MnCl₂, 20mM-sorbitol and 0.3mM required amino acids (isoleucine, valine and leucine in the

present studies). The resuspension medium for transport assays was the same except that sorbitol was 40 mM and amino acids were omitted. Tryptone broth contained 1% tryptone and 0.5% NaCl.

Chemicals

[^{14}C]Proline and [^{14}C]glycine were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Pentachlorophenol was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and pentachlorothiophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, 3,5-dichlorophenol and 3,4,5-trichlorophenol were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 2,4,6-Trichlorophenol was recrystallized and pentachlorothiophenol was washed with chloroform and purified by sublimation.

Growth of bacteria

The procedures of Brummett & Ordal (1977) were used. Spores were inoculated into tryptone broth and grown overnight at 37°C into stationary phase. These bacteria were diluted 1:50 into minimal sorbitol medium and grown to an A_{600} of 0.5 unit (about 1.4×10^8 bacteria/ml). They were washed three times with resuspension medium and resuspended at an A_{600} of 0.2 unit with bubbling at 30°C.

Assay

The procedures of Brummett & Ordal (1977) were used. Pentachlorophenol or one of its analogues (except for pentachlorothiophenol) was prepared freshly for experiment each day by making a solution in chloroform, diluting into resuspension medium, and then bubbling away the chloroform. Pentachlorothiophenol was prepared as described in the legend to Fig. 6.

Bacteria (fully energized since the medium contained sorbitol) were preincubated with inhibitor for at least 5 min and then radioactive amino acid was administered. At the appropriate time a 50 μl sample was withdrawn by using a Finnpiptette, diluted into 5 ml of prewarmed resuspension medium, and filtered (without washing) on Millipore filters. Filters were dried and counted for radioactivity in a scintillation spectrometer. 'Blanks' of bacteria treated with 2.5% (v/v) toluene for each different concentration of amino acid were subtracted from appropriate experimental values. Control experiments showed no accumulation of radioactivity by bacteria treated with toluene. Experiments were also carried out that showed that accumulation of radioactivity was linear throughout the course of the assays (2 min or less; see Figure legends). Finally, there was little metabolism of the transported proline under the conditions of the experiments (where bacteria lacked the amino acids required for growth) since after 45 min the majority

of soluble internal radioactivity was proline as analysed by chromatography of the cytoplasmic contents and was returned to the medium on addition of an excess of non-radioactive proline.

Partition-coefficient measurements

Stock solutions (10 mM) of chlorophenols in ethanol were diluted 400-fold into 4 ml of buffer (10 mM-sodium succinate for pH 6.0, 10 mM-potassium phosphate for pH 7.0, and 10 mM-potassium pyrophosphate for pH 8.5). The absorption spectra of the diluted solutions between 250 and 320 nm were scanned in a DW-2 spectrophotometer to find the absorbance maxima and record the values of absorbance (A_{before}) at that wavelength. The diluted solution (2 ml) was then added to 0.3–0.7 ml of spectrally pure carbon tetrachloride, vortex-mixed, centrifuged at about 3500 rev./min for 2 min, and the absorbance (A_{after}) was read at the same wavelength. The partition coefficient, α , was calculated as:

$$\alpha = \frac{A_{\text{after}}}{A_{\text{before}} - A_{\text{after}}} \times \frac{V_{\text{CCl}_4}}{V_{\text{H}_2\text{O}}}$$

where V_{CCl_4} was the volume of carbon tetrachloride added and $V_{\text{H}_2\text{O}}$, the volume of water, was 2 ml.

Results

Inhibition of proline transport by chlorophenols

Pentachlorophenol is a non-competitive inhibitor of proline transport (Ordal & Goldman, 1975). It seemed likely to us that less highly substituted chlorophenols would also be non-competitive inhibitors, and Fig. 1 confirms this expectation for 2,4-dichlorophenol.

To assess the effect of increasing the pK_a from the low value of 4.7 for pentachlorophenol itself to the high value of 8.2 for 2,4-dichlorophenol, various concentrations of each of several chlorophenols and the K_m concentration (2.3 μM) of proline were incubated with bacteria, and the effect on transport of proline was assessed. Fig. 2 shows Dixon plots for pentachlorophenol, 3,4,5-trichlorophenol, 2,4,6-trichlorophenol and 3,5-dichlorophenol. Values of K_i were obtained from Figs. 1 and 2 by using the equation for non-competitive inhibition:

$$\frac{v}{V_{\text{max.}}} = \frac{s}{(K_m + s) \left(1 + \frac{i}{K_i} \right)}$$

where s is concentration of amino acid and i is the concentration of inhibitor. In the Lineweaver-Burk plot of Fig. 1, the value of K_i was obtained by setting the ratio of ordinate intercepts equal to $1 + i/K_i$, and, knowing the value of i , solving for K_i . In the Dixon plots of Fig. 2, a pair of

Fig. 1. Lineweaver-Burk plot of inhibition of proline transport by 2,4-dichlorophenol

The procedure for inhibition of proline transport by pentachlorophenol is given in the Materials and Methods section. Briefly, bacteria were preincubated with uncoupler for at least 5 min and at zero time radioactive proline was administered. After 40 s 50 μ l of sample was diluted into 5 ml of prewarmed medium and filtered on a Millipore filter. Filters were

values of i and v were read from the graph, s was set equal to K_m and the V_{max} from the graph was doubled to give the true V_{max} of proline transport (velocity being half-maximal when s is the K_m concentration), and the above equation was solved for K_i . These values are recorded in Table 1.

To obtain the pK_a values for each chlorophenol, the value for pentachlorophenol was determined spectrophotometrically by using change in $A_{252.5}$ in increasingly alkaline buffer compared with an isosbestic point at 237 nm. The value for phenol itself was obtained from Hodgman *et al.* (1961). The values for the other chlorophenols were obtained from the Hammett equation (March, 1968; Table 1). It is apparent that the stronger acids (lower pK_a values) are more potent inhibitors than the weaker

dried and counted for radioactivity. A parallel set of samples, for each different concentration of proline used, was treated with toluene and these 'blank' values were subtracted from the values for experimental samples to give values for amount of proline transported. Symbols: ○, + 1 mM-2,4-dichlorophenol; ●, no addition.

Fig. 2. Dixon plots of inhibition of proline transport by chlorophenols

See the legend to Fig. 1 for the method. The concentration of proline was 2.3 μ M. See the text for calculation of K_i . (a) 3,5-Dichlorophenol; (b) 2,4,6-trichlorophenol; (c) pentachlorophenol; (d) 3,4,5-trichlorophenol.

acids (higher pK_a values), implying that the anion is the inhibitory species.

Table 1. Values of pK_a and of pK_i for chlorophenols and pentachlorothiophenol as inhibitors of proline and glycine transport at pH7

Inhibitor	pK_a	pK_i (proline)§	pK_i (glycine)
Pentachlorophenol	4.7*	5.64§	6.16††
3,4,5-Trichlorophenol	6.2†	5.51§	5.94‡‡
3,5-Dichlorophenol	7.1†	4.41§	4.52§§
2,4,6-Trichlorophenol	7.5†	3.28§	3.74
2,4-Dichlorophenol	8.2†	3.49	3.40¶¶
Pentachlorothiophenol	4.3‡	4.74¶	5.54¶¶*
		4.80**	5.67***††

* Obtained by spectrophotometric titration as described in the text.

† Calculated from the Hammett equation $pK_o - pK = \rho \sum \sigma$ (see March, 1968). pK_o refers to the pK of phenol itself, which is 9.9. pK refers to the pK of the chloro-substituted phenol. ρ was calculated from the difference in pK values of phenol and pentachlorophenol, assuming $\sum \sigma$ equals 1.38, and was 3.77. $\sum \sigma$ refers to the summed contributions of substitutions in the *para* (0.24), *meta* (0.37) and *ortho* (0.20) positions.

‡ Obtained from Wilson *et al.* (1971).

§ From Fig. 2.

|| From Fig. 1.

¶ From Fig. 7.

** From Fig. 6.

†† 2 μ M used.

‡‡ 3 μ M used.

§§ 40 μ M used.

||| 0.5 mM used.

¶¶ 0.8 mM used.

*** From Fig. 9.

††† From Fig. 8. To calculate K_i , the line through the data points obtained in the presence of inhibitor was made parallel to the line drawn through the data points obtained without inhibitor, and the ratio of intercepts was used.

Inhibition of glycine transport by chlorophenols

Pentachlorophenol is an uncompetitive inhibitor of glycine transport (Brummett & Ordal, 1977), and Fig. 3 shows that 2,4-dichlorophenol is also.

Experiments similar to those of the preceding section were carried out for glycine transport, except that single concentrations of uncouplers were chosen, rather than a series as in the Dixon plots shown above (Table 2). Again, the anion was found to be the potent inhibitor.

Partition of chlorophenols between water and membrane

As described in the Discussion section, the locus of inhibition of amino acid transport lies within the

Fig. 3. Lineweaver-Burk plot of inhibition of glycine transport by 2,4-dichlorophenol

The method indicated in Fig. 1 was used. Symbols: O, + 1.5 mM 2,4-dichlorophenol; ●, no addition.

Table 2. Values of pK_i for chlorophenols as inhibitors of proline and glycine transport at pH6 and pH8.5

For proline transport, the buffer at pH6 was 0.05M-sodium succinate and the assay was run for 1 min, and the buffer at pH8.5 was 0.05M-potassium pyrophosphate and the assay was run for 2 min. The same was true for glycine transport, except that the buffer at pH8.5 was 0.05M-Tris/HCl, and the assay was run for 1 min.

Inhibitor	Proline transport		Glycine transport	
	pH6	pH8.5	pH6	pH8.5
Pentachlorophenol	5.52*	6.22*	6.22	6.12
3,4,5-Trichlorophenol	5.38*	6.08*	5.92*	5.76*
3,5-Dichlorophenol		4.60§	4.72¶	4.56¶
2,4,6-Trichlorophenol	3.25†	3.73†	4.22**	3.96**
2,4-Dichlorophenol	3.32‡	3.44‡	3.84‡	3.55‡

* 3 μ M used.

† 0.52 mM used.

‡ 0.8 mM used.

§ 39 μ M used.

|| 2 μ M used.

¶ 40 μ M used.

** 0.5 mM used.

Table 3. Partition of chlorophenols between water (buffer) and carbon tetrachloride

Chlorophenol	pH6		pH7		pH8.5	
	α	$\lambda_{\text{max.}}$ (nm)	α	$\lambda_{\text{max.}}$ (nm)	α	$\lambda_{\text{max.}}$ (nm)
Pentachlorophenol	0.068	317.8	0.19	317.8	1.12	317.8
3,4,5-Trichlorophenol	0.26	290.9	0.20	290.6	0.39	305.2
3,5-Dichlorophenol	0.11	282.3	0.17	282.2	0.39	294.9
2,4,6-Trichlorophenol	0.033	293.6	0.094	309.9	0.42	309.9
2,4-Dichlorophenol	0.23	282.5	0.26	283.0	0.24	303.2

Fig. 4. Plots of pK_i (corrected for membrane phase) as a function of pK_a of chlorophenols as inhibitors of amino acid transport. Values of pK_a were taken from Table 1. Values of pK_i were taken from Tables 1 and 2 to which the logarithm of the respective values of α (the partition coefficient) from Table 3 was added. Straight lines were drawn assuming: $\frac{dpK_i}{dpK_a} = -1$ [expressing the assumption that a 10-fold increase in energy of ionization causes a 10-fold decrease in concentration of inhibitory species (anion)]. (a) pH 6.0; (b) pH 7.0; (c) pH 8.5. Symbols: $\circ$, proline transport; $\bullet$, glycine transport.

Fig. 5. Lineweaver-Burk plot of proline transport at pH 6.0 and 8.5. Experiments carried out by the procedure of Fig. 1 except that 0.05M-sodium succinate, pH 6.0, or 0.05M-potassium pyrophosphate, pH 8.5, was used instead of potassium phosphate and incubation was carried out for 60 or 120s respectively, rather than 40s. Symbols: $\circ$, pH 6.0; $\bullet$, pH 8.5.

membrane. We are interested in determining the relative K_i values of the chlorophenols as inhibitors of transport, and, accordingly, we have to correct for the fact that at each pH value each chlorophenol partitions into the membrane phase to a different degree. Therefore we have measured partition of each chlorophenol between buffer and carbon tetrachloride, whose dielectric constant of 2.238 (Hodgman *et al.*, 1961) makes it a good substitute for the low-dielectric hydrocarbon phase of membrane (Table 3). Correction of the data of Tables 1 and 2 to convert aqueous concentrations into membrane-phase concentrations confirms the greater potency of the anionic form of chlorophenols (Fig. 4b).

Variation of pH for inhibition by chlorophenols

To achieve further insight, the experiment was repeated at pH 6 and 8.5 (Fig. 4). Control experiments showed that change of pH did not affect K_m for proline transport and only affected $V_{\text{max.}}$ at pH 8.5 (decrease to 25% of pH 7 value) (Fig. 5). When corrected for concentration of chlorophenols

Fig. 6. Lineweaver-Burk plot of inhibition of proline transport by pentachlorothiophenol

The procedure indicated in Fig. 1 was followed, except that pentachlorothiophenol was dissolved in dimethylformamide and $1\mu\text{l}$ was added to $99\mu\text{l}$ of cell suspension. Briefly, bacteria were preincubated with uncoupler for 5 min and at zero time proline was administered. At 40s $50\mu\text{l}$ of sample was diluted into prewarmed medium and filtered on a Millipore filter. Filters were dried and counted for radioactivity. 'Blank' values with toluene were subtracted from the experimental values. Control experiments showed that amino acid transport was not affected by dimethylformamide at the concentrations used in these experiments. Symbols: ○, $+10\mu\text{M}$ -pentachlorothiophenol; ●, no addition.

Fig. 7. Dixon plot of inhibition of proline transport by pentachlorothiophenol

The procedure indicated in Fig. 6 was followed. Proline concentration was $2.3\mu\text{M}$.

Fig. 8. Lineweaver-Burk plot of inhibition of glycine transport by pentachlorothiophenol

The procedure indicated in Fig. 6 was followed. Symbols: ○, $+6\mu\text{M}$ -pentachlorothiophenol; ●, no addition.

Fig. 9. Dixon plot of inhibition of glycine transport by pentachlorothiophenol

The procedure indicated in Fig. 6 was followed. Glycine concentration was $10\mu\text{M}$.

in the membrane phase, the curves for proline and glycine transport at pH6 were similar to those at pH7, except that 3,4,5-trichlorophenol was relatively more potent than pentachlorophenol at pH6 than at pH7, and at pH7 than at pH8.5. At pH8.5, all points lay fairly close to the same straight line, whereas at pH6 and pH7, the fit was not as good.

Considering the vertical displacement of the straight-line portions of curves at pH6 and at pH7, the chlorophenols are more potent at the higher pH, 2.68 times for glycine and 6.45 times for proline.

Fig. 10. Dixon plot of inhibition of proline transport by *m*-chlorophenol

Proline concentration was $2.3 \mu\text{M}$. The procedure indicated in Fig. 1 was followed.

Fig. 11. Dixon plot of inhibition of proline transport by *p*-chlorophenol

Proline concentration was $2.3 \mu\text{M}$. The procedure indicated in Fig. 1 was followed.

This implies that there exists a functional group that, when protonated, renders the transport resistant to inhibition. The pK values of such groups would then be 6.6 (glycine transport) and 5.8 (proline transport). Raising the pH to 8.5 does not correspondingly increase sensitivity of transport to chlorophenols (i.e. there is not much vertical displacement of curves from pH 7 positions), so that it is apparent that other changes occur as a result of deprotonation of other functional groups.

Inhibition of proline and glycine transport by pentachlorothiophenol

To obtain further evidence in support of the view that the anionic forms of these uncouplers are the

inhibitors, the ability of pentachlorothiophenol to inhibit amino acid transport was measured. Pentachlorothiophenol is virtually insoluble in water, despite the fact that, having a pK of 4.3, it is almost completely ionized at neutral pH (Wilson *et al.*, 1971). It partitions exclusively into membrane as the anion under the conditions of these experiments (Wilson *et al.*, 1971). Like the chlorophenols, it is a non-competitive inhibitor of proline transport (Figs. 6 and 7) and an uncompetitive inhibitor of glycine transport (Figs. 8 and 9). The values of K_i are recorded in Table 1. They indicate that for proline, and especially for glycine, pentachlorothiophenol is a very potent inhibitor of transport.

Inhibition of proline and glycine transport by m-chlorophenol and p-chlorophenol

m-Chlorophenol, whose pK is 8.7, and *p*-chlorophenol, whose pK is 9.1, were assayed for ability to inhibit proline transport. They also inhibit, but by a different mechanism from the more substituted chlorophenols, since the Dixon plots are not linear but higher-order (Figs. 10 and 11).

Discussion

In Brummett & Ordal (1977), we presented evidence that three chemically dissimilar uncouplers interacted at specific sites with the proteins involved in transport of proline and of glycine. We believe that the free energy of this interaction stabilizes particular conformations and hence prevents appropriate conformational changes for transport of glycine or proline to occur. In the present paper, we have explored the nature of this interaction, chiefly by determining whether the uncoupler interacts as anion, protonated neutral species, as either, or as both.

The strategy for this determination was based on testing analogues of one potent uncoupler, pentachlorophenol. One set of analogues, the less highly substituted chlorophenols, are all weaker acids than pentachlorophenol. If the protonated form were the inhibitor, then the weaker acids should have been the stronger inhibitors. If they were equally potent, then all should have been about equally strong. If the anion were the inhibiting species, then the stronger acids should have been more potent inhibitors than the weaker ones. We found that the stronger acids were much more potent inhibitors than the weaker acids (Tables 1 and 2). Pentachlorothiophenol, which is almost completely ionized, was also a potent inhibitor. Yet all are presumed to inhibit by the same mechanism, since both the strongest acid, pentachlorophenol, and the weakest acid, 2,4-dichlorophenol, as well as pentachlorothiophenol, inhibited proline transport non-competitively and glycine transport uncompetitively. It is unlikely that both the

neutral and anionic species as a pair interact, since all the Dixon plots were linear, not higher-order.

There is good reason to believe that the loci of inhibition are within the membrane. Were the sites of interaction at the cell exterior, then pentachlorophenol should have been a much better inhibitor than 2,4-dichlorophenol at pH 6, where the former is an anion and the latter is protonated, but about the same at pH 8.5, where both are mainly anions. However, for both glycine and proline transport, pentachlorophenol was much better at both pH values. Were the sites of inhibition in the cell interior, where the pH is 7.55 (Navon *et al.*, 1977), then pentachlorophenol would have been about twice as good an inhibitor as 2,4,6-trichlorophenol, which is about half-ionized at that pH. However, for the experiment carried out at pH 7, pentachlorophenol was 230 (proline transport) to 260 (glycine transport) times as potent an inhibitor as 2,4,6-trichlorophenol. The wide difference in potency of the strong acids compared with the weaker acids at all pH values measured was very probably a consequence of the destabilization of charged (anionic) species in membrane phase, thus increasing the energy of ionization of each chlorophenol (raising the pK_a of each one).

The deduction that the loci of inhibition lie within the membrane is further supported by the fact that pentachlorothiophenol, which exclusively partitions into membrane (Wilson *et al.*, 1971), was a potent inhibitor. Since, like the chlorophenols, it non-competitively inhibited proline transport and uncompetitively inhibited glycine transport, it probably inhibited by the same mechanism.

Finally, what is the significance of the upward curvature in the Dixon plots of inhibition by *m*-chlorophenol and *p*-chlorophenol, in comparison with the straight-line plots for other inhibitors in the present and previous work (Brummett & Ordal, 1977)? Straight lines indicate that interaction of a monomer with a transport system causes inhibition. Upward curvature indicates that at higher concentrations inhibition is more potent than expected. In experiments on inhibition of proline transport by carbonyl cyanide trifluoromethoxyphenylhydrazone in the absence of an exogenous energy source we observed similar upward curvature, beginning at 80 nM-carbonyl cyanide trifluoromethoxyphenylhydrazone (results not shown). In the presence of an exogenous energy source we observed no such upward curvature even at 1 μ M (Brummett & Ordal, 1977). In the absence of an energy source at 0.3 μ M, ATP concentrations are virtually zero (results not shown); in the presence of an energy source, even at 1 μ M-carbonyl cyanide trifluoromethoxyphenylhydrazone, ATP concentrations are near-normal, and are still 30% at 3 μ M-carbonyl cyanide trifluoromethoxyphenylhydrazone (Ordal & Goldman, 1976). Thus

we believe that upward curvature in Dixon plots of this kind reflect impairment of cellular energy reserves at the high concentrations of inhibitor. Conversely, straight lines in Dixon plots indicate specific inhibition, without impairment of energy reserves. It is apparent that glycolytic degradation of the sorbitol furnishes adequate ATP to fully energize the membrane, despite any dissipation by uncouplers, so that inhibition of transport by uncouplers is due to direct interaction, rather than indirectly from de-energization of the membrane. The deduction that the bacteria were fully energized was further supported by the fact that in the presence of sorbitol and 3.5 μ M-pentachlorophenol or 0.35 μ M-carbonyl cyanide trifluoromethoxyphenylhydrazone transport was slower, but the same final uptake was achieved as in absence of these uncouplers.

In conclusion, these studies support our previous hypothesis that uncouplers may inhibit metabolic processes through specific protein-uncoupler interactions, the free energy of which stabilizes proteins in 'unproductive' conformations. In particular, we have given evidence that it is mainly the anionic form of the uncoupler that binds, at least for pentachlorophenol and its analogues. It is possible that these studies have general significance in that in many instances uncouplers may interact with a protein to stabilize a certain conformation and thus tend to prevent conformational changes needed to carry out its function. Such an interaction can be detected by analysing for competitive, non-competitive and uncompetitive inhibition. That such interactions are commonplace is suggested by the previous study in which three chemically dissimilar uncouplers were found to interact with two transport systems: of six instances tested, all six showed specific interaction. If uncouplers are used to decrease energy reserves of bacterial cells, as they often are, then in instances of specific interaction with the proteins under study the uncoupler should be removed before an experiment on energy-starved cells is begun.

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