

# Effects of Substituted 2-Phenylamino-1,4,5,6-tetrahydropyrimidines on ATP Formation in Isolated Spinach Chloroplasts

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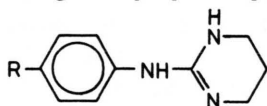
Arylaminoypyrimidines, Photophosphorylation, Electron Transport, Chloroplasts, uncouplers.

The effects of a series of 2-arylamino-1,4,5,6-tetrahydropyrimidines on photosynthesis in spinach chloroplast were studied. The compounds proved to be uncouplers of photophosphorylation, while inhibition of electron transport occurred at higher concentrations. These effects were rather irreversible and the activity increased with lipophilicity. Inhibition of electron transport could not be reversed by trypsin treatment.

Loss of membrane integrity might be the underlying mechanism of these effects.

## Introduction

2-Arylamino-1,4,5,6-tetrahydropyrimidines are chemicals with several biological properties. Some derivatives are plant growth regulators, while others possess fungicidal properties [1, 2].



An interesting growth regulator in this series is 2-(4-*n*-hexylphenylamino)-1,4,5,6-tetrahydropyrimidine (PH 30-17) which can be used to control sucker growth in tobacco plants.

In order to investigate the mode of action of these compounds, we studied the effects of a number of derivatives on photosynthetic reactions in spinach chloroplasts. Inhibition of ATP formation proved the most important effect and the relation between chemical structure and this inhibition was analysed by multiple regression analysis.

Some preliminary results have previously been published [3].

## Methods

### Chemicals

All inhibitors used were synthesized in the Synthesis Department of the Duphar Crop Protection Division according to known methods.

**Abbreviations:** DAD, diaminodurene; DCIP, dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCy, ferricyanide; MV, methylviologen; PD, *p*-phenylenediamine; PMS, phenazine methosulfate; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine.

### Photosynthetic measurements

Broken chloroplasts were isolated as described previously [4]. Unless stated otherwise, photosynthetic processes were measured in a medium of 4 ml containing in  $\mu\text{mol}$ : Tricine/NaOH (pH 8.0) 200,  $\text{MgCl}_2$  4, inorganic phosphate 4, chloroplasts equivalent to 50  $\mu\text{g}$  chlorophyll and appropriate electron donors or acceptors such as FeCy 3, MV 0.04 plus  $\text{NaN}_3$  0.8, or DCIP 0.4. Photosystem I activity was measured in the presence of 40 nmol DCMU and TMPD or DAD plus ascorbate (0.8 and 30  $\mu\text{mol}$ , respectively) were used as donor systems.

For the measurement of photophosphorylation 0.8  $\mu\text{mol}$  ADP were added and the disappearance of inorganic phosphate was determined colorimetrically [5]. Cyclic phosphorylation was studied by using PMS (0.08  $\mu\text{mol}$ ) under nitrogen.

Chloroplasts were treated with trypsin as described by Böger and Kunert [6]: 0.5 mg chlorophyll and 50  $\mu\text{g}$  trypsin (Bovine pancreas, Koch-Light Labs) were incubated in 1 ml grinding medium at 25 °C in the dark for indicated times. No trypsin inhibitor was used but the mixture was added directly to the photosynthetic incubation medium.

To inactivate the water-splitting enzyme chloroplasts were isolated in Tris buffer pH 8.0 and incubated at 40 °C for 4 minutes as described by Böhme and Trebst [7]. Several types of electron donors were used ( $\mu\text{mol}$ ): ascorbate 40,  $\text{NH}_2\text{OH}$  100 or aminophenol 2.0 plus ascorbate 2.0 as recommended by several authors [8, 9].

Electron transport was measured spectrophotometrically or by using a Clark electrode (Gilson Med. Electr.). Inhibitors were added as metha-

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nolic solutions, the final methanol concentration always being 2%.

Although 2-arylamino-1,4,5,6-tetrahydropyrimidines are strong bases, the addition of these chemicals to the incubation medium to a concentration of 1 mM produced a pH shift of only 0.04 unit and in most cases the concentrations used are much less.

Incubation mixtures were illuminated by a projector lamp producing a light intensity of 200 W/m<sup>2</sup> and incubations were carried out at 25 °C.

#### Structure-activity relationships

Biological activities were correlated with physico-chemical parameters according to Hansch and Leo [10] and  $\pi$  values were taken from their tables.

### Results

#### Effects on electron transport and photophosphorylation

When 2-arylamino-1,4,5,6-tetrahydropyrimidines were added to illuminated chloroplasts a strong inhibition of ATP-formation was observed. In some cases such inhibition was accompanied by a stimu-

lation of the electron transport. Fig. 1 shows the dose/response curves of two representatives of these chemicals. The methyl-substituted compound shows a typical uncoupler picture: inhibition of the ATP formation and simultaneous stimulation of the electron transport at a concentration of 0.3 mM. The hexyl-substituted compound (PH 30-17) shows a mixed-type inhibition: at low concentrations inhibition of ATP formation occurs but due to inhibition of the electron transport only a little stimulation of the latter can be observed. At higher concentrations (0.3 mM) 100% inhibition of the electron transport took place.  $pI_{50}$  Values (negative logarithm of the concentration producing 50% inhibition) can be determined from such dose/response curves. Table I shows these  $pI_{50}$  values of PH 30-17 for different electron transport pathways. Photophosphorylation is inhibited by 50% at about 5  $\mu$ M.

The effects of 0.3 mM of PH 30-17 on different types of photoreductions were studied to establish the inhibition site more precisely; see Table II. The inhibition of FeCy reduction cannot be reversed by PD and there is no effect on photosystem I alone (measured as TMPD/asc  $\rightarrow$  MV). Besides normal chloroplasts, trypsin-treated chloroplasts and heated

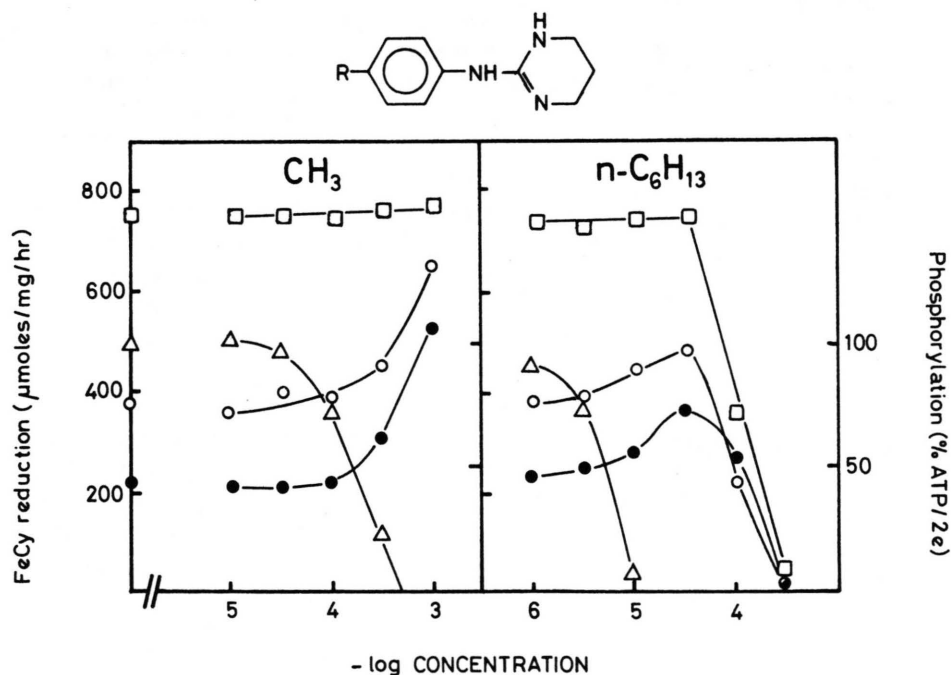


Fig. 1. Hill reaction and photophosphorylation as a function of uncoupler concentration. ● Basal electron transport, ○ phosphorylating electron transport, Δ corresponding phosphorylation, □ uncoupled electron transport (2 mM NH<sub>4</sub>Cl).

Table I.  $pI_{50}$  Values of PH 30-17.

| Electron transport pathway<br>(ADP and $P_i$ present) | $pI_{50}$<br>photophos-<br>phorylation | $pI_{50}$<br>electron<br>transport |
|---|--|------------------------------------|
| $H_2O \rightarrow FeCy$                               | 5.26                                   | 3.96                               |
| $H_2O \rightarrow MV$                                 | 5.45                                   | 3.87                               |
| DAD/asc $\rightarrow$ MV (10 $\mu M$ DCMU)            | 5.45                                   | —                                  |
| PMS ( $N_2$ )   | 5.23                                   | —                                  |

chloroplasts lacking the water-splitting enzyme were used. In heat-treated chloroplasts PH 30-17 has no inhibitory activity if MV is used as acceptor.

Other experiments showed that this chemical was not active as an electron donor for photosystem I and the inhibition of the electron transport could not be reversed by catalytic amounts of TMPD alone.

The time course of trypsin treatment and the dose/response curves with trypsin-treated and control chloroplasts are presented in Fig. 2. Whereas the inhibition of electron transport by DCMU is reversed by trypsin treatment, the inhibition by PH 30-17 is not. Moreover, 0.1 mM of PH 30-17 inhibited the electron transport in trypsin-treated chloroplasts considerably, while the same concentration produced a small stimulation of the electron transport in untreated chloroplasts.

### Reversibility of the inhibition

To investigate whether or not the effects of PH 30-17 were reversible, chloroplasts were pre-incubated in a buffer (50 mM Tricine pH 8.0, 1 mM  $MgCl_2$ ) containing different amounts of this compound. After 10 min the inhibitor was removed by washing and centrifugation at  $30\,000 \times g$  and photosynthetic activities of the chloroplasts were measured in the standard incubation medium. The same experiment was conducted with the uncoupler  $NH_4Cl$ . Table III shows the results.

### Structure-activity relationships

By changing the molecular structure of inhibitors, one can learn which part of the molecule is essential to the inhibition and what type of interactions play a part. We therefore studied the effects of 11 different arylaminotetrahydropyrimidines and two related compounds on cyclic and noncyclic phosphorylation and the reduction of FeCy. The  $pI_{50}$  values of these chemicals are listed in Table IV. The effects on cyclic and non-cyclic phosphorylation are almost identical. The  $pI_{50}$  values increase with increasing length of the hydrocarbon chain indicating the possible role of hydrophobic binding.

Table II. Effects of PH 30-17 on different photoreductions in isolated spinach chloroplasts.

| Pathway   | Basal electron transport, pH 8.0           |                          |                 |
|---|--|--------------------------|-----------------|
|   | control rate<br>( $\mu\text{mol/mg/h}^a$ ) | activity in % of control |                 |
|   |  | 1 $\mu\text{M}$ DCMU     | 0.3 mM PH 30-17 |
| <i>control chloroplasts</i>                     |  |                          |                 |
| H <sub>2</sub> O → FeCy                         | 210  | 6                        | 3               |
| H <sub>2</sub> O → FeCy (0.1 mM PD)             | 363  | 0                        | 0               |
| H <sub>2</sub> O → MV                           | 59   | 0                        | 27              |
| TMPD/asc → MV (10 $\mu\text{M}$ DCMU)           | 180  | —                        | 218             |
| H <sub>2</sub> O → DCIP                         | 42   | 0                        | 17              |
| <i>trypsin-treated chloroplasts<sup>b</sup></i> |  |                          |                 |
| H <sub>2</sub> O → FeCy                         | 220  | 44                       | 0               |
| <i>heated chloroplasts</i>                      |  |                          |                 |
| NH <sub>2</sub> OH → DCIP                       | 12   | 0                        | 14              |
| asc. → MV                                       | 60   | 0                        | 106             |
| aminophenol/asc → MV                            | 97   | 0                        | 95              |

<sup>a</sup>  $\mu mol$  FeCy or DCIP reduced or  $\mu mol$   $O_2$  consumed.

<sup>b</sup> pretreated with trypsin for 2 minutes.

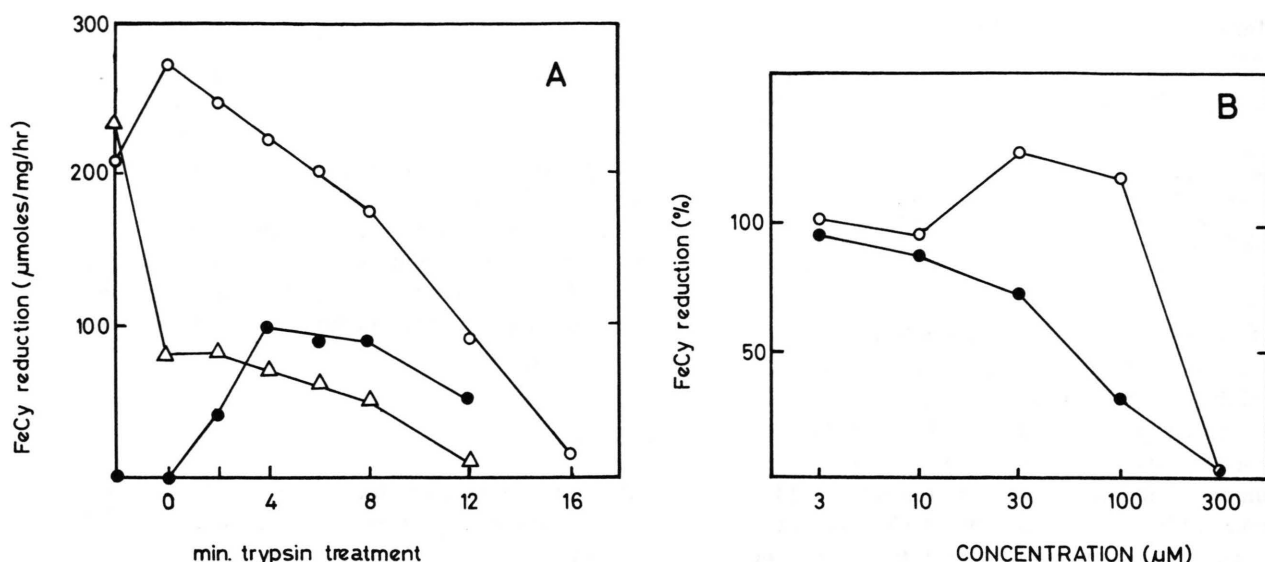


Fig. 2. Effect of trypsin treatment on the inhibition of electron transport by PH 30-17. A. Time-course experiment, ○ control, ● 1 μM DCMU, △ 0.1 mM PH 30-17. B. Dose/response curve, ○ control chloroplasts, ● chloroplasts treated with trypsin for 2 minutes.

The relationship between the lipophilicity parameter  $\pi$  and the uncoupling activity of the first 11 compounds of Table IV was analysed by multiple regression and proved to be quadratic. For the cyclic and noncyclic phosphorylation equations (1) and (2) were found, respectively.

$$pI_{50} = 3.310 + 0.966 \pi - 0.130 \pi^2 \quad (1)$$

(10.075) (− 8.104)

$$n = 11 \quad r = 0.974 \quad s = 0.112 \quad F = 73.75$$

$$pI_{50} = 3.313 + 0.924 \pi - 0.113 \pi^2 \quad (2)$$

(7.833) (− 5.687)

$$n = 11 \quad r = 0.969 \quad s = 0.138 \quad F = 60.89.$$

Table III. Reversibility of the effects of PH 30-17 and  $NH_4Cl$ .

| Inhibitor | Concentration (mM) | FeCy reduction (μmol/mg/h) | ATP/2e ratio |
|-----------|--------------------|----------------------------|--------------|
| Control   |                    | 308 (306)                  | 1.02 (0.65)  |
| $NH_4Cl$  | 1                  | 557 (343)                  | 0.31 (0.67)  |
|           | 10                 | 397 (315)                  | 0.09 (0.65)  |
|           | 100                | 101 (288)                  | 0.00 (0.60)  |
| PH 30-17  | 0.01               | 299 (340)                  | 0.04 (0.54)  |
|           | 0.1                | 185 (353)                  | 0.00 (0.00)  |
|           | 1                  | 24 (3)                     | 0.00 (0.00)  |

Activities after removal of the uncoupler by washing in brackets. Means of two experiments.

In the above presentation  $n$  is the number of compounds,  $r$  is the correlation coefficient,  $s$  is the standard error of the estimate and  $F$  represents the overall statistical significance of the equation. The Student's  $t$ -test values are placed in brackets.

Fig. 3 shows the relationship between  $\pi$  and  $pI_{50}$  values whereas Eqns. (1) and (2) are presented as dotted and drawn lines, respectively.

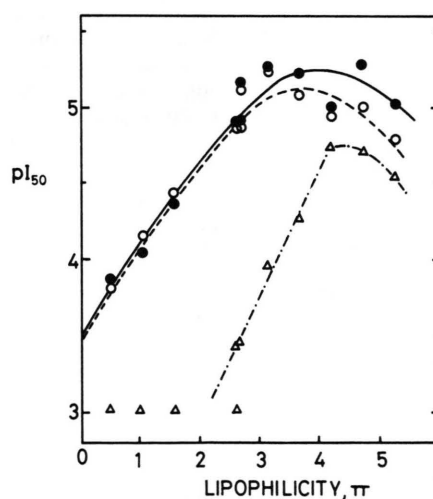
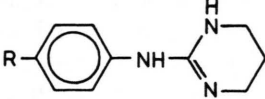


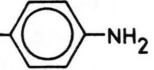
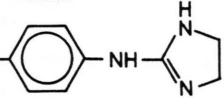
Fig. 3. Relationship between lipophilicity and inhibition of ATP-formation and electron transport. ○---○, Cyclic phosphorylation (Eqn. (1)); ●—●, noncyclic phosphorylation (Eqn. (2)); △, FeCy reduction.

Table IV. Effects of 2-arylmino-1,4,5,6-tetrahydropyrimidines on photophosphorylation and electron transport in spinach chloroplasts.

| Nr. | Structure | pI <sub>50</sub> (M <sup>-1</sup> ) |                           |                    |                        |                    |
|-----|-----------|-------------------------------------|---------------------------|--------------------|------------------------|--------------------|
|     |           | Electron transport                  | Noncyclic phosphorylation |                    | Cyclic phosphorylation |                    |
|     |           |                                     | obsvd                     | calcd <sup>a</sup> | obsvd                  | calcd <sup>b</sup> |



|    |  |      |      |      |      |      |
|----|--|------|------|------|------|------|
| 1  | R = CH <sub>3</sub>  | < 3  | 3.87 | 3.81 | 3.81 | 3.82 |
| 2  | C <sub>2</sub> H <sub>5</sub>  | < 3  | 4.05 | 4.13 | 4.16 | 4.15 |
| 3  | n-C <sub>3</sub> H <sub>7</sub>  | < 3  | 4.37 | 4.43 | 4.43 | 4.45 |
| 4  | n-C <sub>5</sub> H <sub>11</sub>   | < 3  | 4.92 | 4.95 | 4.85 | 4.94 |
| 5  | (PH 30-17) n-C <sub>6</sub> H <sub>13</sub>  | 3.96 | 5.26 | 5.13 | 5.23 | 5.08 |
| 6  | n-C <sub>7</sub> H <sub>15</sub>   | 4.26 | 5.22 | 5.23 | 5.09 | 5.13 |
| 7  | n-C <sub>8</sub> H <sub>17</sub>   | 4.73 | 5.00 | 5.25 | 4.94 | 5.09 |
| 8  | n-C <sub>9</sub> H <sub>19</sub>   | 4.71 | 5.30 | 5.17 | 5.00 | 4.96 |
| 9  | n-C <sub>10</sub> H <sub>21</sub>  | 4.57 | 5.02 | 5.01 | 4.79 | 4.75 |
| 10 | (PH 50-98) c-C <sub>6</sub> H <sub>11</sub>  | 3.45 | 5.14 | 4.97 | 5.13 | 4.96 |
| 11 | C <sub>2</sub> H <sub>4</sub> -C <sub>6</sub> H <sub>5</sub>   | 3.43 | 4.92 | 4.96 | 4.86 | 4.95 |
| 12 | n-C <sub>6</sub> H <sub>13</sub> -  | 3.85 | 4.27 | —    | 4.03 | —    |
| 13 | n-C <sub>6</sub> H <sub>13</sub> -  | 3.98 | 5.16 | —    | 5.11 | —    |

<sup>a</sup> Calculated using Eqn. (2).<sup>b</sup> Calculated using Eqn. (1).

## Discussion

From Fig. 1 and Table I it can be concluded that PH 30-17 produces 50% inhibition of cyclic, non-cyclic as well as pseudo-cyclic phosphorylation at about 5  $\mu$ M, while the methyl-substituted derivative is much less active. Theoretically, inhibition of ATP-formation can be achieved by several mechanisms of which inhibition of energy transfer and uncoupling are the most important. A third mechanism can be the donation of electrons to photosystem I bypassing the energy conservation sites. However, we showed that PH 30-17 does not have electron donating properties. Although the stimulation of the electron transport by PH 30-17 at uncoupling concentrations (10  $\mu$ M) is only limited (see Fig. 1) the inhibition of ATP-formation is probably caused by uncoupling only.

Because PH 30-17 is an inhibitor of electron transport at somewhat higher concentrations, the stimulation of electron which is such a typical feature of

uncouplers, is reduced. In contrast to PH 30-17 the derivatives having short alkyl chains are pure uncouplers, as is illustrated by the methyl-substituted compound in Fig. 1. This compound does not inhibit the electron transport at the concentrations used and a clear stimulation due to uncoupling can be observed. In Table IV the pI<sub>50</sub> values for inhibition of electron transport and uncoupling can easily be compared. Derivatives having a short alkyl chain show a I<sub>50</sub> (electron transport)/I<sub>50</sub> (phosphorylation) ratio greater than 10, while the long chain derivatives have ratios as low as 2 (octyl-derivative).

Inhibition of electron transport by high concentrations of uncouplers has been reported for many types of uncouplers including NH<sub>4</sub>Cl and alkylamines [11–13]. This inhibition probably occurs at the water-splitting site [8], and the results presented in Table II indicate that PH 30-17 behaves similarly. Inhibition of the electron transport by PH 30-17 can be reversed by the addition of the TMPD/ascorbic acid couple (in this case, stimulation of more than 200%,

due to uncoupling, was even observed), but not by adding PD or TMPD alone or by trypsin treatment (Fig. 2A). This suggests an inhibition site before photosystem I but different from the DCMU or DBMIB sites. After trypsin treatment the dose/response curve for PH 30-17 shows only an inhibition of electron transport (Fig. 2B). The curve has a shape comparable to the curve for uncoupled electron transport in Fig. 1. Stimulation of electron transport due to uncoupling by PH 30-17 disappeared totally upon trypsin treatment. Finally, in heated chloroplasts in which the water-splitting enzyme was inactivated, the electron transport could be started by electron donors such as  $\text{NH}_2\text{OH}$ , ascorbic acid and aminophenol plus ascorbic acid. Electron transport supported by the first donor is sensitive to both DCMU and PH 30-17 but electron transport catalysed by the other two donors is sensitive only to DCMU. This indicates an inhibition site for PH 30-17 between the donation sites of  $\text{NH}_2\text{OH}$  and ascorbic acid/aminophenol.

Further experiments concerning the reversibility of the uncoupling and inhibition of electron transport by PH 30-17 showed that these effects are somewhat irreversible. In contrast,  $\text{NH}_4\text{Cl}$  and methylamine were reported to produce fully reversible uncoupling effects [12, 13] which is in agreement with our results in Table III. Partial inhibition or uncoupling by PH 30-17 can be reversed by washing but when a 100% effect is reached the inhibition has an irreversible character. This can be caused by a strong binding of the lipophilic alkyl end of the molecule to phospholipids of the chloroplast membrane or by destruction of the membrane due to the detergent properties of this compound. Because PH 30-17 consists of a lipophilic portion coupled to a strong positive charge at the other end of the molecule, some detergent properties may be expected. Centrifugation studies showed an alteration of the sedimentation behaviour of chloroplasts after treatment with high doses of PH 30-17, indicating loss of membrane integrity.

The most important structural feature of PH 30-17 is the cyclic guanidine portion:  $-\text{NH}-\text{C} \begin{smallmatrix} \nearrow \text{NH}- \\ \searrow \text{N}- \end{smallmatrix}$  which is also present in other uncouplers such as octylguanidine [14, 15] and the energy transfer inhibitor synthalin [16]. Octylguanidine and the fungicide dodecylguanidine (dodine) proved to be inhibitors of phosphorylation in chloroplasts [14, 15] and mito-

chondria [17, 18]. Nevertheless, the real mode of action of these chemicals is believed to be their effects on membrane integrity [19]. Avron and Shavit reported that octylguanidine also uncoupled in a partially irreversible manner [14]. Thus, some similarity between the effects of alkylguanidines and the compounds described in this paper is obvious. However, the possibility that the effect on membrane integrity is a primary effect of these compounds, while the uncoupling is only a secondary effect should not be excluded. Another interesting effect of these chemicals is the inhibition of ergosterol biosynthesis in fungi [20], but this effect can also be caused by loss of membrane integrity.

Table IV clearly shows that the uncoupling activity increases as the number of carbon atoms in the alkyl chain increases, which indicates that the lipophilicity is an important factor. This is expressed mathematically in Eqns (1) and (2) and visualized in Fig. 3. Because the variation in substitution pattern is limited, no conclusions can be drawn concerning electronic and steric effects. Several authors have studied the relationship between uncoupling activity and the chemical structure of amines [12, 13, 21] or uncouplers of other chemical classes [22–26]. Uncoupling by amines is caused by the uncharged species [12, 13] and therefore the activity is dependent on the  $\text{pK}_a$  [13]. In a congeneric series where  $\text{pK}_a$  values are almost identical the activity is determined by lipophilicity [21]. As the compounds **1–11** in Table IV have almost the same  $\text{pK}_a$ 's,  $\pi$  is the only significant parameter. In a study on the inhibition of oxidative phosphorylation by alkylguanidines, Pressman found a linear relationship between  $\text{pI}_{50}$  values and chain length [17].

Further, Table IV shows that the compound having an imidazole ring (**13**) has nearly the same activity as PH 30-17. By contrast, 4-hexylaniline (**12**) is only a poor uncoupler.

In spite of the above considerations the real mechanism of the uncoupling activity of arylamino-tetrahydropyrimidines is unknown, and further experiments, including the effects on ATPase and pH gradients in chloroplasts, are necessary to elucidate their mode of action.

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