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Inhibition of Hill Reaction Produced by Fatty Acid Anilides

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In 1981 in Spain an illness of unknown characteristics appeared which came to affect a great many people; 20,178 according to the report of the Working Committee of the W.H.O. in March, 1983. Said epidemic has been associated with the consumption of rapeseed oil for industrial use, refined and sold illegally; the only foreign substances present in rapeseed oil are the oleoanilides, with practically unknown biological action. In the bibliography there is the data that some anilides and betanapthylamides of acrylic, dichloracetic, isonitrosoacetic and metacrylic acids are capable of inhibiting electronic transport in photosynthesis, the Hill reaction (Baruffini et al 1956). Therefore we have considered it of interest to study the possible inhibitory action of linoleilanilide, quantitatively one of the most important fatty acid anilides in the toxic oil. Said study will allow us to find out not only if fatty acid anilides can possess phytotoxic action, but above all their capacity to interfere with electron transport, which could be of interest with respect to the knowledge of the reactivity of the molecule in a biological medium.

The inhibitory activity of the Hill reaction is perfectly quantifiable as pI_{50} (Baruffini et al 1956). Consequently, in this way the degree of toxicity of a substance whose pathogenic capacity includes this mechanism can be determined.

In this work we shall evaluate the Hill reaction, assessing the reduction of a solution of potassium ferricyanide and the production of ATP (Adenosine triphosphate). We shall also try to establish on which photosystem the oleoanilides exert their action.

MATERIALS AND METHODS

As photosynthetic material, we used fresh picked

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spinach, rapidly processed in the dark in the cold room (2-5ºC) to obtain chloroplasts by the method of Baruffini et al (1956): 5 g of leaves were triturated with sand and suspended in Tris-sucrose buffer, pH 7'9. After filtration through gauze and successive centrifuging at 3,500 r.p.m. a sediment of chloroplasts was obtained, which was resuspended in 2 ml of buffer solution.
The chlorophyll content of the chloroplast fraction was evaluated by the Arnon (1949) method.

The linoleilanilide used was synthesized in our laboratory from linoleic acid and olive oil by heating, obtaining a product which, crystallized in ethanol, had 95% purity.

To evaluate the inhibition of the Hill reaction, and for the determination of pI_{50}, we proceeded as follows. Before 9 hours had elapsed from the moment of preparation of the chloroplast suspension, it was studied, assessing the Hill reaction as reduction of ferricyanide using the method of Baruffini et al (1956).

To make the anilide soluble in the reaction medium it was added in the form of a solution in ethanol. The following concentrations were tested: 25, 100, 200, 300, 400 and 1,600 µg/ml. The course of the reaction was followed minute by minute to a total of 10-12 minutes. The inhibition was expressed as % of a control Hill reaction and with values, graphs of inhibition were drawn. From these the concentrations of anilide which provoke 50% inhibition were interpolated and the molar concentration and pI_{50} calculated.

The evaluation of the inhibition of the photosynthetic formation of ATP is carried out by the determination of ATP in a procedure based on the measurement of the intensity of light produced in a bioluminescent reaction in which the enzyme luciferase, in presence of luciferine, oxygen and magnesium, catalyses the formation of adenyl-luciferine. Subsequently atmospheric O_2 oxidises the adenyl-luciferine to adenyl-oxiluciferine, a process accompanied by the emission of light (Strehler, 1963) which is determined at 562nm (de la Rosa y Galván, 1979).

We carried out the assessments in a Perkin-Elmer Fluorescence spectrophotometer model MPF-3, with the following conditions: emission: 562 nm, excitation: closed, maximum slit, sensibility: 100. A mechanical mixer was connected for the reaction mixture contained in the quartz cuvette.

To localize the site of inhibition, the reaction medium is the same used in the study of the inhibitory effect of the Hill reaction to which is added for each ml., 100 µmols. (3(3,4 dichlorophenyl)-1,1 dimethyl-urea) (DCMU) (inhibitor of Photosystem II), 14 mg. ascorbate and 60 µg of dichlorophenonolindophenol (DPIP) (artificial electron-donor system) (Ortega et al 1976).

The anilide is added in solution in ethanol and in a concentration of 20 µg/ml.

RESULTS AND DISCUSSION

No metabolic transformation of the linoleilanilide has been detected in the reaction system used. It was also demonstrated that no chemical reaction takes place between the linoleilanilide and the potassium ferricyanide.

The results for the inhibition of the Hill reaction are shown in figure 1, and tables 2 and 3. This inhibition increases with the concentration of added anilide following the curve which appears in figure 1. We observe that the extent of the inhibition varies with the time of measurement of the Hill reaction which proves to be more affected in the first few minutes as seen in figure 2. Consequently the pI_{50}, calculated as shown in table 1, also varies with time. The experimental average, that is to say, the pI_{50} for each concentration, independent of time, corresponds to 11.24 ug/ml which assumes a pI_{50} of 4.50. Figure 3 represents the relation between the concentration of linoleilanilide and the extent of the inhibition produced, in semi logarithmic scale. From the linearity of the graph the existence of a dose/effect relationship is deduced.

The inhibition of the photosynthetic formation of ATP is reflected in table 2 which shows, together, the concentrations of ATP produced in the control Hill reaction, the Hill reaction with linoleilanilide and the Hill reaction with DCMU as a model inhibitor, as alos the % of inhibition produced. Linoleil-anilide reduces the photosynthetic formation of ATP by 42.04%.

The localization of the inhibitory effect of linoleil-anilide in photosynthesis can be seen in table 3 which shows the values of inhibition of the production of ATP under different experimental condidions and at different reaction times.

In the light of the results expressed in figure 1 and

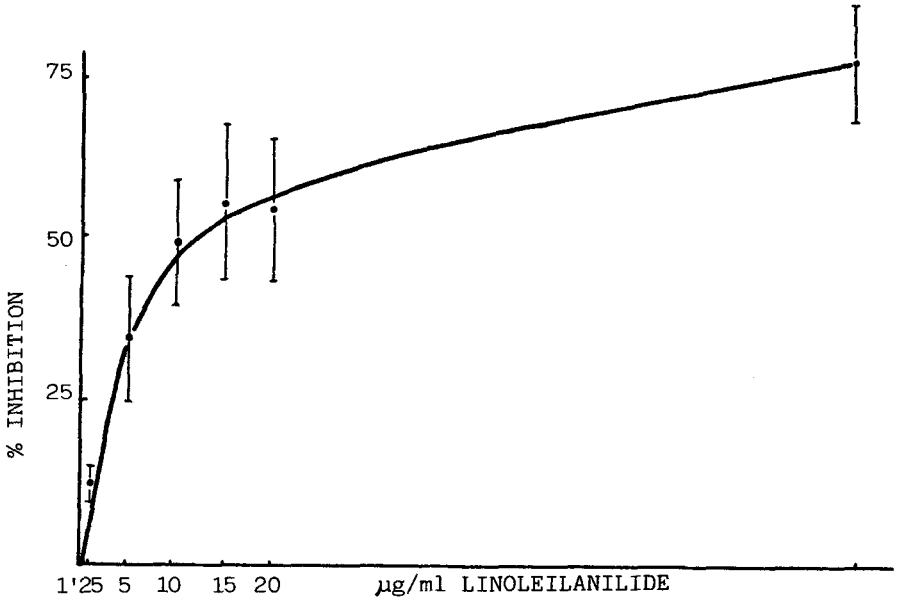

Figure 1. Inhibition of the Hill Reaction by Linoleilanilide.

Table 1. Concentrations of linoleilanilide which produce a 50% inhibition and pI_{50}.

Reaction Time (minutes)	Linoleilanilide Concentrations µg/ml	moles/l	pI_{50}
4	10	2.82×10^{-5}	4.55
6	12.5	3.52×10^{-5}	4.45
8	30	8.45×10^{-5}	4.07
Media Experimental	11.25	3.17×10^{-5}	4.50

table 2 we can state that linoleilanilide inhibits the photosynthetic transport of electrons of the Hill reaction both when measured as reduction of potassium ferricyanide, and when the formation of ATP is determined.

The reduction of potassium ferricyanide diminishes progressively depending on the dose (figure 3) and the time at which the Hill reaction was measured (table 1 and figure 2), proving to be more affected in the first few minutes.

From the values obtained in the determination of the reduction of potassium ferricyanide the potential toxicity (pI) of linoleilanilide has been calculated, obtaining a value of 4.5 (Table 2). Comparing this figure with those obtained by Baruffini et al (1956) in their study on the inhibitory activity of some anilides and their derivatives, we can state that the

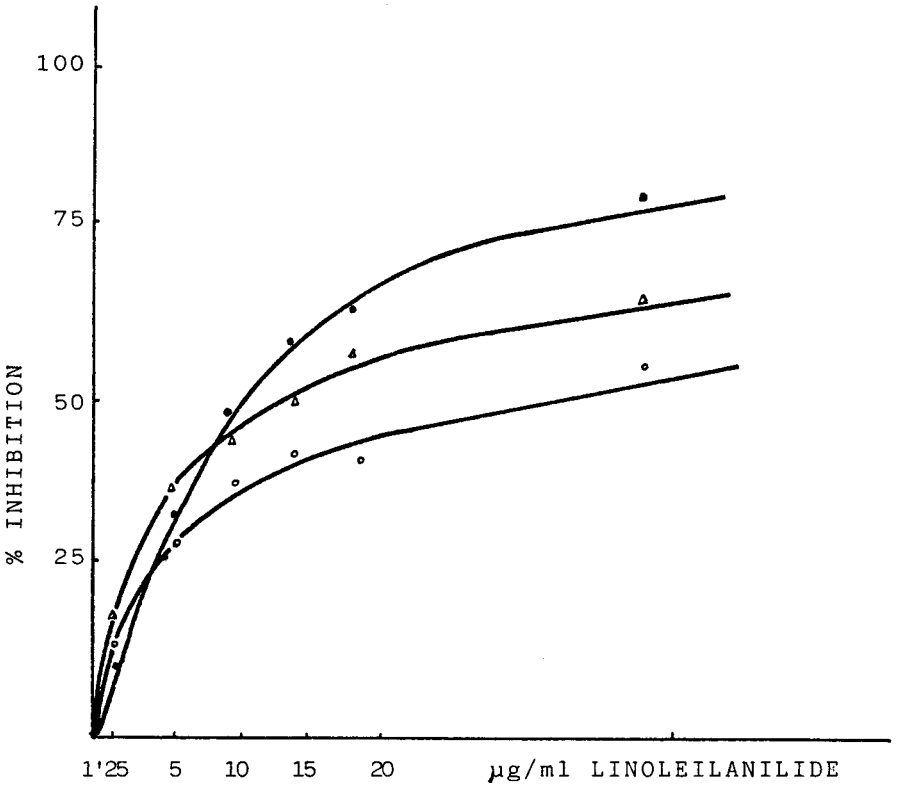

Figure 2. Influence of time in the inhibition of the Hill Reaction by Linoleilanilide.

Table 2. Concentration of photosynthetic ATP and % of inhibition produced in different experimental conditions.

Experimental Conditions	ATP formed (µM)	% Inhibition
Control Hill Reaction	133.53 ± 49.35	0.00
+linoleilanilide (20ug/ml added)	77.40 ± 46.02	42.04
+DCMU (23.3ug added)	33.79 ± 21.72	74.70

oleoanilides present a toxicity, in so far as photosynthetic electron transport is concerned, similar to some acrylanilides, dichlor acetanilide and metacrylanilide. We can situate the toxicity of the linoleilanilides among the anilides as a moderate inhibitor.

Table 3.- Average values of concentration of reduced potassium ferricyanide, in nmoles/ml

Reaction Time (minutes)	Hill Reaction				
	Control	Linoleilanilide (20 µg/ml)	DCMU 100 µmoles = 23,3 µg/ml	DCMU, DPIP Ascorbate	DCMU, DPIP, Ascorbate Linoleilanilide
2	127.3 ± 48.7	11.4 ± 22.7	11.4 ± 13.7	325.0 ± 68.2	286.4 ± 118.9
4	204.8 ± 81.1	14.1 ± 22.3	5.7 ± 13.3	349.7 ± 54.5	318.2 ± 94.4
6	280.8 ± 90.2	30.3 ± 32.1	9.6 ± 15.9	349.3 ± 52.4	324.2 ± 101.1
8	354.6 ± 51.4	27.3 ± 12.9	0 ± 0	350.0 ± 70.7	309.1 ± 128.6
Average Global Values:	237.4 ± 97.2	20.8 ± 26.0	7.7 ± 14.1	340.0 ± 71.3	314.4 ± 97.0

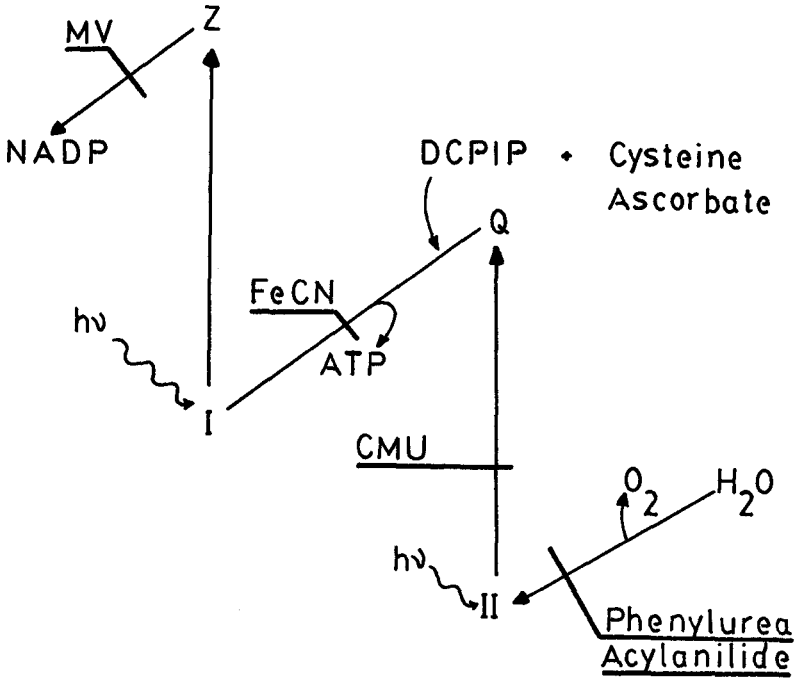

Figure 3. Hill Reaction Inhibition Sites.

The photosynthetic production of ATP is inhibited 42.04% with concentrations of 20 µg/ml of linoleilanilide. With similar concentrations of DCMU (23 µg/ml) considered a potent inhibitor of photosynthesis, the % inhibition is 74.70% which again shows that linoleilanilide is a moderate inhibitor.

The photosynthetic transport of electrons can be inhibited at different points, as shown in figure 3. We deduce that linoleilanilide interrupts the electron flow in photosystem II because on blocking this system with DCMU and leaving photosystem I working alone by the addition of an artificial electron donor system, the linoleilanilide does not block the Hill reaction (Table 3 and figure 3). From this we consider that the action of this substance could be localized in the photosystem II. This fact is in agreement with what is known about the structure/activity relationship in this type of action (Moreland and Hill, 1959; Alcaide and Municio, 1967). The oleoanilides, just like most inhibitors of photosystem II, possess a carbonyl group and an imine hydrogen capable of forming hydrogen bridges with the cyclopentanone ring of the chlorophyll molecule.

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