INHIBITORS OF THE HILL REACTION [1, 2]

NORMAN E. GOOD

PESTICIDE RESEARCH INSTITUTE, UNIVERSITY SUB POST OFFICE, LONDON, CANADA

Many commercially important herbicides seem to kill plants by inhibiting photosynthesis. Wessels and van der Veen have shown that leaves or parts of leaves treated with phenylureas irreversibly lose all ability to assimilate CO_2. They also found that chloroplasts treated with phenylureas are unable to reduce oxidants such as 2,6-dichlorophenolindophenol in the light (20). A wide variety of phenylcarbamates similarly inhibit photochemical reductions by chloroplast (the Hill reaction) although somewhat higher concentrations of these carbamates are required (20, 11). Recently a number of acylanilides have been introduced as herbicides. These, too, interfere with the Hill reaction (12). The phenylcarbamates, phenylureas, and acylanilides are, of course, closely related chemically: all are anilides of carboxyacids. However, the very dissimilar herbicide Simazine [2-chloro-4,6-bis(ethylamino)-s-triazine] also inhibits the Hill reaction and does so at low concentrations (13).

It has been possible to identify more specifically the process which is inhibited in chloroplasts by phenylureas. Bishop discovered that 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibited photosynthesis but not photoreduction in the alga Scenedesmus (3). Since photoreduction differs from photosynthesis in that hydrogen gas replaces water as the ultimate electron donor, it seems reasonable to suppose that DCMU interferes with the mechanism of water oxidation—that is, with the process leading to the production of molecular oxygen. This hypothesis has been supported by experiments with chloroplasts from higher plants. Thus, Jagendorf showed that ATP formation (and therefore probably electron flux) was inhibited in chloroplasts by 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) when flavin mononucleotide (FMN) was the electron carrier but not when N-methylphenazonium ion (PMS) was the carrier (8). Later Krall, Good, and Mayne demonstrated that the FMN catalysed process involved the production and reutilization of molecular oxygen whereas the PMS catalysed process did not (10). Apparently reduced PMS can replace water as electron donor in a reaction analogous to photoreduction. In accordance with Bishop's hypothesis, the process which is independent of the production of molecular oxygen is also resistant to CMU inhibition.

In the work described below the chloroplast-inhibiting herbicides and a large number of related substances were investigated. This report deals with four aspects of the problem:

I. More than 200 acylanilides, thioacylanilides, acylamides, ureas, and thioureas were prepared and identified. About half of these were not described in the readily accessible literature and probably many are new.

II. The work of Wessels and van der Veen (20) and of Moreland et al. (11, 12) on the relationship of inhibitor structure to inhibitor potency has been extended. Inhibition was routinely measured as a decrease in the ability of isolated chloroplasts to reduce ferricyanide in the light.

III. The Jagendorf criterion was used to determine the site of inhibition. Inhibition of the photochemical formation of ATP with FMN as catalyst was compared with the inhibition of ATP formation with PMS as catalyst. This test was applied to representative acylanilides, alkylureas, and triazines as well as to the previously examined phenylureas.

IV. An attempt was made to correlate the bonding potentials of the imino hydrogens of the various inhibitors with inhibitor structure and biological activity. Wessels and van der Veen (20) have suggested that the carbonyl oxygen of the cyclopentanone ring of the chlorophyll molecule may form hydrogen bonds with the imino hydrogen of phenylcarbamates and phenylureas, thus disrupting the photochemical apparatus of the chloroplast. For reasons to be discussed, we do not consider that this particular bond could account for the observed inhibitions. Nevertheless some of the most striking properties of anilides and other amides (high melting points, low vapor pressures, preference for polar solvents, etc.) are directly related to intermolecular hydrogen bonding. Therefore it seems quite probable that these inhibitors become attached by hydrogen bonds to the as yet unidentified active site. Since the formation of a hydrogen bond results in a replacement of one of the characteristic N-H infra-red absorption bands by an N-H ... O band at a longer wave length, the bonding of the imino hydrogen of representative anilides and triazines to various carbonyl groups was observed by infra-red spectroscopy.

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Materials & Methods

I. Syntheses of Inhibitors. Acylanilides were prepared by treating the appropriate free aniline either with acid anhydrides (acetic, propionic, butyric, & isobutyric) or with acid chlorides. The anhydrides or chlorides of the acids were added slowly to a benzene solution of the aniline, and the resulting suspension was boiled for a few minutes. Those anilides which were insoluble in benzene were filtered off, dissolved in alcohol, treated with Norite, and recrystallized from alcohol or, more often, from alcohol and water. Anilides which were soluble in benzene were precipitated by adding *n*-hexane. To bring out the anilides of the higher fatty acids large amounts of hexane and cooling to about −15 C were necessary. The acid chlorides were prepared by heating the acids under reflux with an excess of thionyl chloride and were used without purification after the bulk of the unreacted thionyl chloride had been removed by distillation. Isobutyrylamides were prepared in a similar manner except that no heating of the alkylamine-acid anhydride mixture was required. Formanilides were prepared by heating the anilides under reflux in a large excess of formic acid.

4-Chloro- and 3,4-dichloroanilides of thiopropionic acid were prepared by treating the corresponding oxygen anilides with P_2S_5 (9). The solubility of the thioanilides in dilute KOH was used to facilitate their isolation. They were recrystallized from a solution of benzene and *n*-hexane.

Asymmetrical ureas were prepared either by the action of dimethylcarbamyl chloride on the appropriate amine (3,5-dichloroaniline, 3-chloroaniline, octylamine, benzylamine, cyclohexylamine) at room temperature or by the action of alkylamines on the isocyanates derived from 3,4-dichloroaniline and 2-chloroaniline. Note that unless heat is avoided in the reaction involving dimethylcarbamyl chloride, a rearrangement takes place which results in the formation of symmetrical ureas. The reaction of dimethylcarbamyl chloride with anilines is very slow at room temperature, requiring one to several weeks: with 2-chloroaniline it is prohibitively slow and even at room temperature yields predominantly the symmetrical 1,3-bis(2-chlorophenyl)urea.

Asymmetrical thioureas were prepared by the action of alkylamines on 3,4-dichlorophenyl isothiocyanate.

Symmetrical diphenylureas and diphenylthioureas (most of which were too insoluble to test) were prepared by prolonged heating of the appropriate aniline with dimethylcarbamyl chloride (see above) or dimethylthiocarbamyl chloride.

Alkylamines, anilines, and acids used in this investigation were obtained from commercial sources except 3,4-dichlorophenylacetic acid which was prepared by the Arndt-Eistert synthesis (1) from 3,4-dichlorobenzoyl chloride. The phenyl isocyanates and phenyl isothiocyanates were prepared by slowly adding the corresponding aniline to solvents containing an excess of phosgene or thiophosgene (2).

The melting point of each of the potential inhibitors was determined on a melting point block. This block had been calibrated with standard pure substances having well-recognized melting points. Melting points so obtained were identified with those in the literature whenever possible. The only major difference involved 1-benzyl-3,3-dimethylurea (found 76–77°, literature 166°); the substance described in the literature (19) is probably 1,3-dibenzylurea, mp 168°. The melting point of another of our products, N-isobutyryl-2,6-dimethylaniline, could not be established. This substance softened and sublimed over a range of more than 100°. Some of the substituted ureas also had abnormal (double?) melting points. In these instances we have followed the literature in reporting only the higher temperature. (table I).

When no description of the substance could be found (or if there was reason to question the literature) the identity of the substance was confirmed by elemental analysis. (table I).

II. Determination of Relative Potencies of Inhibitors. Chloroplasts from young pea plants (*Pisum sativum* L.) were isolated in the following manner: Freshly picked leaves were ground in a chilled mortar with an ice-cold buffer consisting of 135 g sucrose, 3.0 g tris(hydroxymethyl)aminomethane, and 1.0 g sodium chloride per liter, adjusted to pH 7.95 with 1 N sulfuric acid. The homogenate was filtered through glass wool into ice-encased centrifuge tubes. After centrifugation for 3 minutes at about 4,000 times gravity, the supernatant was discarded and the chloroplasts were resuspended in more cold buffer. The suspension was transferred to other ice-encased centrifuge tubes and was again centrifuged for 2 minutes at 4,000 times gravity. The chloroplasts were then suspended in a minimum volume of buffer and the suspension was filtered through glass wool again to remove clumps. The final dense suspension was stored briefly in a flask which was deeply immersed in chopped ice and water.

The reaction mixture (final volume 2.0 ml) consisted of the above mentioned buffer, methylamine hydrochloride (24 μmoles), potassium ferricyanide (1.0 μmole), and an amount of the chloroplast suspension containing about 30 μg chlorophyll. Ferricyanide reduction was followed spectroscopically in an apparatus described elsewhere (6). Reaction temperatures were held at about 15 C by cooling the cuvette holder with rapidly circulating tap water. An intense beam of light from a 300 w projection lamp was passed through a red glass filter and directed against the frosted side of the reaction vessel, a 1 cm cuvette. The inhibitors to be tested were dissolved in alcohol. Different concentrations were prepared, usually by a process of serial dilution. After it had been ascertained that 5% alcohol alone had very little effect on the rate of ferricyanide reduction, these alcoholic inhibitor solutions were added to the reaction mixture in amounts not exceeding 0.1 ml. With each inhibitor the concentration which halved the reaction rate was determined. For convenience

TABLE I

AMIDES AS INHIBITORS OF HILL REACTION
GENERAL FORMULA: R-NH-CX-R^1

No.	R	X	R^1	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
	Part I—Anilides from unsubstituted aniline							
1	Phenyl	O	Methyl	3.7	113–114	114		
2	"	O	Chloromethyl	5.0	133–134	134		
3	"	O	Dichloromethyl	4.4	118	118		
4	"	O	Trichloromethyl	5.0	94	94		
5	"	O	Ethyl	4.7	105	103		
6	"	O	1-Chloroethyl	4.1	89–90	92		
7	"	O	1,1-Dichloroethyl	4.4	99–100	101		
8	"	O	Propyl	4.0	94–95	95		
9	"	O	2-Propyl	4.2	105–106	105		
10	"	O	Phenyl	4.0	161–162	160		
11	"	O	2-Methyl-1-propenyl	5.2	127–129		N 8.04	N 8.00
12	"	O	Dimethylamino	**5.2**	129–130	127–129		
13	"	S	Dimethylamino	2.0	132–133	127		
14	"	O	2-Propoxy	4.1	87–88	90		
	Part II—4-Chloroanilides							
15	4-Chlorophenyl	O	H	2.5	101–102	102		
16	"	O	Methyl	4.3	178–179	179		
17	"	O	Chloromethyl	4.7	169–170	169		
18	"	O	Dichloromethyl	4.7	137–138		Cl 44.5	Cl 44.6
19	"	O	Trichloromethyl	4.6	127–128		Cl 52.2	Cl 52.0
20	"	O	Ethyl	4.5	137	141		
21	"	S	Ethyl	3.2	77–78		Cl 18.3 S 16.0	Cl 17.8 S 16.1
22	"	O	1-Chloroethyl	4.7	112		Cl 32.1	Cl 32.5
23	"	O	1,1-Dichloroethyl	5.1	94–95		Cl 42.0	Cl 42.1
24	"	O	Propyl	4.7	102–103	104		
25	"	O	2-Propyl	5.2	148–150	153		
26	"	O	Phenyl	4.5	192	192–193		
27	"	O	2-Methyl-1-propenyl	5.0	121–122		Cl 17.0	Cl 17.0
28	"	O	2-Methyl-2-propyl	4.5	148–149		Cl 16.7	Cl 16.8
29	"	O	Dimethylamino	6.3	169	171		
30	"	S	4-Chloroaniline	4.5s	178–179		Cl 23.8 S 10.8	Cl 23.9 S 10.7
	Part III—3-Chloroanilides							
31	3-Chlorophenyl	O	H	3.2	55–56			
32	"	O	Methyl	4.5	76	79		
33	"	O	Chloromethyl	5.0	99–100		Cl 34.9	Cl 34.8
34	"	O	Trichloromethyl	5.0	101–102		Cl 52.4	Cl 52.0
35	"	O	Ethyl	5.5	87–88	88–89		
36	"	O	Propyl	5.3	45–46		Cl 17.6	Cl 17.9
37	"	O	2-Propyl	4.8	112		Cl 17.8	Cl 17.9
38	"	O	2-Methyl-1-propenyl	5.0	112–113		Cl 16.9	Cl 16.9
39	"	O	3-Chloroaniline	5.0	246	245	Cl 23.2	Cl 23.0
40	"	O	Dimethylamino	6.3	139–141		Cl 17.9	Cl 17.9
41	"	O	2-Propoxy	4.4				
42	"	O	4-Chloro-but-3-yn-1-oxy	5.1	75–76			

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

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TABLE Ia

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R^1

No.	R	X	R^1	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
	Part IV—2-Chloroanilides							
43	2-Chlorophenyl	O	Trichloromethyl	3.7	62		Cl 51.8	Cl 52.0
44	"	O	Ethyl	2.6	92–93	91		
45	"	O	1-Chloroethyl	2.3	59		Cl 32.3	Cl 32.5
46	"	O	Propyl	2.0	80		Cl 17.7	Cl 17.9
47	"	O	2-Propyl	2.2	93–94		Cl 17.7	Cl 17.9
48	"	O	2-Methyl-2-propyl	2.5	76		Cl 16.6	Cl 16.7
49	"	O	2-Methyl-1-propenyl	3.4	88–89		Cl 16.9	Cl 16.9
50	"	O	Dimethylamino	3.3	92–93	95		
	Part V—3,5-Dichloroanilides							
51	3,5-Dichlorophenyl	O	H	3.2	127		Cl 37.5	Cl 37.3
52	"	O	Methyl	4.0s	186	186–187		
53	"	O	Trichloromethyl	5.7	121–122		Cl 58.1	Cl 57.8
54	"	O	Ethyl	5.7	118–120		Cl 32.8	Cl 32.7
55	"	O	2-Chloroethyl	4.5	97–98		Cl 41.9	Cl 42.1
56	"	O	Propyl	4.6	85–86		Cl 30.9	Cl 30.6
57	"	O	2-Propyl	4.8	134–135		Cl 30.5	Cl 30.6
58	"	O	2-Methyl-1-propenyl	4.7	106–107		Cl 29.0	Cl 29.0
59	"	O	Dimethylamino	6.0	163–165		Cl 30.2	Cl 30.4
	Part VI—2,4-Dichloroanilides							
60	2,4-Dichlorophenyl	O	Methyl	2.5s	143–144	144		
61	"	O	2-Propyl	2.8s	124–125	124		
	Part VII—3,4-Dichloroanilides							
62	3,4-Dichlorophenyl	O	H	3.5	108–109	110–112		
63	"	O	Methyl	5.5	122–123	121		
64	"	O	Chloromethyl	6.5	106–107		Cl 44.7	Cl 44.7
65	"	O	Trichloromethyl	6.5	124–126		Cl 57.8	Cl 57.7
66	"	O	Bromomethyl	6.6	99–101		N 4.98	N 4.96
67	"	O	Ethyl	6.8	91–92	91–92		
68	"	O	1-Chloroethyl	6.2	127–129	133–134		
69	"	O	1,1-Dichloroethyl	6.0	110–112		Cl 49.3	Cl 49.5
70	"	S	Ethyl	4.3	71–72		Cl 29.5 S 13.4	Cl 30.4 S 13.7
71	"	O	2-Chloroethyl	5.2	112–113		Cl 42.0	Cl 42.1
72	"	O	2-Propenyl	6.7	120–122			
73	"	O	2-Methyl-2-propyl	6.2	145–146		Cl 28.9	Cl 28.8
74	"	O	Propyl	5.7	79–80	77–78		
75	"	O	2-Propyl	6.2	132–134	135		
76	"	O	3-Pentyl	5.5	125	125		
77	"	O	2-Butyl	6.3	112–113		Cl 28.8	Cl 28.9
78	"	O	Butyl	6.5	73.5	71.5–73		
79	"	O	2-Methyl-1-propyl	5.0	85–86	82–87		
80	"	O	2-Pentyl	7.0	105–106	108–109		
81	"	O	Pentyl	6.5	75–76		Cl 27.3	Cl 27.3

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

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(Continued from Ia)

TABLE Ib

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R^1

No.	R	X	R^1	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
Part VII—3,4-Dichloroanilides (Continued)								
82	"	O	Hexyl	5.2	61.5	60–61.5		
83	"	O	Heptyl	6.0	42		Cl 24.4	Cl 24.6
84	"	O	Octyl	5.4	69–70		Cl 23.4	Cl 23.5
85	"	O	Nonyl	5.4	70–71		Cl 22.5	Cl 22.4
86	"	O	Phenyl	5.2	145–146		Cl 26.7	Cl 26.7
87	"	O	2-Chlorophenyl	4.8	152–153		Cl 34.8	Cl 34.5
88	"	O	4-Chlorophenyl	5.7	172–173		Cl 35.5	Cl 36.0
89	"	O	2,4-Dichlorophenyl	5.5	156–157		Cl 42.1	Cl 42.4
90	"	O	3,4-Dichlorophenyl	6.0	227–228		Cl 42.3	Cl 42.4
91	"	O	Cyclohexyl	6.0	137–138		Cl 26.0	Cl 26.1
92	"	O	Benzyl	6.8	132		Cl 25.3	Cl 25.3
93	"	O	3,4-Dichlorobenzyl	5.6	186–187		Cl 40.3	Cl 40.7
94	"	O	Phenoxymethyl	5.0	141–142		Cl 24.0	Cl 24.0
95	"	O	2,4-Dichlorophenoxymethyl	5.5	160–161		Cl 39.0	Cl 39.0
96	"	O	2-Methyl-1-propenyl	5.9	103		Cl 29.0	Cl 29.0
97	"	O	3-Phenylpropyl	5.1	74–75		Cl 23.1	Cl 23.0
98	"	O	Trans-2-phenylethenyl	5.2	179–181		Cl 24.7	Cl 24.3
99	"	O	2,4,5-Trichlorophenoxymethyl	5.2	141–142		Cl 43.4	Cl 44.5
100	"	O	2-Naphthylmethyl	5.4	157–158		Cl 21.6	Cl 21.6
101	"	O	1-Naphthylmethyl	5.6	170–172		Cl 21.7	Cl 21.6
102	"	O	Methylamino	7.0	156–157	155.5		
103	"	O	Ethylamino	6.2	175–176	179.5		
104	"	O	Propylamino	5.8	128–129		Cl 28.2	Cl 28.7
105	"	O	Butylamino	6.7	121–122		Cl 26.8	Cl 27.1
106	"	O	Hexylamino	5.5	104–105		Cl 24.9	Cl 24.5
107	"	O	Cyclohexylamino	5.5	183–184	188	Cl 24.6	Cl 24.6
108	"	O	Benzylamino	5.5	171–172		Cl 24.0	Cl 24.2
109	"	O	2-Hydroxylethylamino	4.8	137–138		Cl 28.6	Cl 28.5
110	"	O	Dimethylamino	7.5	156–157	158–159		
111	"	O	Diethylamino	6.8	111–112		Cl 26.3	Cl 26.2
112	"	O	Dipropylamino	4.7	96–97		Cl 24.2	Cl 24.5
113	"	O	Di-(2-propyl)amino	5.3	130–131		Cl 24.4	Cl 24.5
114	"	O	Piperidino	6.8	172–173		Cl 26.1	Cl 26.1
115	"	O	Morpholino	6.4	153–154	157.5	Cl 25.5	Cl 25.8
116	"	O	Di-(2-hydroxyethyl)amino	4.5	153–154	157.0	Cl 24.3	Cl 24.2
117	"	S	Dimethylamino	4.5	161–162	166.0		
118	"	S	Methylamino	3.7	148–150	147.5		
119	"	S	Diethylamino	4.3	95–96		Cl 25.6	Cl 25.6
120	"	S	Ethylamino	3.8	114–115		Cl 28.5	Cl 28.5
121	"	O	Amino	4.7	152–154	155.5		
122	"	S	Amino	3.3	209–210	203–204		
Part VIII—Miscellaneous anilides of isobutyric acid								
123	2,3-Dichlorophenyl	O	2-Propyl	3.0s	108–109		Cl 30.4	Cl 30.6
124	2,5-Dichlorophenyl	O	"	3.0s	137–139		Cl 30.4	Cl 30.6
125	2,4,5-Trichlorophenyl	O	"	4.5	145–146		Cl 39.9	Cl 40.0
126	2,4,6-Trichlorophenyl	O	"	5.0s	151–152		Cl 39.4	Cl 40.0
127	4-Bromophenyl	O	"	5.0	151–152	150–151		

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

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(Continued from Ib)

TABLE Ic

AMIDES AS INHIBITORS OF HILL REACTION

GENERAL FORMULA: R-NH-CX-R¹

No.	R	X	R¹	pI_{50}*	MELTING POINTS		ELEMENTARY ANALYSES	
					FOUND	LITERATURE	FOUND %	CALCULATED %
Part VIII—Miscellaneous anilides of isobutyric acid (Continued)								
128	2-Methylphenyl	O	2-Propyl	2.3	115–116	115–116		
129	4-Methylphenyl	O	"	4.5	108–109	108		
130	3-Methylphenyl	O	"	4.8	82–83	85		
131	2-Methoxyphenyl	O	"	2.0	44		N 7.19	N 7.25
132	4-Methoxyphenol	O	"	5.0s	109–111		N 7.26	N 7.26
133	4-Nitrophenyl	O	"	4.0	167–169		N 13.4	N 13.45
134	3-Nitrophenyl	O	"	3.8	93		N 13.5	N 13.45
135	3-Chloro-4-methylphenyl	O	"	5.8	146–147		Cl 16.8	Cl 16.8
136	2-Methyl-3-chlorophenyl	O	"	2.5s	142–143		Cl 16.8	Cl 16.8
137	2-Methyl-4-chlorophenyl	O	"	2.5s	163–164		Cl 16.8	Cl 16.8
138	3-Nitro-4-methylphenyl	O	"	4.8	106–107		N 12.5	N 12.6
139	1-Naphthyl	O	"	2.0s	147–149		N 6.57	N 6.58
140	5,6,7,8-Tetrahydro-2-naphthyl	O	"	5.0	102		N 6.57	N 6.45
141	4-Dimethylaminophenyl	O	"	3.0	157–158		N 13.8	N 13.6
142	2,6-Dimethylphenyl	O	"	2.7	?		N 7.26	N 7.35
Part IX—Alkylamides & alkylureas								
143	Cyclohexyl	O	2-Propyl	3.3	116–117		N 8.32	N 8.30
144	Benzyl	O	"	2.3	91–92		N 8.05	N 7.92
145	Octyl	O	Dimethylamino	5.6	27–28		N 13.7	N 14.0
146	Cyclohexyl	O	"	4.8	156–157		N 16.5	N 16.4
147	Benzyl	O	"	3.6	76–77		N 15.7	N 15.7

*pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction.

these concentrations have been expressed on a logarithmic scale. Thus the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition is referred to as the pI_{50}. The inhibitors with the larger pI_{50}'s are the more potent and a difference in pI_{50} of one unit represents a tenfold difference in potency.

Several points on the concentration-inhibition curve were established for each compound in order to avoid spurious conclusions which might arise from an inadvertent saturating of the medium with inhibitor. (With saturated or transiently supersaturated solutions, adding more inhibitor does not necessarily increase the effective concentration and may even decrease it by initiating crystallization.) Solubility problems did occur with a number of substances. In fact, many of the compounds prepared and tested are not included in this report because it was impossible to achieve concentrations high enough to cause 50 % inhibition. Other substances were doubtful, but by utilizing the fleeting phenomenon of supersaturation often it was possible to obtain the desired level of inhibition. However, to indicate our reservations we have added the letter "s" after the pI_{50} value listed in table I whenever the true inhibitor concentration was in doubt. Fortunately most of the highly active inhibitors presented no problem, either because their water solubilities, although very small, were adequate to supply the extremely low inhibitor requirement or because, at the very great dilution involved, crystallization was so slow as to render the supersaturated state quasi-stable.

III. INHIBITION OF ATP FORMATION. Chloroplasts (60–90 μg chlorophyll) were illuminated in a reaction medium consisting of the pH 7.95 buffer, ATP (10^{-3} M), K_2HPO_4 labeled with P^{32} (10^{-2} M), magnesium sulfate (3×10^{-4} M), hexokinase (3.0 mg), glucose (2×10^{-2} M), either FMN or PMS (10^{-4} M), a trace of horse liver catalase to hasten the decomposition of the hydrogen peroxide formed on oxidation of the reduced flavin, and various amounts of inhibitor in 0.2 ml of alcohol. The total volume was 5.0 ml. The reactions were carried out

in test tubes (15 mm I.D.) supported in a wire rack immersed in a large rectangular glass water bath. Under the bath was a mirror. Light from a 500 w incandescent lamp was directed down on the bath and light from two 300 w reflector lamps was directed against each side of the bath. The temperature of the bath was held constant ± 1 C during the 10 minutes of illumination either by adding ice or by circulating tap water. The reaction temperature in different experiments varied from 3 C to 20 C, although most of the experiments were conducted at about ten Centigrade. After illumination, the reaction mixture was poured into 10 % perchloric acid and the unreacted orthophosphate was extracted as phosphomolybdate by the method of Nielsen and Leninger (15). The organic phosphate (glucose-6-phosphate synthesised by hexokinase from glucose & labeled ATP) was measured as the residual radioactivity of the acidified medium.

IV. Hydrogen Bond Formation. Various acylanilides, acylamides, alkylureas, phenylureas, and triazines were dissolved in chloroform (approximately 0.067 or 0.5 M), in chloroform + 5 % acetone (0.067 M), and in carbontetrachloride (0.067 M). There is a characteristic absorption band of the N-H bond at 2.9 μ (wave number = 3,440 cm^{-1}) and a corresponding absorption band of the N-H ... O bond near 3.0 μ. Consequently the absorption spectrum between 2.6 and 3.2 μ of each substance in each solvent system was determined with a Perkin-Elmer Model 21 infra-red spectrophotometer.

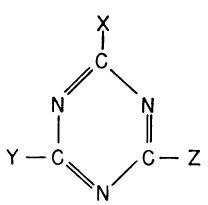
Results

The substances with which the report deals may be classified on the basis of their chemical structures into three unequal groups:

I. Amides having the general formula R-NH-CX-R^1 where X is oxygen of sulfur, R and R^1 are alkyl or aryl, substituted or unsubstituted; R^1 may also be alkylamino, dialkylamino, or arylamino. (See tables I & II).

II. Miscellaneous compounds resembling group I) in that they are also amides but not fitting the general formula, e.g., N-methylanilides, sulfonamides.

III. A small number of symmetrical triazines having the general formula:



where X is chlorine or methoxy, Y and Z are ethylamino, or diethylamino. (See table III).

A. Inhibition of Ferricyanide Reduction. There are several considerations which may throw doubt on comparisons of inhibitor potencies. It is conventional to express the activity of the inhibitor in terms which involve its molar concentration. Now a) if the enzyme-inhibitor reaction is essentially irreversible or b) if the inhibitor's solubilities are such that it partitions between water and the biological material in a manner which strongly favors the latter, then the significant parameter becomes the amount of inhibitor in each chloroplast and not the original concentration of inhibitor in the suspension medium. Unfortunately, the real condition is frequently intermediate and neither the overall inhibitor concentration nor total amount of inhibitor added is uniquely significant. This may make kinetic analysis of the situation almost impossible. We have followed the popular trend and expressed inhibitor potencies as pI_{50}'s, that is, $\log_{10}$ of the reciprocal of the molar concentration causing 50 % inhibition. However, all the inhibitor studies reported in tables I, II, and III were made with the same or nearly the same proportion of chloroplasts to medium. Therefore, the concentration data expressed as pI_{50}'s may be converted into data expressed as amounts of inhibitor per chloroplast or per μg chlorophyll without changing in any way the order of potencies. Of course, this still leaves undecided the question of the proportion of the applied inhibitor actually in the chloroplasts (which proportion may be quite different with different inhibitors) and it must be recognized that these and all similar values for inhibitor potency are the outcome of at least two unrelated properties of the inhibitor: its inherent effectiveness as an enzyme poison and the coefficient of its partitioning between water and chloroplasts.

In order to obtain the maximum Hill reaction rates in our test system, we were very careful to use saturating light intensities and to uncouple the phosphorylating electron transport system with methylamine (6). Under these conditions the uninhibited rates were of the order of 1 millimole ferricyanide reduced per mg chlorophyll per hour.

The reproductibility of the pI_{50}'s in our test varied with different substances. In comparing the potencies of the various substances, it is probably safe to consider a difference of 0.2 as of borderline significance and a difference of 0.3 as quite significant.

I. Acylanilides, Thioacylanilides, Acylamides, Phenylureas, Phenylthioureas, Alkylureas, & Phenylcarbamates Having General Formula R-NH-CX-R^1 (table I).

Part I. Derivatives of unsubstituted aniline. The unsubstituted anilides are not nearly as effective as many of the substituted anilides described below. Among the unsubstituted anilides, the acetyl derivative (No. 1) is a poor inhibitor while the chloroacetyl (No. 2), the trichloracetyl (No. 4) and the β,β-dimethylacrylyl (No. 11) are all fairly good and about equally potent. The well known herbicide, phenyldimethylurea (No. 12), has the same activity as the better acyl derivatives but its sulfur analog (No. 13) is almost inactive. Note that the only carbamate (No. 14) is not very effective as a Hill reaction in-

hibitor although it is a commercial herbicide. The probable mode of action of the phenylcarbamates will be compared with the mode of action of the other anilides in the discussion.

Part II. Derivatives of 4-chloroaniline. These exhibit a wider range of activities than do the unsubstituted anilides; the potencies of the various derivatives do not follow an entirely parallel pattern. The formyl (No. 15) and the thiopropionyl (No. 21) derivatives are particularly poor. The α,α-dichloropropionyl (No. 23), the isobutyryl (No. 25), and the β,β-dimethylacrylyl (No. 27) derivatives are reasonably good. The dimethylcarbamyl derivative (No. 29), (4-chlorophenyldimethylurea, CMU or monuron), is at least 10 times more potent than its nearest competitor.

Part III. Derivatives of 3-chloroaniline. Again the sequence of relative potencies is different in some details. The formyl derivative (No. 31) has very low activity. The acetyl derivative (No. 32) and the isopropylcarbamate (No. 41) are rather poor. The 3-chloro-analog of CMU (No. 40) is, like CMU, outstandingly better than the other members of this group.

Part IV. Derivatives of 2-chloroaniline. Without exception these anilides are feeble inhibitors.

Part V. Derivatives of 3,5-dichloroaniline. Although the average potency is about the same as in the para- and meta-substituted series, these disubstituted anilides do not differ as widely. Again the dimethylurea (No. 59) has the highest activity, but only by a narrow margin.

Part VI. Derivatives of 2,4-dichloroaniline. The two substances in this class resemble 2-chloroanilides. Apparently a chloro group in the ortho position interferes seriously with the mechanism of inhibition.

Part VII. Derivatives of 3,4-dichloroaniline. The 3,4-dichloroanilides are the most active inhibitors we have investigated. Indeed, there is not a single instance of another anilide or alkylamide exceeding the corresponding 3,4-dichloroanilide in potency. Consequently, it seemed appropriate to use 3,4-dichloroaniline as the common moiety in a large number of substances to determine how the structure of the rest of the molecule affected the inhibitory functions. Admittedly, this investigation was not very systematic but, unfortunately, no principles on which to base a systematic study were apparent. Even now, widely applicable generalizations relating activity to the structure of the acyl moiety of the anilides elude us. However, it is possible to make certain generalizations of limited applicability:

Among the anilides of aliphatic acids, inhibitor activity is a periodic function of the chain length of the acid (See fig 1). There is a maximum at the propionyl derivative (No. 67), another maximum

TABLE II

ANILIDES OF ISOBUTYRIC ACID AS HILL REACTION INHIBITORS

No.	POSITION OCCUPIED ON BENZENE RING					pI_{50}*
	2	3	4	5	6	
9						4.2
25			Cl			5.2
37		Cl				4.8
47	Cl					2.2
61	Cl		Cl			2.8
57		Cl		Cl		4.8
75		Cl	Cl			6.2
123	Cl	Cl				3.0
124	Cl			Cl		3.0
125	Cl		Cl	Cl		4.5
126	Cl		Cl		Cl	5.0s
127			Br			5.0
128	CH_3					2.3
130		CH_3				4.8
129			CH_3			4.5
142	CH_3				CH_3	2.7s
132			CH_3O			5.0s
131	CH_3O					2.0
133			NO_2			4.0
134		NO_2				3.8
135		Cl	CH_3			5.8
136	CH_3	Cl				2.0
137	CH_3		Cl			2.5s
138		NO_2	CH_3			4.8
141			$(CH_3)_2N$			3.0

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration of inhibitor giving 50 % inhibition of ferricyanide reduction. "s" following pI_{50} indicates doubts concerning the actual amount of inhibitor in solution.

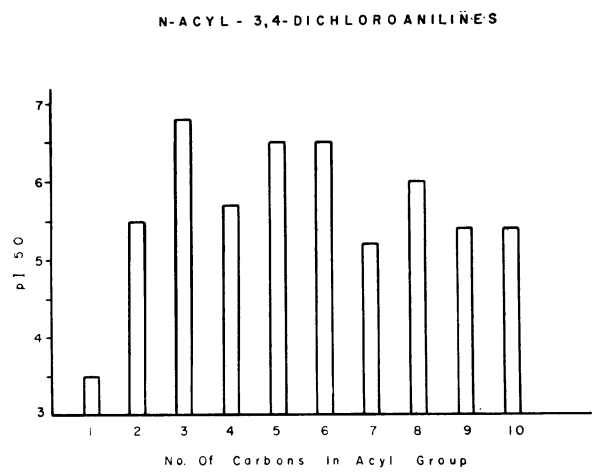


FIG. 1. The effectiveness of 3,4-dichloroanilides as inhibitors of the reduction of ferricyanide by illuminated chloroplasts. pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition. The abscissa numbers refer to the carbons in the normal acyl moiety.

which includes the valeryl (No. 78) and the caproyl (No. 81) derivatives, and a third lower maximum at the octanoyl derivative (No. 83). Branched or substituted aliphatic acids somewhat resemble the parent straight chain acid. Thus the methylpropionic (isobutyric) anilide (No. 75), like the propionyl derivative, is considerably more active than the butyric anilide (No. 74), while α-methylbutyric acid yields an anilide (No. 77) which is intermediate (perhaps because it is also α-ethylpropionic acid?). In this connection, an interesting substance is the 3,4-dichloroanilide of α-methylvaleric acid, the herbicide Karsil (No. 80). This is one of the most effective inhibitors of the Hill reaction described. It is conceivable that Karsil owes its unusual potency to the fact that α-methylvaleric acid is also α-butylpropionic acid; in a sense Karsil sits on both the first and second maxima shown in figure 1.

Replacing a methyl group in the aliphatic acid by a chlorine atom, despite the inevitable major rearrangement of electron densities, results in surprisingly little change in the activity of the anilide. The chloroacetanilide (No. 64) resembles the highly active propionyl derivative (No. 67), the anilide of β-chloropropionic acid (No. 71) is similar to the anilide of *n*-butyric acid (No. 74), and the α-chloropropionyl derivative (No. 68) has the same activity as the isobutyryl derivative (No. 75). On the average, however, the chloro- acids form 3,4-dichloroanilides of slightly lower activity than do their methyl counterparts. The effect of replacing a methyl group by a bromine atom seems to be similar since the bromacetanilide (No. 66) is also comparable to the propionic anilide in activity.

Polar groups sharply reduce the activity of the inhibitor. Thus, 3-(3,4-dichlorophenyl)-1-(2-hydroxyethyl)- and 3-(3,4-dichlorophenyl)-1, 1-bis(2-hydroxyethyl)- ureas (No. 109 & No. 116) are poor inhibitors. On the other hand, the dehydrated version of the latter compound, in which the two hydroxyl groups are replaced by a relatively non-polar ether bridge (No. 115), is 100 times more active than its polar counterpart. It is uncertain whether this inactivity of polar substances should be explained in terms of enzyme affinities or on the basis of partitioning and penetration; inactivity could result from the fact that such inhibitors fail to enter the lipid-rich chloroplasts in significant amounts.

The thiopropionyl-3,4-dichloraniline (No. 70) and the 3,4-dichlorophenyl-thioureas (No. 117 to No. 120) are from 300 to 2,000 times less effective than the oxygen analogs.

The important herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea or DCMU (No. 110) is the most inhibitory substance we have encountered. In this respect, the 3,4-dichloroanilides resemble the other anilides and alkylamids examined: the dimethylcarbamyl derivative is always the most active of the derivatives tested. However, other 3,4-dichloroanilides are also very good inhibitors; some of their activities approach that of DCMU. Among these are the corresponding monomethylurea (No. 102), the diethylurea (No. 111), the anilide of α-methylvaleric acid (Karsil, No. 80), the anilides of propionic (No. 67) and phenylacetic (No. 92) acids, and the piperidino compound (No. 114).

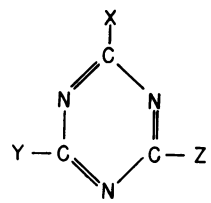
Part VIII. Anilides of isobutyric acid (table II). Isobutyric acid was chosen to contribute the common acyl moiety when a study of the influence of the amine moiety on inhibitor potency was undertaken. The reasons for this choice were the availability of isobutyric anhydride and the simplicity of its use in forming amides, the desirable crystal characteristics of most of the amides, and the moderately high activity of most of the isobutyranilides already tested.

The data, most of which are brought together in table II, may be summarized as follows: When chlorine atoms, bromine atoms, methoxy groups, or methyl groups replace the para- and meta-hydrogens of the aniline, there is a marked increase in activity. The effect is greatest with chlorine, slightly less with bromine and methoxy, and again slightly less with methyl. Meta-para disubstituted compounds are doubly activated while meta-meta compounds are not. On the other hand, any ortho substitution causes a striking reduction in activity. The adverse effect of an ortho-chloro can be overcome in part by adding a meta-para pair as in the 2,4,5-trichloranilide (No. 125). Surprisingly, two ortho-chloro atoms reduce the activity much less than one does. This may be seen by comparing the 2,4-dichloroanilide (No. 61) with the 2,4,6-trichloroanilide (No. 126). Moreland and Hill have observed a similar relationship between derivatives of 2-chloro- and 2,6-dichloroaniline (11). The same is not true of two ortho methyl groups; the 2-methylanilide (No. 128) and the 2,6-dimethylanilide (No. 142) are both exceedingly poor inhibitors. Nitro groups, either meta or para, reduce the activity by a small amount while the dimethylamino group in the para position almost abolishes the inhibitory ef-

TABLE III

TRIAZINES AS HILL REACTION INHIBITORS

GENERAL FORMULA



X	Y	Z	pI_{50}*	TRADE NAME
Cl	Ethylamino	Ethylamino	6.4	Simazine
Cl	Isopropylamino	Isopropylamino	6.3	Propazine
Cl	Ethylamino	Diethylamino	4.7	Trietazine
Cl	Isopropylamino	Diethylamino	4.9	Ipazine
Cl	Isopropylamino	Ethylamino	6.6	Atrazine
CH_3O-	Ethylamino	Ethylamino	5.7	Simetone
CH_3O-	Isopropylamino	Isopropylamino	5.8	Methoxypropazine

* pI_{50} is the $\log_{10}$ of the reciprocal of the molar concentration giving 50 % inhibition of ferricyanide reduction.

fect (No. 141). The 3,4-cycloalkyl-substituted anilide, N-isobutyryl-5,6,7,8-tetrahydro-2-naphthylamine (No. 140) is a good inhibitor as might have been predicted, and the 2,3-disubstituted anilide, N-isobutyryl-1-naphthylamine (No. 139) has the low activity which is almost always associated with ortho-substituted substances.

The steric and other effects of ortho substitution will be discussed below under the topic of hydrogen bond formation.

Part IX. Alkylamides & alkylureas. The greater part of this paper deals with acylanilides and phenylureas, partly for historical reasons and partly because they seem to be more active than any alkylamide or alkylurea yet tested. Indeed the amides formed from the smaller alkylamines, such as propylamine, ethylamine, and methylamine, are completely inactive. However, *n*-octylamine (No. 145) and cyclohexylamine (Nos. 143, 146) yield ureas and amides which are fair inhibitors. No derivatives of higher amines have been prepared to date, nor has the possibility of using branched or substituted aliphatic amines been investigated.

II. N-METHYLANILIDES & SULFONAMIDES. N-methyl-N-chloracetylaniline was the only secondary amide tested during this investigation. (Melting points; found 68 C, literature 70 C). It was a very poor inhibitor with a pI_{50} of 2.3. This agrees with the observation of other workers who tested other N-methylaniline derivatives (20, 11). Apparently an imino hydrogen is required for inhibition.

Several sulfonamides were prepared and tested. Two of these, the *p*-toluene-sulfonyl derivatives of *n*-octylamine (mp 55 C) and 3,4-dichloroaniline (mp 144 C), were fair inhibitors having pI_{50}'s of 4.5. Since no further tests were made on these compounds, it is not known if sulfonamides and the amides of carboxyacids inhibit the same biological processes.

TABLE IV

INHIBITIONS OF ATP FORMATION IN FMN & PMS CATALYSED SYSTEMS

No.	NAME	pI_{50} WITH FMN (SPECIFIC INHIBITION)	pI_{50} WITH PMS (UNSPECIFIC INHIBITION)
2	N-Chloracetylaniline	4.3	< 2.0
29	3-(4-Chlorophenyl)-1,1-dimethylurea (Monuron, CMU)	6.2	< 4.0
63	N-Acetyl-3,4-dichloroaniline	4.8	3.5
69	N-Propionyl-3,4-dichloroaniline	6.2	4.0
64	N-Chloracetyl-3,4-dichloroaniline	5.8	4.5
65	N-Trichloracetyl-3,4-dichloroaniline	6.3	4.8
80	N-(2-Methylvaleryl)-3,4-dichloroaniline (Karsil)	7.0	4.9
110	3-(3,4-Dichlorophenyl)-1,1-dimethylurea (Diuron, DCMU)	7.2	< 4.0
145	3-Octyl-1,1-dimethylurea	4.6	3.2
	2-Chloro-4,6-bis(ethylamino)-*s*-triazine (Simazine)	5.6	< 3.5
	2-Chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine (Atrazine)	6.2	< 3.5
	2-Chloro-4,5-bis(2-propylamino)-*s*-triazine (Propazine)	5.7	< 3.5

III. Herbicidal Triazines (table III). The most active triazine inhibitors are about as effective as the average 3,4-dichloroanilide and about 10 times less active than the best of the anilides. Not until a more diverse group of triazines has been examined will it be possible to establish reliable rules for predicting the potency of these inhibitors from their molecular structures. On the basis of the limited information provided here, two tentative generalizations are presented: Chlorine atoms are appreciably better than methoxy groups, and two imino hydrogens are very much better than one. It is interesting to speculate as to the significance of the common requirement for imino hydrogens in these apparently very different types of molecules—the triazines and the anilides.

Fig. 2

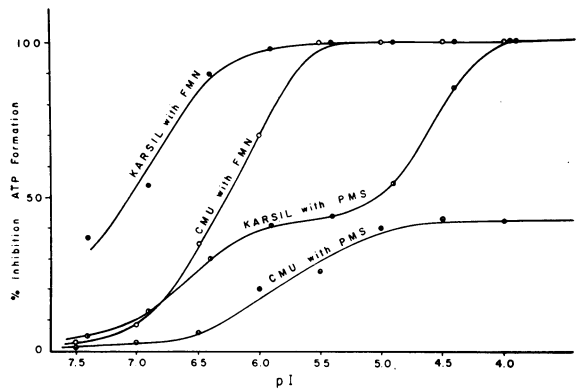


Fig. 3

Propazine

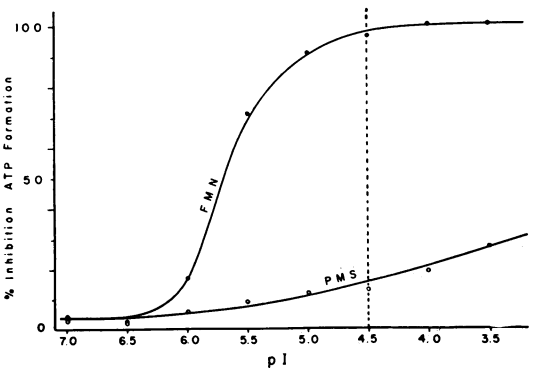


Fig. 2. The inhibition of photophosphorylation by the 3,4-dichloroanilide of α-methylvaleric acid (Karsil) and the 4-chloroanilide of dimethylcarbamic acid (CMU). Electron transport was mediated either by riboflavin-5-phosphate (FMN) or by N-methylphenazonium ion (PMS). pI is the $\log_{10}$ of the reciprocal of the inhibitor concentration.

Fig. 3. The inhibition of photophosphorylation by 2-chloro-4,6-bis(isopropylamino)-*s*-triazine (Propazine). Concentrations to the right of the dotted line represent supersaturated solutions. FMN, PMS, and pI have the same significance as in figure 2.

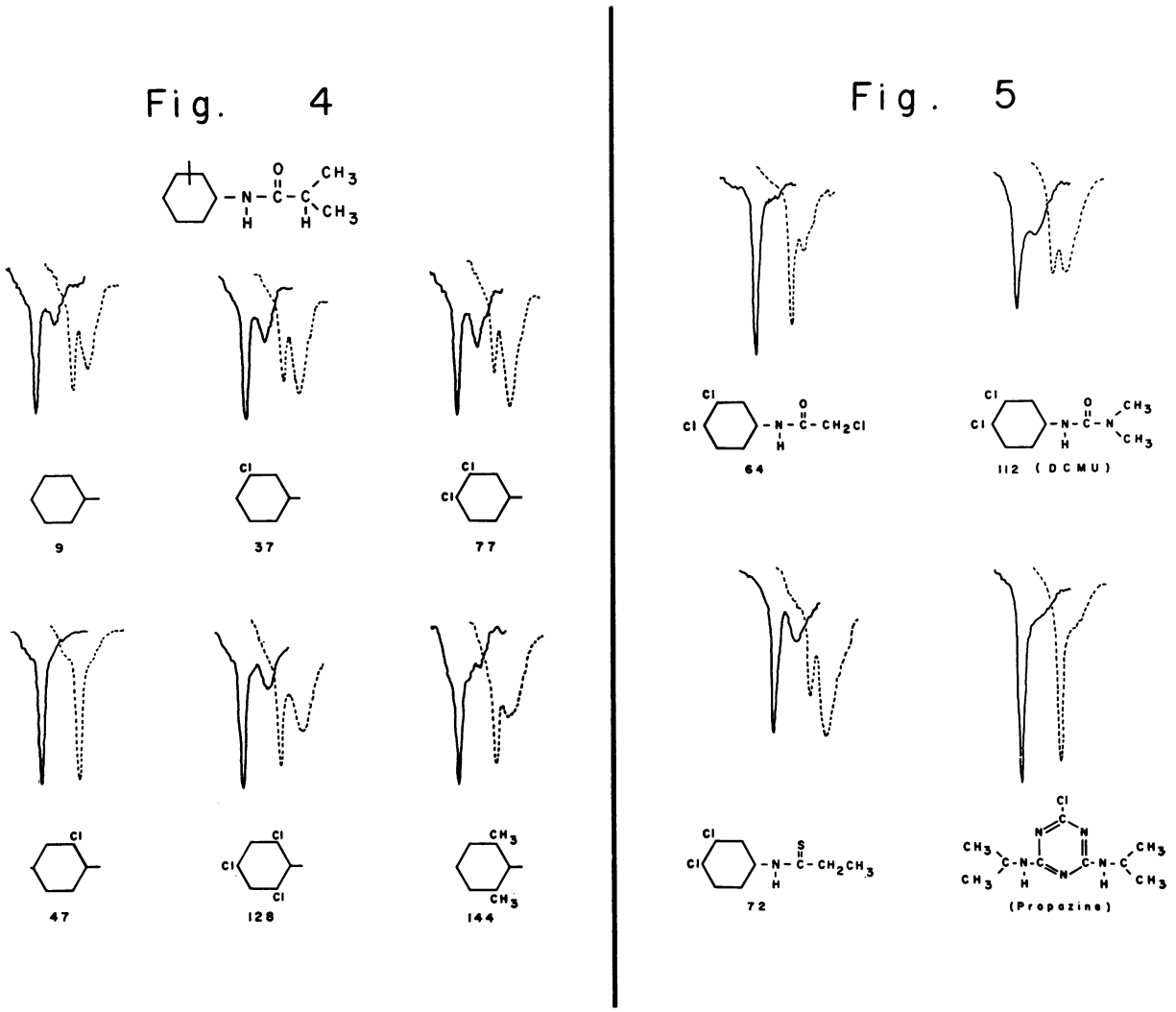
B. *Localization of Site of Inhibition.* (Figs 2 & 3 & table IV). It has been known for several years that CMU inhibits the formation of ATP by illuminated chloroplasts when either ferricyanide or FMN is the electron acceptor but not when *N*-methylphenazonium ions (PMS) are the acceptors. It was not surprising, therefore, to find that the chemically related acylanilides such as Karsil (No. 80) exhibited a similar behavior. Actually Karsil has two inhibitory functions, one requiring at least 100 times more inhibitor than the other. (See the bimodal Karsil curve in fig 2). Apparently the chloroplast preparation employed for figure 2, unlike most preparations, was unable to by-pass completely the oxygen-producing system in the presence of PMS. Consequently at very low Karsil concentrations all of the FMN-catalysed ATP formation and a part of the PMS-catalysed ATP formation were inhibited. With increasing inhibitor concentration no further increase in the inhibition of the PMS-catalysed system occurred until quite high concentrations had been reached. Probably the curve for CMU inhibition also would have risen again had it been possible to achieve the requisite high concentration. We shall refer to these two types of inhibition as specific (at low concentrations) and unspecific (at high concentrations).

In table IV is shown the specific and unspecific potency of several inhibitors. [The reader should be warned that the data on unspecific inhibition (pI_{50}'s with PMS as catalyst) are quantitatively unreliable because of the limited solubilities of the inhibitors: concentrations quoted are calculated on the basis of the amount of inhibitor added to the medium and ignore the possibility of saturation of the medium. This problem was particularly severe with the very insoluble DCMU & triazines.] If the Jagendorf criterion is a reliable guide to the nature of the process inhibited, we must conclude that each of the twelve inhibitors listed in table IV—and presumably most of the large number of inhibitors with which this paper is concerned—are primarily inhibitors of the mechanism of water oxidation. As already indicated, the chemical similarity of the phenylureas, phenylcarbamates, and acylanilides is such that a common mechanism of inhibition was not unexpected. However, the similar pattern of inhibition with the triazines was a surprise. Errors arising from the solubility problems mentioned above cannot have misled us since Simazine, Propazine (fig 3), and Atrazine inhibited ATP formation in the FMN system completely at concentrations which caused at most a few percent inhibition of the PMS system.

C. *Hydrogen Bond Formation* (Figs 4 & 5). The inhibiting substances described in this paper are very stable compounds. They react only with powerful chemical reagents and many of them resist degradation by the enzymes of plants and bacteria. Pre-

sumably, they are absorbed and held on a catalytic surface such as the active site of an enzyme by physical forces rather than by chemical reactions. [Note that phenylurethane, the classical narcotic or surface-blocking inhibitor of Warburg (18), belongs to the class of anilides with which we have been dealing. Indeed, it was one of the chloroplast inhibitors investigated by Wessels and van der Veen (20)]. A likely binding force, one able to hold substances such as these reasonably firmly, is the electrostatic attraction between the positive imino hydrogen atom of the inhibitor and the negative carbonyl oxygen of a polypeptide chain. With the amide and anilide inhibitors, this force could be augmented by the reciprocal attraction between the inhibitor's carbonyl oxygen and one of the protein's imino hydrogens.

In non-aqueous solutions of anilides, dimers and higher aggregates are produced, the imino hydrogen forming an electrostatic bond with the carbonyl of another like molecule. There is, therefore, an amount of bonding which depends on the concentration of the anilide, on the electron densities throughout the molecule which determine the electro-positivity of the hydrogen and the electro-negativity of the oxygen, and on steric considerations which may limit the accessibility of either the hydrogen or the oxygen. To determine the relative bonding potentials of the imino hydrogens of the various inhibitors without the complication of variations of the carbonyl oxygen, we added an excess of acetone to some of the solutions. With an excess of a common carbonyl compound present, differences in the amount of bonding should de-



FIGS. 4 & 5. The absorption spectra of various anilides and Propazine between 2.6 μ and 3.2 μ. The solid lines show the absorption of 0.67 M solutions in chloroform and the dotted lines show the absorption by the same substances when the chloroform solvent contained 5 % acetone. For clarity of illustration the dotted curves have been displaced to the right; actually the narrower peaks on the left of both the solid and dotted curves represent absorption by the N-H bond and are at the same wave length. The broader peaks to the right, which are invariably higher in the dotted acetone curves, represent absorption by the N-H . . . O bond.

pend exclusively on differences in the nature and position of the imino hydrogen. To investigate the characteristics of the imino hydrogens of triazines, which have no carbonyl oxygen of their own, it was obviously also necessary to add an outside source of electronegative groups such as acetone.

Hydrogen bonding was measured as a decrease in the N-H absorption in the region about 3,440 cm^{-1} and the appearance of N-H . . . O absorption about 3,340 cm^{-1}. Twenty-five acylanilides, thioacylanilides, phenylureas, phenylthioureas, and triazines were studied. The findings are summarized in the following generalizations:

I. When the acyl moiety is acetyl, propionyl, or isobutyryl, anilides form hydrogen bonds readily. This tendency is increased in both the 3- and 4-chloroanilides and is even greater in the 3,4- and 3,5-dichloroanilides. The increase in hydrogen bond formation is primarily due to changes in the hydrogen since the same increase occurs in the acetone-containing solvent. Para- and meta-methyl substitution of the aniline has a similar but much smaller effect. All these observations parallel the inhibition data and consequently support the hypothesis that hydrogen bonding plays an important role in the mechanism of inhibition.

II. Also in support of the hypothesis is the fact that the inactive 2-chloroanilides form hydrogen bonds neither between like molecules nor with acetone. This also applies to the 2,4-dichloroanilide (No. 61) and the 2,4,5-trichloroanilide (No. 125). Moreover, the 2,4,6-trichloroanilide (No. 126), which as we have noted, is an unexpectedly effective inhibitor, forms hydrogen bonds to about the same extent as the unsubstituted anilides. The inactive 2-methylanilide (No. 128) and the equally inactive 2,6-dimethylanilide (No. 142) do form hydrogen bonds, but rather reluctantly and the N-H . . . O absorption band is widened and shifted toward the N-H band.

III. Both 4-nitro (No. 133) and 4-dimethylamino (No. 141) anilides form hydrogen bonds but not as freely as the unsubstituted anilides; these substitutions also reduce inhibition, very markedly in the latter case.

IV. On the other hand, halogen substitution of the acyl moiety reduces the tendency to form N-H . . . O bonds without causing a corresponding diminution of inhibition potency. In chloroform solutions the highly active trichloracetyl-3,4-dichloroaniline (No. 65) gives only a very small peak at 3,340 cm^{-1} and the highly active chloracetyl-3,4-dichloroaniline (No. 64) gives none. Even in the acetone solvent the hydrogen bonding tendency is very much reduced.

V. The thioanalogs of acylanilides and of phenylureas have an absorption peak at about 3,340 cm^{-1}. In fact the spectra of the thio compounds and the corresponding oxygen compounds are indistinguishable in the region between 2.6 and 3.2. I was surprised, since sulfur atoms (as in H_2S) do not ordinarily form this type of bond with hydrogens. However, it is well known that the sulfur atoms in compounds such as thioureas and thioamides are exceptionally electronegative. The tendency of the imino hydrogen of thiopropionyl-3,4-dichloroaniline (No. 70) to form bonds with the carbonyl oxygen of acetone is apparently the same as in the oxygen anilides. (See fig 5).

VI. The N-H . . . O peak in the most powerful inhibitors of all, the phenyldimethylureas, is lower than in the acylanilides; both the N-H and N-H . . . O peaks are moved through about 30 wave numbers towards shorter wave lengths.

VII. The imino hydrogens in the triazines do not form bonds with the carbonyl oxygen of acetone to an appreciable extent.

Discussion

Structure & Activity. A variety of substances, including compounds with widely different molecular configurations, are powerful inhibitors of the Hill reaction. Apparently these inhibitors interfere with the same overall process since they prevent the reduction of ferricyanide and FMN by illuminated chloroplasts without seriously hindering PMS-catalysed photophosphorylation. The steric requirements for high activity follow a consistent pattern among the different classes of anilides and alkylamides and, therefore, there can be little doubt that these act at a common site. There is, however, no convincing reason for believing that the triazines must inhibit at the same site. Possibly several catalysed reactions not required in PMS-mediated phosphorylations are involved in the reduction of ferricyanide and FMN. Consequently, it is possible that the poisoning of any one of several catalysts (enzymes?) would explain my experimental results. On the other hand, if the general formula is broadened for the anilide and amide inhibitors, R-NH-CX-R′, to include not only X equals O but also X equals N-, the triazines may be accommodated; moreover the results of experiments employing combinations of phenylureas and triazine are consistent with a single site of inhibition (unpublished). In view of this uncertainty regarding the functional relationship of the triazines to the other inhibitors, the triazines will be disregarded in the following discussion of structure and activity.

Even among the anilides, compounds with quite different structures (e.g. DCMU & Karsil) have similar high activities. However, the apparent low level of specificity on the part of the inhibited catalyst can scarcely be a manifestation of a general low affinity; so few molecules of DCMU are present in an inhibited preparation that a considerable proportion of them must reach, and remain at, the sensitive sites. Although quite different classes of amide inhibitors may share the property of inhibiting at low concentrations, within any one class closely similar compounds may differ sharply. For instance, *N*-valeryl-3,4-dichloroaniline (No. 78) is 30 times more active

than N-isovaleryl-3,4-dichloroaniline (No. 79). Steric considerations seem to be more important in determining the effectiveness of these inhibitors than are factors affecting the distribution of electron densities. Thus chloroacyl-anilines closely resemble the corresponding methyl-substituted acyl compounds. Moreover, para-and meta-substitution of the aniline moiety are equivalent and are opposite in effect to ortho-substitutions; electronically the para and ortho substituted anilines are related and contrast with the meta. This preponderance of steric influences is consistent with a mechanism of inhibition in which the active site is physically obstructed; the important thing is that the inhibitor fit the enzyme well. The great chemical stability of these anilides and the reversibility of the inhibition caused by them (20) argue the same type of inhibition. Electron distributions are more pertinent to chemical reactivity and, therefore, may be expected to play a bigger part in determining the effectiveness of those inhibitors actually reacting with the catalytically active site, e.g., organophosphate inhibitors.

Almost the only feature common to all the Hill reaction inhibitors investigated is the presence of an imino hydrogen. Moreover, there is good reason to believe that this imino hydrogen must not only be present but also accessible if a substance is to have significant inhibitory action. In ortho-chloroanilides, the bulky acyl groups almost certainly lie in the plane of the benzene ring and remote from the also bulky chlorine atom. Such an orientation would force the imino hydrogen into close proximity with the chlorine atom where it would be shielded from interactions with adjacent molecules. Consequently ortho-chloroanilides may be expected to, and do, resemble N-methylanilides in having low melting points, low biological activities, and no hydrogen bonding. In contrast, the imino hydrogen of 2,6-dichloroanilines would be forced into a position away from the plane of the benzene ring and midway between the two chlorines. In this position the imino hydrogen should be, and apparently is, available for hydrogen bond formation and for whatever other functions may be involved in producing the inhibition.

If one were permitted to select his data, it would be possible to obtain a striking correlation between the activity of inhibitors and the tendencies of their imino hydrogen to form bonds with carbonyl oxygen atoms. Substitutions on the benzene ring of the anilides have completely parallel effects on these two properties. For instance, the series 3,4 > 3- and 4->2,4,6-> unsubstituted > 2- applies equally to the hydrogen bonding and the inhibitory action of the chloroanilides. Unfortunately, this beautiful correlation does not apply at all when the acyl moiety is varied. For example, the biologically active chloroacylanilides form hydrogen bonds most reluctantly, and the extremely potent phenyldimethylureas (DCMU, CMU, etc.) form hydrogen bonds much less freely than do the moderately active isobutyrylanilides and the weakly active acetylanilides. Since the author is inclined to follow Wessels in believing that hydrogen bonds should play an important role in anilide inhibitions, he finds these exceptions puzzling. Of course, in this study we may have presented the inhibitor molecules with inappropriate carbonyl oxygens; it may be that hydrogen bonding with an oxygen (or nitrogen?) in a very unusual situation is required if the catalytic site is to be blocked, and, therefore, the *general* bonding potential of the imino hydrogen may be of limited interest. The converse fact that many inactive or almost inactive substances readily form hydrogen bonds is irrelevant since obviously many requirements other than the capacity to form hydrogen bonds must be satisfied if a substance is to be a strong inhibitor.

Site of Action & Photosynthetic Unit. The Bishop-Jagendorf-Krall interpretation of phenylurea inhibition seems plausible in the light of our present knowledge of photosynthesis. Nevertheless, we would do well to remember that our information concerning the mechanisms of photophosphorylation and oxygen production is practically non-existent. Consequently the phosphorylation measurement described above cannot establish either with precision or with certainty the loci of the inhibitions. All that we can say with confidence is this: The anilides (which include phenylureas), the alkylamides, and the triazines have similar modes of action and nothing that we now know is inconsistent with the hypothesis that the mechanism for the oxidation of water to molecular oxygen is the process which is primarily affected.

Wessel's suggestion that the imino hydrogens of phenylureas form hydrogen bonds with the carbonyl oxygen of the cyclopentanone ring of chlorophyll (20), encounters two serious objections. In the first place such a mechanism of inhibition should equally affect photosynthesis, photoreduction, the Hill reaction with ferricyanide or FMN, and the Hill reaction with PMS. As we have seen, this is not the case. In the second place, the better inhibitors are much too effective. The number of moles of DCMU or Karsil required for nearly complete inhibition is smaller than the number of moles of chlorophyll present, by a factor of at least 100. Moreover, this factor of 100 does not allow for the fact that not all of the inhibitor can have reached the site of inhibition. Actually we have shown that at least two-thirds of the DCMU does not even leave the medium under the conditions of our experiments. (This was done both by varying the amount of chloroplast material & by preincubating the medium with chloroplasts which were subsequently removed & discarded). Since the chloroplasts do not remove all of the inhibitor from the medium it follows that the inhibitor-sensitive sites do not remove all of the inhibitor from the other parts of the chloroplasts. In fact, one would expect a higher concentration of inhibitor in the body of the chloroplast than in the medium in view of the solubility characteristics of DCMU and the lipoidal nature of the structural components of the plastids. Consequently, it must be concluded that an unknown but possibly quite small proportion of the inhibitor reaches

its target. This target cannot be chlorophyll unless we postulate one uniquely situated chlorophyll molecule among hundreds or perhaps among thousands. Since excitation energy certainly can be transferred from chlorophyll molecule to chlorophyll molecule many times, there is no reason why there might not be a few special chlorophyll molecules associated with enzymes or substrates to act as a trap for this mobile excitation energy, but it surely stretches credulity also to attribute unique bonding powers to the cyclopentanone carbonyl of these particular molecules.

Nevertheless, the above considerations suggest a possible relationship of the inhibited site to the photosynthetic unit. On the basis of kinetic data [flash saturation of photosynthesis (5), etc.] it has been postulated that a number of chlorophyll molecules collaborate in some way, funnelling their excitation energy or an intermediate chemical product of the excitation energy through a common channel. This photosynthetic unit may have a morphological basis or it may have a purely statistical basis in nature: according to Rabinowitch (16) "the now most plausible interpretation of these phenomena is in terms of an enzymatic component ordinarily present in concentrations 1/300 to 1/2400 that of chlorophyll". The interesting point is that the unit may consist of about 300 molecules of chlorophyll, and the number of units is, therefore, of the same order as the number of molecules of DCMU which we can reasonably expect to be at the inhibited sites when inhibition is practically complete. Unfortunately, the number of photosynthetic units is uncertain and estimates of the number of effective inhibitor molecules are necessarily very crude. Therefore, there is a high probability that the correspondence of the numbers is altogether fortuitous.

Herbicidal Activity & Mechanism of Killing. We are now experimenting to determine the effectiveness of these compounds as inhibitors of the in vivo process of photosynthesis. We are employing excised bean leaves according to the method of Minshall (14). Our preliminary results indicate that the effectiveness of the chemicals in the intact organ is only roughly correlated with in vitro potency. Thus, the thioureas are much too active in vivo and the better acylanilides are not nearly active enough. On the basis of analogy to the well known conversion of P = S to P = O in organophosphate metabolism, we suggest that the thioureas may be fairly rapidly transformed into their oxygen analogs in the plant. The unexpected low toxicity of the acylanilides could be explained in at least two ways: These are generally less polar substances and they may be preferentially absorbed by lipids, never reaching the sensitive site; alternatively the acylanilides may be hydrolysed enzymatically. If the activations of the thioureas and the inactivations of the acylanilides are indeed enzymic reactions, and if the enzymes involved are not universally distributed, there would seem to be some hope of discovering among the thioureas and acylanilides, herbicides with appreciable species specificity.

The herbicidal activity of the phenylcarbamates is not completely understood. It has long been known that these substances interfere with cell division, especially in grasses (4), and it was taken for granted that the plants were thereby killed. However, Moreland and Hill (11) have shown recently that most of the herbicidal phenylcarbamates also inhibit the Hill reaction and therefore they may inhibit photosynthesis. In fact both mechanisms may be operative and both may contribute to the death of the plant but the author is inclined to favor the original hypothesis for several reasons. Of these, the most compelling is the fact that isopropyl(2-chlorophenyl)-carbamate and isopropyl(2-methoxyphenol) carbamate are typical carbamate herbicides (17). From our work and the work of Moreland and Hill, it seems most improbable that such ortho-substituted anilides could be effective inhibitors of photosynthesis.

The manner in which the phenylureas, acylanilide, and triazine herbicides kill plants is not as obvious as one might think. Naturally, interference with the photosynthetic apparatus must ultimately lead to death of the plant through starvation. However, there is evidence that a more active process is involved since the phenylureas are toxic to sugar fed algae in the light but not in the dark (7). Since in this case there can be no question of starvation, it is presumed that deleterious oxidation processes accompany the accumulation of an oxidized photo-product, the photoproduct which would be reduced, in the absence of inhibition, by water.

Summary

I. A large number of substances having the general formula R-NH-CX-R′ were obtained commercially or prepared and identified. These included acylanilides, acylamides, thioacylanilides, phenylureas, alkylureas, and phenylthioureas.

II. A study was made of the effectiveness with which these and miscellaneous other related compounds inhibit the reduction of ferricyanide by illuminated chloroplasts. A similar study was made of the inhibitory activity of several herbicidal triazines. The observations made possible certain generalizations:

A. Substitution of the hydrogen on the 3-, 4- or 5- positions of the benzene ring of the aniline derivatives by Cl, Br, CH_3O, or CH_3 increases the inhibitory effect. Substitution of the ortho position practically abolishes inhibition. Other parts of the molecule being equal, the inhibitory activity of the chloroanilides follows this sequence: 3,4-> 3,5- and 3- and 4- > 2,4,6- > unsubstituted and 2,4,5- > 2,5- and 2,3 > 2-. Para- and meta-nitro groups reduce activity slightly and the *p*-dimethylamino group reduces activity sharply.

B. The effects of modifying the acyl moiety of the anilides are too complex to classify. Steric considerations seem to be paramount since the chloro acids and their methyl analogs yield anilides of comparable potency. Polar groups reduce activity.

C. Chloracetyl-N-methylaniline, an anilide which lacks an imino hydrogen atom, is scarcely inhibitory.

D. Among the triazines, the substances with two imino hydrogens are more inhibitory than those with one.

III. The acylanilides, phenylureas, and triazines, like CMU, inhibit chloroplast reactions when ferricyanide or FMN is the electron acceptor but not when PMS is the acceptor. This probably means that the mechanism inhibited by all of these chemicals is the mechanism normally responsible for the oxidation of water to molecular oxygen.

IV. Attempts to relate inhibitor potency to the bonding tendency of the imino hydrogen were only partially successful; the role of hydrogen bonding in the mechanism of inhibition remains uncertain. Substitutions on the aniline moiety which favor hydrogen bond formation also increase the effectiveness of the inhibitor. However, modification of the acyl moiety did not reveal a similar correlation; the imino hydrogens of chloracetyl- and trichloracetyl-3,4-dichloroaniline form bonds with carbonyl oxygens to a very limited extent although both substances are excellent inhibitors. The same is true of the triazines.

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