fraction 3 would act as resistance factors in inhibiting insect feeding.

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# Development of *s*-Triazine Anticytokinins and Their Quantitative Structure–Activity Relationship

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A new, non-adenylate series of anticytokinins, $N^2$-substituted 2-amino-4-chloro-6-(ethylamino)-*s*-triazines, has been developed. The activity in terms of the $I_{50}$ value of the most potent members was (0.3–0.5) $\times 10^{-6}$ M when examined by the tobacco (*Nicotiana tabacum* L.) callus assay in the presence of 0.05 $\times 10^{-6}$ M kinetin. The design of the molecule was made on the basis of insight into the active structure obtained from a cytokinin receptor map drawn previously. Quantitative analysis of their structure–activity relationship showed that the mode of their binding to the receptor was in important ways the same as for previously known anticytokinins, the structure of which resembles the adenylate cytokinins.

Five structural classes of anticytokinins, 7-substituted 3-methylpyrazolo[4,3-*d*]pyrimidines (Hecht el al., 1971; Skoog et al., 1973), 4-substituted 7-(β-D-ribofuranosyl)-(Iwamura et al., 1974, 1975), 4-substituted 2-methyl-(Iwamura et al., 1979a), and 4-substituted 2-(methylthio)pyrrolo[2,3-*d*]pyrimidines (Skoog et al., 1975), and 4-substituted 2-(methylthio)pyrido[2,3-*d*]pyrimidines (Iwamura et al., 1979b), have been developed in the past 15 years, and they have been used to study the biochemical mechanisms of their agonists, cytokinins (Hamaguchi et al., 1985; Iwamura et al., 1979a; Skoog et al., 1973; Tanimoto and Harada, 1982–1984, 1986). All of them are immediately similar in structure to a naturally occurring class of cytokinins, $N^6$-substituted adenines. Anticytokinins with a structural resemblance to diphenylureas, another class of cytokinins, or a nonadenylate structure, are not yet known.

The quantitative analysis of the structure-activity relationships of $N^6$-substituted adenine and diphenylurea cytokinins and anticytokinin-active pyrrolo- and pyrido-[2,3-*d*]pyrimidines has helped us to draw a cytokinin receptor map (Iwamura et al., 1980, 1983, 1985) that shows visually the framework of the receptor or receptor cavity into which an active compound should be accommodated and also the difference in the binding modes of agonists and antagonists. We selected from among all *s*-triazine structures non-adenylate candidates that may fit the receptor and exhibit activity, we hope an antagonistic one. The structures of $N^2$-substituted 2-amino-4-chloro-6-(ethylamino)-*s*-triazines thus prepared and tested are shown in Figure 1, together with the cytokinins and anticytokinins mentioned above. Figure 2 reproduces the receptor map.

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MATERIALS AND METHODS

**Chemicals.** The preparation of compounds **1–6**, **8**, **10–14**, and **16–18** was previously reported (Mitsutake et al. 1986). Triazines **7**, **9**, and **19** were synthesized by the reaction of 2,6-dichloro-4-(ethylamino)-*s*-triazine (Thurston et al., 1951) with *n*-decyl-, 2-methoxyethyl-, and 4-phenylbenzylamines, respectively, in the presence of $NaHCO_3$ in water at about 50 °C. $N^2$-(*n*-Decyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**7**): mp 162–163 °C. Anal. Calcd for $C_{15}H_{28}N_5Cl$: C, 57.40; H, 8.99; N, 22.31. Found: C, 57.60; H, 9.22; N, 22.59. $N^2$-(2-Methoxyethyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**9**): mp 172 °C. Anal. Calcd for $C_8H_{14}N_5OCl$: C, 41.47; H, 6.09; N, 30.23. Found: C, 41.71; H, 6.23; N, 30.27. $N^2$-(4-phenylbenzyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**19**): mp 240 °C. Anal. Calcd. for $C_{18}H_{18}N_5Cl$: C, 63.62; H, 5.34; N, 20.61. Found: C, 63.82; H, 5.19; N, 20.39. $N^2$-Phenyl-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (**15**) was prepared by the reaction of ethylamine with 2,6-dichloro-4-anilino-*s*-triazine produced from cyanuric chloride

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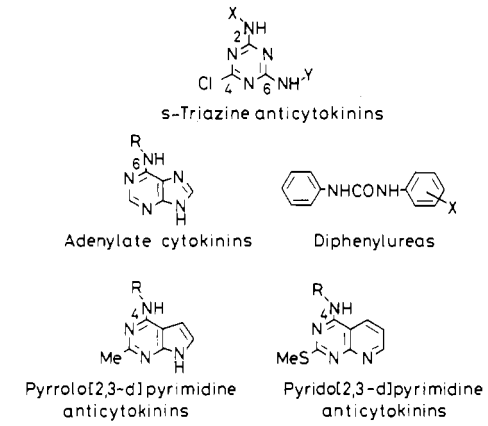



**Figure 1.** Structures of *s*-triazine derivatives and cytokinin- and anticytokinin-active compounds.

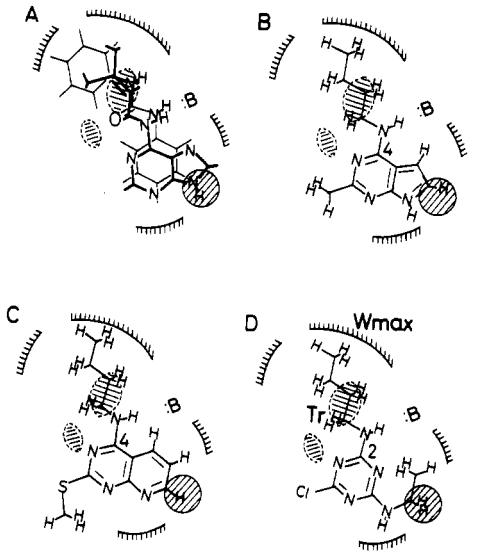



**Figure 2.** Cytokinin receptor map showing the complexes with active compounds. The solid lines with a fringe represent the steric interaction sites or receptor walls located on the page plane, and the ovals are those located above or below it. The shaded circle shows the hydrophobic region, and :B shows the electronic interaction site or the basic group of the receptor. Key: A, cytokinin-active 6-(3-methyl-2-butenylamino)purine ($N^6$-isopentenyladenine, bold lines) and *N,N'*-diphenylurea (light lines); B, anticytokinin-active 4-(*n*-butylamino)-2-methylpyrrolo[2,3-*d*]pyrimidine; C, anticytokinin-active 4-(*n*-butylamino)pyrido-[2,3-*d*]pyrimidine; D, anticytokinin-active $N^2$-(*n*-butyl)-2-amino-4-chloro-6-(ethylamino)-*s*-triazine (4). In A, the ends of the bars of the structures represent hydrogen atoms and the double bonds of the aromatic rings are omitted. C was reproduced with permission from Iwamura et al. (1985). Copyright 1985 The American Chemical Society.

and aniline (Thurston et al., 1951); mp 209–210 °C. Anal. Calcd. for $C_{11}H_{12}N_5Cl$: C, 52.91; H, 4.84; N, 28.05. Found: 53.18; H, 4.78; N, 27.79.

**Tobacco Callus Test.** Compounds to be tested were added in different concentrations ((0–4) × $10^{-5}$ M) with 0.05 × $10^{-6}$ M kinetin to the basal medium as specified previously (Linsmaier and Skoog, 1965). The medium was adjusted to pH 5.6 with 1 N NaOH and the medium autoclaved at 1.0 kg/cm$^2$ for 15 min. Three callus pieces of about 10 mg fresh weight derived from *Nicotiana tabacum* L. var Wisconsin No. 38 were implanted on the agar surface and maintained at 28 °C in darkness for 4 weeks; then the average fresh weight was found. The range of experimental error was within ±30%. The anticytokinin activity was expressed by the $I_{50}$ value, which is the molar concentration at which is obtained 50% of the callus growth on the medium with 0.05 × $10^{-6}$ M kinetin but without test compounds.

***Brassica* Growth Test.** A commercial cotton pad was cut, stuffed into a glass tube (3-cm diameter × 12 cm height), moistened with 2 mL of sample solution, and autoclaved at 120 °C for 15 min. The surface of the pad was then sown with five seeds of Chinese cabbage (*Brassica rapa* L. var Komatsuna) that had been surface-sterilized by being soaked in about 2.5% sodium hypochlorite solution for 30 min and washed several times with sterilized distilled water. The sample solution contained less than 0.3% of $Me_2SO$ into which the test compounds (**11** and **12**) were dissolved. There was little effect on plant growth of 0.3% $Me_2SO$ compared with growth without the organic solvent. The concentrations of the test compounds used were 1 × $10^{-5}$ and 4 × $10^{-5}$ M; the compounds were sparingly soluble at higher concentrations. In each run of experiments, at least three replicas of the tubes were used for a given concentration of a compound. The fresh weight was found after incubation for 8 days at 28 °C in darkness. The experiments were repeated three times, and the results were averaged and reported by percentages of the control growth.

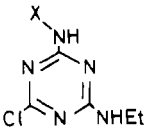
## RESULTS

**Design and Anticytokinin Activity.** Figure 2 reproduces the receptor map drawn after quantitative structure–activity analyses of adenylate and urea cytokinins (Iwamura et al., 1980) and adenylate anticytokinins (Iwamura et al., 1983, 1985). The solid lines with a fringe represent the steric interaction sites or receptor walls located on the page plane, and the ovals are those located above or below it. The shaded circle shows the hydrophobic region and :B shows the electronic interaction site or the basic group of the receptor.

The analysis of cytokinins suggests that the imino group of adenines and that in the urea bridge of diphenylureas interact with a common basic site (:B) of the receptor. A hydrophobic substituent enhances cytokinin activity when it is located at a meta position of diphenylureas, whereas such a hydrophobic effect is not observed for $N^6$-substituent of adenines. Thus, the base moiety, rather than the side chain moiety, of adenines is considered to occupy the region where the hydrophobic site exists. Moreover, when one benzene ring of diphenylureas is replaced by various alkyls, their steric requirement bears analogy to that of $N^6$-substituents of adenines. Based on these, the structural correspondence at the site of action of the chemically different classes of compounds is thought to be as shown in Figure 2A. In anticytokinin-active 4-substituted pyrrolo- and pyrido[2,3-*d*]pyrimidines shown in Figure 2B, C, the steric requirement for 4-substituents is more strict than that for the $N^6$-groups of adenines. The $N^4$-substituents are thus considered to be located close to the receptor wall that faces them. In other words, the arrangement of the molecules on the receptor is thought to be somewhat different between the agonists and antagonists. Parts B and C of Figure 2 were drawn so as to satisfy this condition, but not to spoil the plausible electronic interaction between the $N^4$-imino group and the basic site of the receptor.

On the basis of the above information, we considered $N^2$-substituted 2-amino-4-chloro-6-(ethylamino)-*s*-triazines to be the compounds that could fit the receptor in a manner similar to that for the adenylate anticytokinins. The situation is shown schematically in Figure 2D, $N^2$-substituent locating at the site where the 4-substituent of adenylate compounds comes on and 2-ethylamino group being at the site where the pyrrole or pyridine moiety occupies.

**Table I. Structure, Activity, and Steric Parameters of *s*-Triazines**



| no. | X | anticytokinin act. $I_{50}$, $10^{-6}$ M | anticytokinin act. $pI_{50}$ obsd | anticytokinin act. $pI_{50}$ calcd[a] | $\Delta pI_{50}$ | steric parameters $W_{max}$, Å | steric parameters $T_r$, Å |
|---|---|---|---|---|---|---|---|
| 1 | Et | 8.91 | 5.05 | 5.04 | 0.01 | 2.97 | 3.80 |
| 2 | *n*-Pr | 5.25 | 5.28 | 5.63 | −0.35 | 3.49 | 3.80 |
| 3 | *i*-Pr | 1.10 | 5.96 | 5.94 | 0.02 | 3.17 | 5.05 |
| 4 | *n*-Bu | 0.44 | 6.36 | 5.92 | 0.44 | 4.54 | 3.80 |
| 5 | *i*-Bu | 1.05 | 5.98 | 5.94 | 0.04 | 4.45 | 3.80 |
| 6 | *n*-amyl | 5.13 | 5.29 | 5.71 | −0.42 | 4.94 | 3.80 |
| 7[b] | *n*-decyl | >39.8 | <4.40 | −5.22 | | 8.78 | 3.80 |
| 8 | $CH(Me)(CH_2)_4CH_3$ | 17.4 | 4.76 | 5.02 | −0.26 | 5.96 | 5.05 |
| 9 | $CH_2CH_2OCH_3$ | 2.00 | 5.70 | 5.95 | −0.25 | 4.40 | 3.80 |
| 10 | c-Pr | 1.29 | 5.89 | 5.75 | 0.14 | 3.24 | 4.51 |
| 11 | c-Bu | 0.29 | 6.54 | 6.47 | 0.07 | 3.82 | 5.01 |
| 12 | c-Hx | 0.47 | 6.33 | 6.27 | 0.06 | 3.49 | 5.05 |
| 13 | $CH_2$-c-Pr | 0.47 | 6.35 | 6.31 | 0.04 | 4.43 | 4.51 |
| 14 | $CH_2$-c-Hx | 4.79 | 5.32 | 4.88 | 0.44 | 5.67 | 3.80 |
| 15[b] | Ph | 1.91 | 5.72 | 5.02 | 0.70 | 3.11 | 3.40 |
| 16[b] | $CH_2CH_2Ph$ | >3.98 | <5.40 | 5.91 | | 4.57 | 3.80 |
| 17[b] | $CH_2(CH_2)_2Ph$ | >3.98 | <5.40 | 0.33 | | 7.47 | 3.80 |
| 18[b] | $CH_2(CH_2)_3Ph$ | >3.98 | <5.40 | 4.27 | | 6.02 | 3.80 |
| 19[b] | $CH_2C_6H_4$-*p*-Ph | >39.8 | <4.40 | −12.0 | | 9.99 | 3.80 |

[a] Calculated by eq 1. [b] Compounds not included in the analysis but their activity predicted by eq 1.

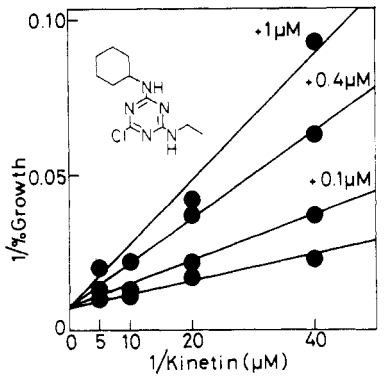



**Figure 3.** Reciprocal of the growth rate of tobacco callus plotted as a function of the reciprocal of the concentration of kinetin alone (bottom line) and in the presence of compound **12**.

The structures of *s*-triazines thus prepared are summarized in Table I, together with the assay results, $I_{50}$ (M) values and their negative logarithmic values, $pI_{50}$. Most potent were *n*-butyl (**4**), cyclobutyl (**11**), cyclohexyl (**12**), and cyclopropylmethyl (**13**) compounds, the $I_{50}$ (M) being $(0.3–0.5) \times 10^{-6}$ M ($pI_{50} \approx 6.4$). The compounds with a larger or smaller substituent had weaker activity, suggesting the participation of steric factors in fitting to the receptor.

To examine their competitive, antagonistic nature, we applied the method of Lineweaver and Burk (1934), which has been used to confirm an antiauxin characteristic (McRae and Bonner, 1953) as well as the anticytokinin nature of pyrrolo- and pyrido[2,3-*d*]pyrimidine derivatives (Iwamura et al., 1979, 1983). The result of the treatment on compound **12**, one of the highest active members of the class, is shown in Figure 3, where the reciprocal of the growth response was plotted against the reciprocal of the concentration of added kinetin. That the resultant set of straight lines have a common intercept suggests that the *s*-triazines share the site of action with the cytokinins. Although this treatment was not carried out on all compounds, the results strongly suggest the antagonistic nature of the series of compounds, together with the following fact that the structure–activity relationship of the *s*-triazines is expressed by a single, common eq 1.

**Table II. Effects of *s*-Triazines on the Growth of Chinese Cabbage Seedlings in Darkness**

| no. | concn, $10^{-5}$ M | germin rate, % | dec in fr wt, % |
|---|---|---|---|
| 11 | 1 | 96.0 (±3.7)[a] | 18.7 (±7.6)[a] |
| | 4 | 99.0 (±2.0) | 25.5 (±3.9) |
| 12 | 1 | 94.3 (±7.8) | 15.3 (±7.9) |
| | 4 | 98.3 (±2.7) | 20.5 (±5.6) |
| control | | 97.9 (±5.7) | 0.0 |

[a] Mean (± SD).

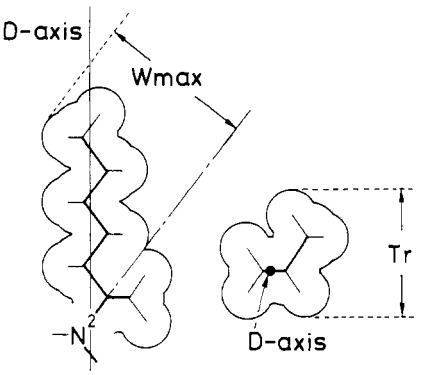



**Figure 4.** Schematic representation of the steric parameters for $N^2$-alkyl substituents of *s*-triazines. The ends of the bars of the molecular models represent hydrogen atoms.

Appropriately substituted *s*-triazines are herbicidal. The activity is believed to be due to the inhibition of the photosystem II electron flow in chloroplasts, and most of the compounds listed in Table I have this activity to some extent (Mitsutake et al., 1986). In herbicidal action or against intact plants, however, the anticytokinin characteristic may participate, especially in preemergent treatment. We examined the effect of compounds **11** and **12**, the highest and next highest active members in the tobacco test, on the germination and growth of Chinese cabbage (*B. rapa* L. var Komatsuna) in darkness. Table II summarizes the results, showing that the effects on germination are slight, and those of growth later are not so strong as to be herbicidal. The anticytokinin characteristic probably is not immediately involved in the killing of plants.

**Structure–Activity Relationship.** Quantitative analysis was done of the *s*-triazines listed in Table I to identify the structural conditions common to the present and previous series of anticytokinins.

*Steric Parameters.* In analyses of pyrrolo- and pyrido[2,3-*d*]pyrimidine anticytokinins (Iwamura et al., 1979a,b, 1983, 1985), we found that the size, especially the maximum width, of their $N^4$-substituents is important in binding to the cytokinin receptor as well as in the expression of the anticytokinin activity. On the basis of the insight that the same or similar steric factor(s) is operative at the structurally various $N^2$-sites of the *s*-triazine derivatives as well, we defined the steric parameters as shown in Figure 4. The $W_{max}$ is the maximum width of the $N^2$-substituents measured from the bond axis in the direction in which the longest chain extends in the fully extended (staggered) conformation. To express the bulkiness of the upward or downward direction (or both), we first defined the *D*-axis that bisects the zigzag main chain and then measured the thickness of the right-hand part of the substituents, $T_r$, in the direction perpendicular to the zigzag plane. Similarly, the thickness of the left-

hand part was defined. It was, however, not significant in the regression analysis of these compounds and thus not indicated in the figure. These dimensional parameters were calculated with a computer program based on the partial bond lengths (covalent radii), bond angles, and van der Waals radii of the Corey–Pauling–Koltun molecular models (Asao and Iwamura, 1985). The values were expressed in angstroms and are listed in Table I.

*Analysis.* Equation 1 is the result obtained for compounds **1–6** and **8–14** with $N^2$-alkyl substituents. In the equation, $n$ is the number of the compounds analyzed, $s$ is the standard deviation, and $r$ is the correlation coefficient. The figures in parentheses are the 95% confidence intervals.

$$pI_{50} = \underset{(\pm 2.23)}{4.68}W_{max} - \underset{(\pm 0.25)}{0.55}(W_{max})^2 + \underset{(\pm 0.37)}{0.51}T_r - \underset{(\pm 5.51)}{5.96} \quad (1)$$

$$n = 13,\ s = 0.31,\ r = 0.88$$

The significance of both the $W_{max}$ and its squared term means that the activity is parabolically related to the $W_{max}$ and that there is an optimal steric condition for activity, the value being calculated to be about 4.3 Å. The $T_r$ term with a positive coefficient suggests that the thickness of the right-hand side of the $N^2$-substituents is favorable for activity. The squared correlation coefficient between the $W_{max}$ and $T_r$ is 0.02, indicating the independence of the variables in the correlation.

The hydrophobicity in terms of the logarithm of the partition coefficient between 1-octanol/water, log $P$, was not significant, suggesting that the permeability to the site of action was not so important for this set of compounds. The electronic property of the $N^2$-substituents was also insignificant, perhaps because of its slight variation in alkyls. The phenyl derivative **15** was not included in the analysis, since its $pI_{50}$ value always deviated from the calculated one through analyses and since the mode of steric interactions is somewhat different for the phenyl and alkyl substituents in analyses of adenylate anticytokinins (Iwamura et al., 1983, 1985). Equation 1 predicts considerable potency for compounds **16** and **18**, as shown by Table I, but their activity could not be measured exactly because of their sparing solubility. Compound **17** was also sparingly soluble. compounds **7** and **19** had little activity even at $4 \times 10^{-5}$ M; this can be explained by the set of parameters in eq 1, with the predicted $pI_{50}$ values being below −5 and −12, respectively.

## DISCUSSION

The *s*-triazines presented in this paper were active as cytokinin antagonists (anticytokinins) in the tobacco callus assay. The significance of the $W_{max}$ and its squared term in eq 1 suggests that a spatial wall exists in the $W_{max}$ direction about 4.3 Å (the optimum value) apart from the $N^2$-bond axis. Similarly, the $T_r$ term in eq 1 suggests that there is a wall located upward (or downward). The suffixes $W_{max}$ and $T_r$ in Figure 2D indicate these sites, and the results show that the steric features important for activity of *s*-triazines are shared with those of other classes of anticytokinins. Although eq 1 did not make clear the electronic effect of the substituents for this set of compounds, the drawings suggest that the $N^2$-hydrogen atom may interact with the basic group :B. The 6-ethylamino group is thought to have a resemblance to the pyrrole or pyridine moiety of the adenylate compounds with respect to the receptor fit.

The activity in tobacco callus test of the present *s*-triazines is as high as that of pyrido[2,3-*d*]pyrimidine derivatives (Iwamura et al., 1979b, 1985) but a few times less than that of pyrrolo[2,3-*d*]pyrimidine compounds (Iwamura et al., 1979a, 1983), the most potent class of anticytokinins known so far. The optimization of the structure within the congeneric series is under way, as well as efforts to develop other new structures within the framework indicated by Figure 2. Some of the present compounds have novel flower-inducing activity in *Asparagus officinalis* L. (Abe et al., 1987), although how this activity is related to anticytokinin properties is presently not known. Active compounds with a new skeletal structure such as the compounds presented here may act differently in species other than tobacco.

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# Applicator Exposure to Fluvalinate, Chlorpyrifos, Captan, and Chlorothalonil in Florida Ornamentals[1]

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The exposure of a tractor driver applying pesticides to Florida ornamentals was assessed. The chemicals applied were fluvalinate, chlorpyrifos, captan, and chlorothalonil. Total-body exposure rates, estimated from external exposure pads, were low. Exposure rates followed application rates and were larger when the applicator pulled a boom sprayer than when he pulled a span sprayer. Pesticide on the hands of the ungloved applicator and air samples from his breathing zone were monitored. No significant difference between exposure to the right and left hands was found. The distribution of pesticide on the applicator depended strongly on which spraying device was used. Except for chlorothalonil, tank mixture samples were about 50% weaker in pesticide concentration than would be expected on the basis of complete mixing.

The pesticide exposure of greenhouse applicators is a current regulatory interest of the U.S. Environmental Protection Agency (U.S. EPA). The U.S. EPA is specifically faced with the task (1) of assessing the pesticide exposure of greenhouse applicators and (2) for pesticide label requirements, suggesting protective clothing that is both effective and comfortable. This study is a first step toward providing the data necessary for these evaluations.

The questions addressed by this study were as follows: (1) What was the *potential* for dermal exposure to the applicator; i.e., at what rate did pesticide accumulate on the body (excluding hands) of the applicator, unprotected by clothing of any kind? We term this estimated total-body accumulation rate (ETBAR) and measure it in micrograms per hour. Also, did the ETBAR depend upon the rate of pesticide leaving the spray nozzles, the kind of pesticide applied, and/or the method of application? (2) How was the ETBAR distributed over the anatomy of the applicator, and upon what factors did this distribution depend? (3) What was the accumulation rate of pesticide on the hands of the applicator? Was there a relationship between worker hand preference and exposure to the right and left hands? Did hand exposure depend upon the pesticide effluent rate, compound applied, and/or application method? (4) What was the atmospheric contamination from the pesticide in the breathing zone of the worker as he applied the compound? Did it depend upon the compound type, its effluent rate, or the application method? (5) How did samples of the spray mixture, taken pre- and postapplication, compare in pesticide concentration with that presumed to exist in the tank based on the tank mixture, the pesticide label, and an assumption of thorough mixing?

## MATERIALS AND METHODS

**Study Site.** The study was conducted in 1985 at a commercial greenhouse facility at Cortez, FL, devoted primarily to growing chrysanthemums. The subject monitored was a tractor driver who pulled either a boom sprayer or a span sprayer. The chemicals applied were fluvalinate, chlorpyrifos, captan, and chlorothalonil (Table I), usually in some combination.

**Experimental Subject.** The subject chosen for this study was a 30-year-old male, height 173 cm, weight 54 kg, who was left-handed. His estimated body surface area was 1.62 $m^2$ (Gehan and George, 1970). He was instructed to follow his normal application procedure and wore no gloves, coveralls, boots, etc., but did wear a respirator. His outer clothing consisted of a short-sleeved cotton work shirt, denim trousers, and leather shoes.

**Application Method.** The subject drove a tractor that usually pulled a hydraulic boom sprayer. However, because of equipment failure, he pulled a span sprayer on Aug 14, 1985. With a span sprayer, the spray mixture is

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