involving alternate ionization pathways is used then the significance of the observed rate constant for the breakdown of the enzyme-substrate complex is altered. In fact the observed rate constant equals the true rate constant multiplied by the factor K_A/K_1 and incorporation of this in equation (12) makes it identical with Alberty & Massey's formula. Hence their procedure for calculating K_1 and K_2 from the pH dependence of V is valid on either model of enzyme activity.

SUMMARY

1. The theory of kinetics of the reaction between chloroacetamide and amino thiols developed by Lindley (1960) has been further investigated.

2. The reaction between four cysteine peptides and chloroacetamide has been studied experimentally.

3. For three of the peptides it appears probable that $pK_{SH} > pK_{NH_3^+}$; for the fourth peptide, glutathione, $pK_{NH_3^+} > pK_{SH}$.

4. Some general aspects of the determination of pK of complex ions by kinetic methods are discussed and their relevance to some aspects of enzyme kinetics is pointed out.

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The Relationship of Structure to Activity in Plant-Growth-Regulating Compounds

2. GROWTH-REGULATING ACTIVITY OF SUBSTITUTED AMIDES OF 2,4-DICHLOROPHENOXYACETIC ACID*

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During the course of an investigation on the plant-growth-regulating activities of a number of derivatives of 2,4-dichlorophenoxyacetic acid, considerable differences in activity were shown by closely related substituted amides of this acid. Since these differences in activity could not be accounted for by known factors, the scope of the investigation was widened to gain further information on the mode of action of the amides of plant-growth-regulating acids.

There does not appear to be agreement among workers in this field as to whether the amides of auxin acids are active auxins as such or whether hydrolysis to the acid is necessary before activity is shown. In an attempt to answer this question the rates of hydrolysis of a number of these amides were determined in alkaline solution (Johnston, 1961).

* Part 1: Johnston (1961).

The auxin activities of these compounds have now been determined in order to find out whether any relationship exists between the activities of the amides and their chemical stabilities or any other chemical properties which can be related to their stabilities.

EXPERIMENTAL

The activities of the amides were determined by comparing their physiological effects on intact growing plants with that of 2,4-dichlorophenoxyacetic acid as a reference compound.

Test plants. Haricot beans were grown in normal soil in cardboard cartons approximately 2½ in. diam., two or three plants/carton. The plants were grown under standardized conditions and only healthy plants of the same size, age and stage of development were used. They were used when the first two leaves were expanding.

Test solutions. The compounds to be tested were dissolved in a mixture of methylcyclohexanone (80 parts by

Table 1. *Auxin activities of N-substituted amides of 2,4-dichlorophenoxyacetic acid compared with their rates of hydrolysis in 0.22N-potassium hydroxide in 90 % (v/v) ethanol*

Compound no.	$\text{Cl}_2\text{C}_6\text{H}_3 \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NR} \cdot \text{R}''$		$t_{\frac{1}{2}}$ (50°) (min.)	Auxin activity (2,4-dichlorophenoxyacetic acid = 100)
	R'	R''		
I	-H	-H	50	98 ± 7
II	-H	-CH ₃	330	65 ± 8
III	-H	-C ₂ H ₅	710	39 ± 4
IV	-H	-CH(CH ₃) ₂	5800	41 ± 4.5
V	-H	-CH ₂ ·CH ₂ ·OH	220	2 ± 3.5
VI	-H	-C ₆ H ₅	260	84 ± 8.5
VII	-H	-C ₆ H ₄ ·CH ₃ (<i>p</i> -)	330	63 ± 6
VIII	-H	-C ₆ H ₄ ·NO ₂ (<i>p</i> -)	< 20	3 ± 3
IX	-H	-C ₆ H ₄ ·NO ₂ (<i>m</i> -)	70	5 ± 4
X	-H	-C ₆ H ₄ ·NH ₂ (<i>p</i> -)	250	4 ± 2.5
XI	-H	-C ₆ H ₄ ·NH ₂ (<i>m</i> -)	185	6 ± 2
XII	-H	-C ₆ H ₄ ·Cl (<i>p</i> -)	190	61 ± 6
XIII	-H	-C ₆ H ₃ Cl ₂ (3,4-)	100	41 ± 7.5
XIV	-CH ₃	-CH ₃	50	8 ± 2
XV	-CH ₂ ·CH ₂ ·OH	-CH ₂ ·CH ₂ ·OH	< 20	5 ± 4
XVI	-CH ₃	-C ₆ H ₅	130	37 ± 5.5
XVII	-C ₆ H ₅	-C ₆ H ₅	30 (40°)	4 ± 4
XVIII	-H	-C ₆ H ₄ ·NH·CO·CH ₃ (<i>p</i> -)	—	45 ± 1.5
XIX	-H	-C ₆ H ₄ ·NH·CO·CH ₃ (<i>m</i> -)	—	73 ± 9

weight) and an emulsifying agent (20 parts by weight) to give stock solutions containing 5% acid equivalent (0.226M) or 1.25% acid equivalent (0.0565M) depending upon the solubility. As standards for comparison, solutions of pure 2,4-dichlorophenoxyacetic acid of the same molar concentrations were made in the same solvent system. When these stock solutions were diluted with water for the actual test, fine dispersions of the test compounds were obtained. Separate control tests showed that, at the concentrations used in the tests, the solvent-emulsifier system was not phytotoxic.

Test procedure. The stock solutions were diluted with water to give dispersions containing the test substances in concentrations in the range 20–2500 p.p.m. acid equivalent. The test plants in the cartons were then sprayed under standard conditions with these test dispersions by using a standardized compressed-air spray. The dose per carton of plants was 1.0 ml. Five series, each of eight cartons of plants, were sprayed in a batch, four of these series with different test substances, the fifth being treated with equivalent concentrations of 2,4-dichlorophenoxyacetic acid. After 7–8 days in standard climatic conditions the effects of auxin upon the test plants were assessed and compared with the series treated with the standard 2,4-dichlorophenoxyacetic acid and with untreated controls. The visible effects of the treatments varied only in degree from one compound to another, and no test substance produced effects of a type which were not produced by the reference 2,4-dichlorophenoxyacetic acid. Since the tested compounds are all relatively simple derivatives of the one parent acid it may be assumed that the effects assessed are symptoms of truly comparable growth factors and not toxicity. Some instances of toxic symptoms were observed but these were traced to contamination of the solvent system and were not observed when the tests were repeated with purified solvent. Visible auxin effects such as foliar damage, epinasty, callus formation and changes in turgor were assessed for each test compound and rated on a

5-point scale according to severity. The sum of these ratings for each test series was compared with that obtained from the series treated with standard auxin, and the results were finally expressed as a percentage of the activity of 2,4-dichlorophenoxyacetic acid. Photographic records were also kept and used as an additional check on day-to-day variations. The tests on the amides were replicated four times and the final ratings are the mean of the replicates. Comparison between runs on the reference 2,4-dichlorophenoxyacetic acid showed good reproducibility; the maximum scatter of these runs was ± 5%.

RESULTS

The results obtained from the activity tests are shown in Table 1. The chemical stabilities of the compounds, as determined by their rates of hydrolysis by 0.22N-potassium hydroxide in 90 % (v/v) ethanol (Johnston, 1961), are indicated by their half-life values ($t_{\frac{1}{2}}$).

DISCUSSION

Several workers have stated what they consider to be the essential structural requirements for an active auxin molecule (Koepfli, Thimann & Went, 1938; Muir & Hansch, 1951, 1953; Smith & Wain, 1952). The one structural requirement common to all these theories is a carboxyl group, or a group that can be converted readily into a carboxyl group, in the side chain of the auxin molecule. The implication is that all derivatives of the carboxyl group, such as the amides, must be converted into the free acid before showing activity (Fawcett, Taylor, Wain & Wightman, 1956). On the other hand Seely, Fawcett, Wain & Wightman (1956)

showed that indol-3-ylacetamide is not hydrolysed to the free acid by all plant tissues, wheat and celery tissue being incapable of producing the free acid in any significant quantity from the amide.

The considerable differences in activity shown by closely related amides of 2,4-dichlorophenoxyacetic acid (Table 1) cast doubt on the supposition that the amides are not active as such, but require to be hydrolysed to the free acid before showing activity. This doubt is strengthened by the fact that 2,6-dichlorophenoxyacetamide is an active auxin, but the corresponding acid is not (van Overbeek, 1959). If hydrolysis of the amides to 2,4-dichlorophenoxyacetic acid were a necessary prelude to auxin activity, the apparent activities of the amides would depend on their rates of hydrolysis in the plant, provided that penetration into and transport within the plant are not overriding factors. No experimental evidence has been obtained on penetration or transport rates of the amides. The fact that there is neither a correlation between solubilities in common organic solvents or water nor between the size of the molecules and the activities of the compounds suggests that these factors are not limiting. It is evident from the results obtained that no relationship exists between activity and the rates of hydrolysis in alkaline solution *in vitro*. These rates of hydrolysis are, of course, not necessarily a guide as to what may happen in the plant itself, but yield information about the electronic status of the molecules.

Base-catalysed hydrolysis of amides involves a nucleophilic attack of the hydroxyl ion on the carbonyl carbon atom, and any substituent on the nitrogen atom that withdraws electrons from the $-C(=O)\cdot N=$ portion of the molecule will increase the rate of hydrolysis and vice versa (Ingold, 1953). The rates of hydrolysis of the amides will therefore be inversely proportional to the electron availability in this part of the molecule.

van Overbeek (1959) suggested that the function of the carboxyl group of an auxin is to take part in an extended hydrogen-bonding system at the receptor point in the cytoskeleton, or enzyme matrix of the cell, of the plant. The ability of an atom or group to form hydrogen bonds depends on its electronic status; generally this ability is possessed only by atoms which possess a lone pair of electrons. The results of the present work suggest strongly that electron availability at a particular point in the side chain, rather than a carboxylic acid function, is an essential structural feature of an active auxin.

The apparent anomalies in the results quoted in Table 1 can be satisfactorily resolved if the requirements for an active auxin molecule as originally stated by Smith & Wain (1952) are modified to read as follows: (a) an unsaturated ring system;

(b) a side chain containing an atom or group of atoms which can function as an electron donor or as a nucleophilic centre [Smith & Wain (1952) suggested that a carboxylic acid group was essential]; (c) at least one hydrogen atom on the carbon atom adjacent to the carbon carrying or forming part of this group; (d) a definite spatial relationship between these essential units to allow the correct positioning of the auxin molecule in relation to the receptor groups of the cytoskeleton.

If the results of the present investigation are examined in the light of this statement, there are five categories of compound to be considered:

Free acid. The carboxyl oxygen atoms possess lone pairs of electrons and, as such, the electron availability of the carboxyl group is high. There is, in addition, no steric hindrance to offset this.

Unsubstituted amide. Rapid hydrolysis indicates a relatively low electron availability on the carbonyl carbon atom. The high activity of this compound is due to high electron density on either the amide nitrogen or the carbonyl oxygen atoms of the resonance structures of the amide:

As with the acid, there is no steric hindrance.

Monosubstituted amides with moderate to high activity and with high electron availability as shown by low hydrolysis rates. This group includes the methyl, ethyl and isopropyl amides (II, III, IV), the anilide, toluides and the chloroanilides (VI, VII, XII, XIII). Electron availability on the $-C(=O)\cdot N=$ moiety is high owing to the donor influence of the *N*-substituents but with the increasing size of the substituent steric hindrance about the amide nitrogen also increases; this tends to reduce activity below what might otherwise be expected. The *p*-chloroanilide will also fall into this category since the chlorine can, when required by electrophilic reagents, act to some extent as a donor, e.g. in aromatic substitution reactions. The 3,4-dichloroanilide is less active since a greatly reduced donor effect will result from this configuration.

Amides of low activity and with low electron availability as shown by rapid hydrolysis. Two monosubstituted amides, the nitroanilides VIII and IX, fall into this category on account of the powerful electron-withdrawing effects of the nitro group. The disubstituted amides XIV, XV, XVI and XVII presumably have reduced electron availability owing to resonance effects in the $-NR'R''$ grouping, and the activity is closely related to this electron availability. Steric hindrance about the nitrogen atom is also considerable.

Amides of low activity despite high electron availability as shown by low rates of hydrolysis. The hydroxyethyl amide (V) and, in particular, the two amino anilides (X and XI) appear to contradict the hypothesis since, despite high electron density in the $-C(:O) \cdot N=$ area due to the effects of donor groups, their activity is negligible. This lack of activity may, however, be satisfactorily explained by the probability that the donor groups in the substituents on the nitrogen atom will compete with the correctly positioned amide-nitrogen donor. Since these extraneous donor groups are not sterically hindered they can interact with the electrophilic receptor group of the plant cytoskeleton to the virtual exclusion of the amide auxin centre. This will result in an incorrect spatial relationship of the remainder of the molecule with the other receptor groups, so that auxin activity is not obtained.

Convincing evidence that this interpretation is correct is obtained when the competing donor groups of the amino anilides are blocked by acetylation. The resulting acetamido anilides XVIII and XIX show auxin activity of a high order, nearly comparable with the anilide VI. Since acetylation will not remove all the donor characteristics of the amino groups it cannot be expected that the activity will reach the same value as that of the anilide. The observed activities of the acetylated products are, in fact, of the order of magnitude that could be expected from compounds containing two donor groups of similar character.

The results of this investigation lead to two conclusions: (a) there is no evidence that amides of 2,4-dichlorophenoxyacetic acid require to be hydrolysed to the free acid before manifesting auxin activity; (b) it is not essential that an auxin molecule should contain a carboxylic acid group in the side chain, but activity does depend on the existence of a suitable donor group or nucleophilic centre in the side chain, and this centre must have a particular spatial relationship with the other essential groups of the molecule as envisaged by Smith & Wain (1952). This dependence of activity upon spatial relationships within the molecule is demonstrated by the deactivating effect of extraneous, incorrectly positioned donor groups which compete with the active auxin centre.

SUMMARY

1. The auxin activities of a number of *N*-substituted amides of 2,4-dichlorophenoxyacetic acid have been determined. Wide variations in activity are shown by closely related amides.

2. It is suggested that these amides are active auxins as such and do not of necessity require hydrolysis to the free acid within the plant before manifesting activity.

3. The observed activities of these amides, taken in conjunction with their rates of hydrolysis in alkaline solution, can, with certain apparent exceptions, be related to the electronic status of the $-C(:O) \cdot N=$ portion of the molecules.

4. The theory is advanced that an essential structural feature of an active auxin molecule is an electron-donating group or nucleophilic centre at a particular position in the side chain of the molecule, and not, as previously believed, a carboxylic acid group. The low activity of apparent exceptions to this rule is explained as being due to competing donor groups which lead to misalignment of the molecule on the receptor centres in the plant.

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