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ON THE EXTRACTION AND FORMATION

OF NATIVE AUXINS IN PLANTS

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INTRODUCTION

The many studies on indigenous plant growth promoting substances which have ensued since the monumental work of Went (64) opened the field for development have generally been concerned with the following problems: (1) how the substance is extracted from plants, (2) what substance is extracted, (3) the state and nature of the substance in the plant, (4) the quantity of the substance extracted, (5) the effects exhibited in the plant by the substance, and (6) the mechanism whereby the substance exerts these effects.

The apparent simplicity of these problems is misleading. Auxin occurs in extremely small quantities in plants [e.g. 1.6×10^{-4} µg. per Avena coleoptile (67)]. This renders removal of the auxin and its subsequent identification extremely difficult; even the quantitative determinations depend largely upon bioassay methods which are not precisely quantitative.

The situation is further complicated by the many states in which auxin exists in plants. In addition to the free acid auxin, indole-3-acetic acid (IAA), reported by many workers (20, 23, 27, 29, 30, 43, 59, 60, 67) the presence of two non-acidic auxins, indole-3-acetonitrile

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The definitions accepted by Leopold (39) for the terms auxin, growth regulator, and hormone are used throughout this dissertation; the expression plant growth promoting substance will be used synonymously with the term auxin.

(IAN) and indole-3-acetaldehyde (IAc), is indicated. IAN has been demonstrated with certainty in Crucifers (28, 61) and a few other plants (5); the presence of IAc has been strongly suggested (20, 24, 31, 63). The ethyl ester of IAA has been reported (45, 54), as have various presumed intermediates in IAA formation such as tryptamine (65) and indole pyruvic acid (50).

During the extraction of auxin, the free form is assumed to be gradually released, either in a purely chemico-physical way, or enzymatically from one of the various bound forms. IAA-protein complexes have been reported (66, 68, 69) and precursor and/or postcursor complexes of IAA are well known (8, 9, 49); it is also known that IAA can be adsorbed on various proteins (19). The problem is further complicated by the fact that tryptophane, which occurs in many plant proteins and exists to some extent in the free form, is easily converted to IAA, either chemically (22, 46) or enzymatically (67, 70).

To obscure the auxin picture still further there exists an IAA oxidase complex of enzymes (51, 52, 62) which continuously destroys IAA; and a naturally occurring inhibitor of that enzyme complex also exists (52). The IAA oxidase has recently been shown to behave like an induced enzyme, rising in response to increased exogenous auxin levels (17, 44). There are also found in many plants non-auxin related inhibitors (24, 25, 26) and auxin antagonists (40, 42).

General agreement is lacking as to the actual nature of these forms of auxin or their physiological significance in the plant. Consequently, there is no standard method for the removal of these substances

from plants. Auxin has been collected from diffusate into agar blocks or other media. More commonly the tissue is immersed in an auxin solvent such as ethanol, chloroform, carbon tetrachloride, water, or especially diethyl ether (35, pp. 565-570). The auxins obtained by these different methods may represent a variety of different forms and these different forms may undergo further changes during extraction. Thus the extraction of auxin from plants has occasioned great difficulty. This then leads to the anomalous situation in which advances are being made daily in problems 2-6 enumerated in the first paragraph of this section, but their validity is always overshadowed by the rather uncertain approaches to the basic problem of initially obtaining the auxin. The initial portion of the following study was begun in an attempt to devise, if possible, a satisfactory procedure for extracting auxin from any plant tissue.

The scope of this investigation includes only the first three problems listed at the beginning of this section. A particularly puzzling problem is the formation, identity, and significance of non-acidic auxin which has been found from time to time (10, 20, 24, 31, 63). Work on its formation was undertaken with the view that it might yield some further insight into the biogenesis of IAA from tryptophane. With these goals in mind this study was carried out in the Botanical Laboratory of the University of Bergen, Norway, from September, 1953 to June, 1954; and intermittently at the University of Oklahoma at Norman, Oklahoma, from September 1954 to June, 1955.

MATERIALS AND METHODS

EXTRACTION AND FORMATION OF AUXIN

Tissue. An unknown strain of yellow field maize with starchy endosperm was used as a source of tissue. Large, uniform kernels were selected and the endosperms, together with portions of the seed and fruit coat, were removed with a pair of diagonal cutters. Care was taken to ensure that none of the embryo was included. As the endosperm did not appear to lose its growth promoting activity upon storage, a large quantity was coarsely ground in a coffee grinder and stored in a desiccator. This afforded uniform samples throughout the experimentation. Samples of 10 gms. each were used in three early extractions, but in all later extractions 2 gm. samples were consistently used.

In other experiments peas of a Danish variety, Dippes mai, were planted in river sand in clay pots. After 9 days growth in the dark with occasional exposure to dark-room red light (see Bioassay of Auxin), the plants were harvested. From the plants in which the fourth internode was just commencing to elongate, the third internode was removed and placed in a large petri dish lined with moist filter paper. In each extraction fifty internodes (ca. 5 gms.) were harvested, moved to a cold (1° C.) room, cut into about 1 cm. lengths, and quickly weighed. Extraction was commenced immediately.

Extraction Solvents. Mallinckrodt's "Ether Anhydrous, Analytical Reagent," British "Analar," or a local Norwegian ether were all equally satisfactory. Since none contained more than 0.01% alcohol no trouble was encountered in subsequent partitioning of extracts. These ethers were purified as described by Larsen (35, p. 568) and stored at below-freezing temperatures.

In all cases in which water was the extraction solvent or a component of the solvent system, freshly-boiled, glass-distilled water was used.

Enzyme Inhibitor. A 100 p.p.m. solution of sodium diethyldithiocarbamate [Na-DEDT following the abbreviation scheme of Goksøyr (18)] was used for some extractions. Terpstra (53) reported that this compound does not cause a curvature in the Avena test itself, nor does it influence the curvature caused by IAA. These results were confirmed for a concentration of 100 p.p.m., but stronger concentrations interfered with curvature responses caused by IAA (Table 1).

Temperature and Light. Two temperatures, 1° C. and 22° C., were employed for extractions. The temperature was controlled in each instance to within 0.5° C. All extractions were carried out under red light (Corning filter No. 2424).

Extraction Procedure

1. Maize Endosperm. The standard procedure was to place 2 gms. of endosperm in a 50 ml. Erlenmeyer flask, add 4 ml. of freshly-boiled water (which had been cooled to the extraction temperature) and 10 ml. of diethyl ether. The flask was stoppered and agitated at high speed for 57 minutes on a mechanical agitator. Then the

TABLE I

RESPONSE OF AVENA COLEOPTILES TO SYNTHETIC IAA AND SYNTHETIC IAA + Na-DEDT

IAA (µg. per Liter)	Na-DEDT (p.p.m.)	Degrees Curvature
40	0	18.0
40	100	18.2
40	10,000	0

ether was poured off, and the last three minutes of the hour of extraction were devoted to rinsing. The standard rinsing procedure consisted of pipetting 2 ml. of ether into the aqueous endosperm, shaking briefly, and then decanting the ether. This was repeated for a total of three rinsings. The rinse ether was combined with the original 10 ml. and the resulting extract was either made up to 25 ml. in a volumetric flask or reduced in volume by evaporation at 50° C. and made up to 10 ml. If the extraction was continued longer than one hour, fresh ether was added after rinsing. The amounts of solvent and endosperm, the container, the agitation, the time of extraction, and the rinsing procedure were all varied at one time or another and these changes from the standard procedure are noted in the discussions of the appropriate experiments. When water alone was used as a solvent the aqueous endosperm was centrifuged and the centrifugate immediately shaken in a separatory funnel with 1½ times its volume of ether for three periods of 4, 3, and 2 minutes each. The aqueous extract was acidified with HCl to approximately pH 3.0 to obtain a more favorable partition coefficient. For convenience in centrifugation 50 ml. centrifuge tubes were used as extraction vessels in extractions requiring several changes of water.

In some cases an aqueous extract of maize was immediately hydrolyzed by boiling at pH 9.6 [obtained by using M/20 borate buffer described by Avery, Berger, and Shalucha (3)]. After cooling and acidification the hydrolyzed extract was partitioned with ether as described above. A second portion of the same original extract was first acidified and shaken out with ether, thus obtaining the ether soluble substances, and then the remainder was hydrolyzed. This procedure enables one to

obtain information concerning the relative amounts of total, ether soluble, and hydrolyzable auxin from an aqueous extract of endosperm.

2. Pea Internodes. In each extraction 50 third internodes of pea were covered with 20 ml. of ether or water in a 100 ml. Erlenmeyer flask. In the first water extractions performed at 22° C. it was found that the internodes continued to grow. Therefore, in all subsequent water extractions the tissue was rapidly frozen in a freezing chest, thawed and extracted. In certain extractions 100 p.p.m. of Na-DEDT was added. The extraction flask was placed on an agitator and shaken at the desired temperature for 57 minutes. The solvent was then poured off and the tissue rinsed three times with 2 ml. portions of solvent. The rinse solvent was combined with the original extract and, in ether extractions, the resulting mixture was made immediately to volume in a 25 ml. volumetric flask, evaporating if necessary. In all water extractions the auxin was first partitioned from water into ether as previously described for aqueous maize endosperm extracts, and then partially evaporated and made to volume.

If the extraction was to be continued the pea internodes were covered again with 20 ml. of solvent and the entire procedure was repeated. The internodes were often left for intervals longer than an hour. Also, in some cases 24 hours elapsed before the initial solvent change. These variations from the standard procedure are indicated in the presentation of results.

Purification of Extracts. Crude extracts obtained as described above were purified by partitioning between ether and water (35, pp. 571-572). Tartaric acid was first used to achieve a pH value suitable

for regeneration of the acid fraction. However, Larsen (36) in renewing his previous warning (38) points out that tartaric acid is not suitable for this purpose in many auxin experiments. He reported that while the effect of HCl was only slight, increasing concentrations of tartaric acid caused reduced response of test plants to synthetic IAA. In the present investigation tartaric acid influenced the apparent auxin yield from maize, probably by affecting the Avena curvature test (Table 2). Other experiments performed by the writer show that tartaric acid (either d or dl) causes a diminished curvature response to synthetic IAA. Bonde (11) stated that microscopic sections of Avena coleoptile, to which a tartrate-containing agar block was applied, showed extensive cell injury. Therefore, Terpstra's (53) method of acidification with HCl was adopted.

Bioassay of Auxin

The Avena coleoptile curvature test was employed for most of the bioassays. Grains of Swedish Victory oats were obtained from Sveriges Utsädesförening, Svalöf, Sweden. Cultivation in vials of soil and preparation of the test plants employing the double decapitation method were done exactly as described by Larsen (35, pp. 579-581).

Since the plants are highly sensitive to wave lengths of light shorter than 550 millimicrons, they were grown in a dark room and test procedures carried out by the light from a 15 watt bulb screened with a Corning No. 2424 filter. Such a filter excludes shorter wave lengths of light thereby preventing phototropic curvatures, and increasing the sensitivity of the coleoptiles to auxin.

Agar for the bioassay was prepared according to Larsen (35,

TABLE 2

THE EFFECT OF THE ACID USED IN PURIFICATION OF THE EXTRACT UPON APPARENT YIELD OF ETHER SOLUBLE ACIDIC AUXIN IN ONE HOUR EXTRACTIONS OF MAIZE ENDOSPERM. ACTIVITY EXPRESSED IN MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Dilution of Crude Extract With Ether Before Shaking Out	Dilution of Crude Extract With Ether After Shaking Out	Tartaric Acid		Hydrochloric Acid	
		µg. eq. IAA	No. of Tests	µg. eq. IAA	No. of Tests
1 to 6	200 Times	3500	2	3500	1
1 to 40	30 Times	3100	4	3450	1
1 to 150	8 Times	2300	4	3100	4
1 to 800	1.5 Times	950	1	----	---
1 to 1200*	----	(3400 µg. eq., 1 test)			

* Crude extract without purification.

pp. 585-586) and auxin was incorporated into the agar by Larsen's (35, pp. 587-588) modification of the ether dropping method of Boysen-Jensen (12). Blocks of 4 $mm.^3$ (2 x 2 x 1 mm.) were prepared by means of a 5-bladed cutter. Advantages of this block size were discussed by Larsen (35, pp. 588-590). The agar block was then applied on one side of the decapitated Avena coleoptile. Test plants respond to auxin by curving away from the agar block. Growth inhibitors cause curvature toward the agar block. The angle which the straight base of the coleoptile made with the tangent to the curved portion at its extreme upper end was taken as a measure of the response. The angle was measured on the convex side of the coleoptile. A curvature measuring chart modified from Purdy (35, p. 584) was used. The computation of degrees curvature by this method was fully described by Larsen (35, p. 584).

The standard solution for all the auxin bioassays was prepared with pure crystalline IAA. On three occasions aqueous standard solutions (35, pp. 590-591) were used for comparison but, in general, solutions in ether were employed (35, p. 591). An activity curve of IAA was obtained by plotting curvatures against concentration (Figure 1).

In considering the variability of the Avena curvature test or any test in which the result is expressed as a mean of the responses of a number of individual test organisms, one must distinguish between the variability among individuals used on a given occasion and the variability among means obtained on different occasions. Using the writer's data Larsen (35, pp. 593-594) demonstrated that variations among occasions was no greater than the variation among individual test plants.

Curvature readings are expressed as IAA µg. equivalents per

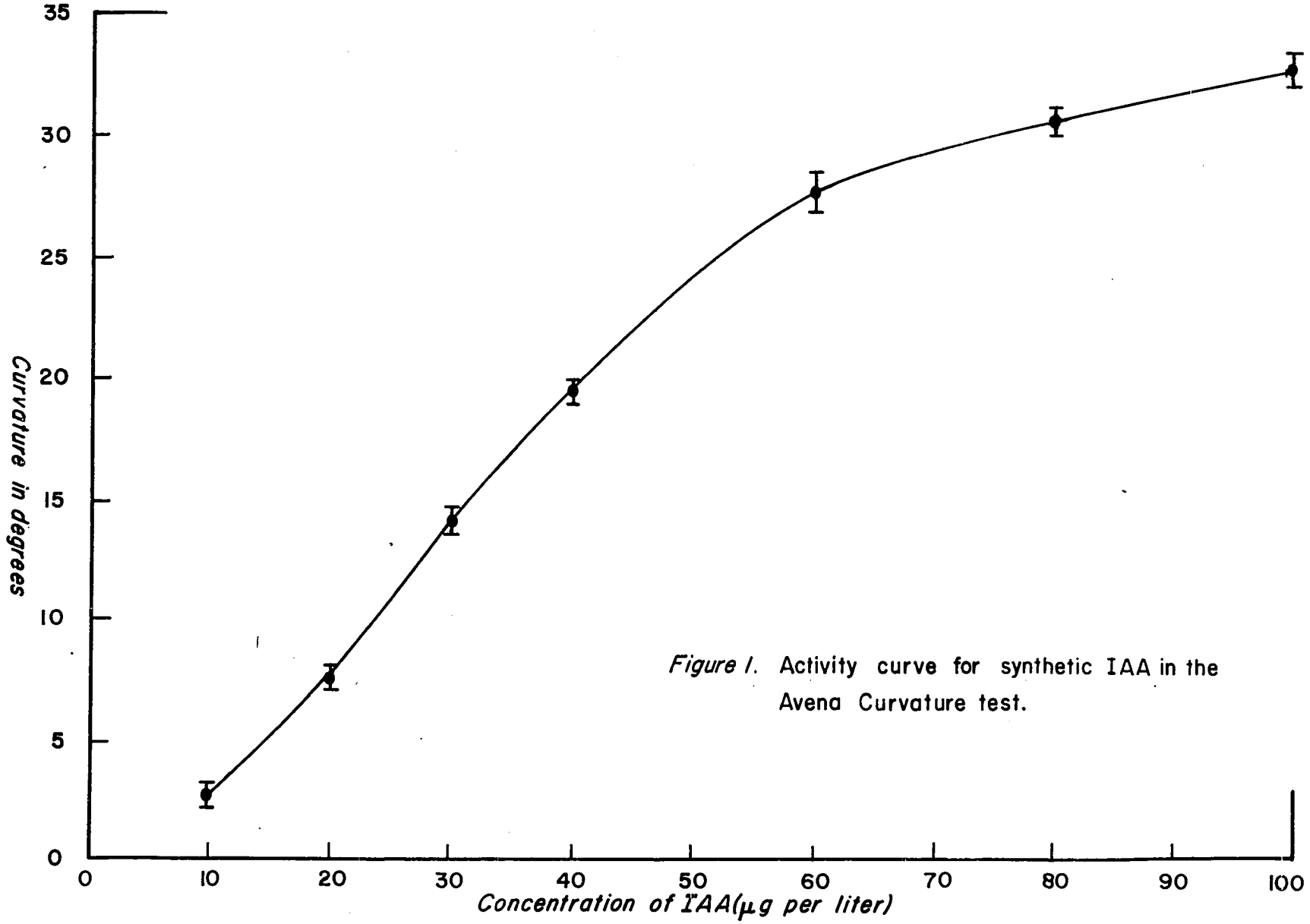

Figure 1. Activity curve for synthetic IAA in the Avena Curvature test.

kilogram of tissue. The method of calculation and a discussion of the merits of such a value are given by Larsen (35, pp. 597-599).

Identification Procedure

The initial separation into acidic and non-acidic fractions was described under Purification of Extracts. Further separation was accomplished by paper chromatographic techniques. Strips of Whatman No. 1 paper 30 cm. long and 2 cm. wide were prepared. In the center of each strip 3 cm. from one end a small dot was made with a pin. Twenty centimeters from this dot a pencil line was drawn across the strip. The strips were stretched on a wooden frame with thumb tacks placed below and above the 20 cm. portion through which the separation occurs. The ether extract to be separated was applied dropwise with a micropipette on the pin-prick. An effort was made to keep the dropped area no more than 1 cm. in diameter with the pin-prick as the center. After the extracts were dry the upper ends of six such strips were clipped to a glass rod suspended on a small wooden scaffold and 0.5 cm. of the lower ends was allowed to dip into a large petri dish filled with the appropriate partition solvent. This ascending solvent system was then covered with a bell jar and allowed to run in a temperature controlled room (22° C.) equipped with red lights. The partition solvents used were the following: (1) glass-distilled water, freshly boiled and cooled; (2) isopropanol (10 parts), ammonia, sp. gr. 0.88 (1 part), water (1 part); (3) 70% ethanol.

A wick of cloth was placed in the petri dish to help saturate the atmosphere with solvent. In addition, with water or 70% ethanol,

the bell jar was partially lined with filter paper wetted with solvent. The isopropanol-ammonia-water solvent contained so little water the filter paper would not adhere to the glass.

When the front of the partition solvent had reached the 20 cm. mark the strip was removed, pinned back on the wooden frame, and allowed to dry. The paper strips were then cut into sections of desired length, shredded, and placed in 50 ml. Erlenmeyer flasks or 30 ml. test tubes. Ten milliliters of ether were added and they were agitated two hours on the agitator. The ether was then poured off and the paper rinsed three times with 2 ml. portions of ether. This eluate was condensed to 10 ml. and tested with the Avena curvature test.

On some strips relatively large quantities of synthetic and natural substances were placed. Large amounts of concentrated non-acidic auxin, pure crystalline IAA, pure crystalline IAN (courtesy of Dr. E. R. H. Jones, University of Manchester, England), and synthetic preparations of a non-acidic auxin, presumably IAc, were used. The latter compound was prepared from tryptophane and ninhydrin (or isatin) according to Larsen (31, pp. 85-86). The yields of active substance were quite low (about 0.5%) and all efforts to increase them by varying the proportion of either reagent, or by varying the time of refluxing were to no avail. Strips believed to contain at least 0.2 µg. of some indole compound were sprayed with a modified Salkowski reagent consisting of a mixture of 80 ml. of 10% H_2SO_4 by volume, and 2 ml. of 0.5 M. $FeCl_3$. They were then dried at 60° C. for 3-6 minutes and the characteristic color reactions observed. Some strips were also viewed under ultraviolet light in an effort to locate fluorescent indole compounds.

RESULTS AND DISCUSSION

Maize Endosperm

Extraction of Auxin.

1. Extractions of one hour duration. To obtain information concerning the release or formation of auxin, extractions should be carried out over prolonged periods of time. However, for many of the points under consideration in establishing an extraction technique it is sufficient, or even preferable, to limit extraction time to one hour.

Of the many solvents which will extract auxin, diethyl ether and water are probably the two most frequently used. These two solvents and combinations of the two were used as extractants of air dry (water content about 9%)maize endosperm. Extracting with dry ether alone yielded no auxin at either temperature (Table 3). This was no surprise as Link, Eggers, and Moulton (41) had reported that dry ether did not extract auxin from lyophilized tissue, even though the auxin, once extracted, was highly soluble in dry ether; Thimann and Skoog (57) using dry Lemna powder obtained no auxin unless water was added to the extracting ether; Wildman and Muir (71) later reported that dry ether did not extract auxin from lyophilized tobacco ovaries.

With water alone there was a series of increasing auxin yields

TABLE 3

THE EFFECT OF VARIOUS AMOUNTS OF ETHER, WATER, AND ETHER PLUS WATER AS EXTRACTANTS UPON ETHER SOLUBLE AUXIN YIELD IN ONE HOUR AGITATED EXTRACTIONS OF MAIZE ENDOSPERM. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Sample	Extraction Technique		22° C.		1° C.	
	Water (ml.)	Ether (ml.)	µg. eq. IAA	No. of Extractions	µg. eq. IAA	No. of Extractions
1	0	10 (dry)	0	1	0	3
2	0	25 (dry)	0	1	0	1
3	4	0	600	1	400	1
4	10	0	1700	1	1100	3
5	25	0	2300	1	1600	2
6	10	10	2400*	4	1250*	1
7	10	50	3000*	1	1700*	1
8	4	10	3300	5	1900	7
9	4	50	3350	1	2100	2

* It is probable that part of the auxin remained in the aqueous phase in these extractions.

with increasing amounts of water at either temperature, though the actual amounts varied with temperature (Table 3). The amount of water seemed to limit the extraction of auxin (samples 3, 4, 5). Since the auxin was obtained rather slowly, it appears that one was testing not only the extracting capacity of these solvents, but also the effects of the solvents upon the formation of auxin. As even the 25 ml. portion of water (sample 5) may have been limiting it became of interest to find the minimum volume of water necessary to achieve a rapid total extraction of auxin. A practical limitation to increasing water volume indefinitely arises in that all aqueous extracts are subsequently partitioned with ether to insure that one is dealing only with the ether soluble substances and to facilitate incorporation of auxin into agar by the ether dropping technique. Therefore various combinations of ether and water were next employed (samples 6-9). A mixture of 10 ml. of water and 10 or 50 ml. of ether gave unsatisfactory results (samples 6 and 7). With sample 6 the ratio of solvents was such that an emulsion was formed which rendered separation of the aqueous endosperm phase and the ether phase difficult. In both samples 6 and 7 it was suspected that the standard rinsing technique was inadequate to recover much of the auxin dissolved in the ether, which in turn was dispersed throughout the aqueous phase. Therefore, it is likely that the values of auxin activity for these samples should be revised upward. A more satisfactory combination was 4.0 ml. of water (twice the weight of the endosperm) and 10 or 50 ml. of ether (samples 8 and 9). Extractions with these combinations of solvents gave the highest yield of auxin, higher than with water alone (samples 3, 4, 5), and much higher than the negligible amount extracted with dry ether

alone (samples 1 and 2). It must be noted here that agitation was performed upon all samples and Erlenmeyer flasks no smaller than 100 ml. were used as extraction vessels. This afforded a constant mixing of ether and water throughout the extraction period. Addition of more ether had a slight effect at 1° C. and essentially none at 22° C. (sample 9). It would appear then that with dry tissue the water volume is indeed limiting. One can extract a small amount of tissue with a great volume of water or one can extract with a small amount of water and a little ether. The water still presumably does the extracting and auxin is merely partitioned into the ether. The ether is intimately mixed with the water by agitation and thus "cleans" the water of auxin. This is, in effect, extracting with virtually an unlimited amount of water.

Further evidence that tends to corroborate this conclusion has been compiled (Table 4). Agitation of the extraction flask did not increase the yield of auxin when water was used alone as the extractant. However, agitation definitely did increase the yield of auxin when ether and water were the extractants (samples 1 and 2). Without agitation about the same amount of auxin was obtained as with that quantity of water alone. That an increase in yield was the result of agitation, and not increased aeration brought about by the agitation, was borne out by sample 3 in which there was no increase in yield when a 0.05% solution of methylene blue, a hydrogen acceptor, was employed in the unshaken flask. Furthermore, this was indicated in a more direct approach in samples 4 and 5. In these samples the tissue and extractants were placed in a 28 x 112 mm. vial and in sample 4, a stirrer was inserted with the blades at the interface of the aqueous endosperm-ether layer. The shaft of the

TABLE 4

THE EFFECT OF AGITATION, AERATION, AND CONTAINER SIZE UPON ETHER SOLUBLE AUXIN YIELD IN ONE HOUR EXTRACTIONS OF MAIZE ENDOSPERM AT 1° C. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Part	Sample	Water (ml.)	Ether (ml.)	Agitation Treatment	Container	µg. eq. IAA	No. of Extractions
A (Agitation and Aeration)	1	4	10	Shaken	50 ml. Erlenmeyer	1900	7
	2	4	10	Unshaken	50 ml. Erlenmeyer	1050	1
	3	4*	10	Unshaken	50 ml. Erlenmeyer	1000	1
	4	10	25	Stirred	28 x 112 mm. Vial	1850	1
	5	10	25	Unstirred	28 x 112 mm. Vial	450	1
B (Container Size)	6	4	10	Shaken	30 ml. Bottle	<250	2
	7	4	10	Stirred	28 x 112 mm. Vial	1900	1
	8	4	25	Shaken	30 ml. Bottle	<250	1
	9	4	25	Shaken	50 ml. Erlenmeyer	700**	2
	10	4	25	Shaken	1000 ml. Erlenmeyer	2000	1
	11	4	25	Stirred	28 x 112 mm. Vial	2150	1
	12	4	50	Stirred	100 ml. Erlenmeyer	2100	2

* Four milliliters of 0.05% methylene blue solution.

** Comparable extracts made at 22° C. showed a similar low yield under these extraction conditions.

stirrer was covered to a depth of 5-6 cm. with ether, thus affording relatively anaerobic conditions for the endosperm in both cases. A high yield was obtained in the stirred vial (sample 4) and a low yield in the similar unstirred vial (sample 5).

Other evidence indicated that even though the volume of water and ether was suitable for extraction of the endosperm, and even though the extraction flask was agitated, one must use a large enough flask to ensure adequate mixing of the ether and water. This was discovered when it was observed that the yield of auxin with 4 ml. of water and 25 ml. of ether was lower than with 4 ml. of water and 10 or 50 ml. of ether (Table 4). This apparent anomaly was explained by further experiments which showed that in containers too small for agitation to result in effective mixing of the ether and aqueous phase, there was a decrease in auxin yield. In samples 1 and 9, a 50 ml. Erlenmeyer flask was the extraction vessel. Adequate mixing occurred only in sample 1 with the lesser volume. The 29 ml. of liquid of sample 9 filled the flask so that adequate mixing did not occur during agitation. This problem did not arise with sample 12 because the larger initial volume had already required a larger extraction vessel. After making this observation, tests were run with different containers (all of which were agitated) and the results confirmed the hypothesis. With a 30 ml. bottle (samples 6 and 8) a negligible auxin yield was obtained. Though the bottle was shaken during the agitation, the endosperm packed down into the bottom and proper mixing could not occur. Placing the endosperm in a long vial was satisfactory, however, if adequate (anaerobic) stirring was carried out (samples 7 and 11). Thus, small containers of any shape, if properly agitated, are

satisfactory extraction vessels.

In another type of experiment in which the volume of ether was varied, extractions were set up with the standard technique (see Materials and Methods), except ether changes and rinsings were performed every 10 minutes instead of every hour, thus giving six changes and six rinses each hour. This treatment resulted in no appreciable increase in auxin yield at 22° C. and only a slight increase at 1° C. over the standard procedure (Table 5). This technique is essentially the same as extracting with more ether initially (e.g. 50 ml.) and compares favorably with the value obtained when 50 ml. were used. There would seem to be no great advantage in changing the solvent repeatedly as long as other aspects of the standard procedure are observed.

As trapping of the auxin into ether from water involves its partitioning between two immiscible solvents, experiments were initiated to determine the effectiveness of the standard rinsing procedure in removing the remaining free auxin from the water upon cessation of extraction. Using standard extraction techniques at 22° C. with three parallel samples, 3300 µg. equivalents of auxin per kilogram of tissue were obtained for the rinsed sample and about 15% less (2800 µg. equivalents) for each of the two unrinsed samples. In one of the unrinsed samples, after the ether was poured off, the remaining aqueous endosperm was centrifuged and the centrifugate shaken out with twice its volume of ether for 4, 3, and 2 minutes in a separatory funnel. This more thorough rinsing treatment yielded only an additional 550 µg. equivalents, giving a total of 3350 µg. equivalents. Thus, the standard rinsing procedure appeared to remove most of the auxin from the water. In these experiments

TABLE 5

COMPARISON OF A SINGLE CHANGE OF SOLVENT AND SIX CHANGES OF SOLVENT DURING ONE HOUR UPON ETHER SOLUBLE AUXIN YIELD IN MAIZE ENDOSPERM. ACTIVITY EXPRESSED IN MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Extractants		Rinsing Treatment	22° C.		1° C.	
Water (ml.)	Ether (ml.)		µg. eq. IAA	No. of Extrac-tions	µg. eq. IAA	No. of Extrac-tions
4	10	3 x 2 ml. Ether	3300	5	1900	7
4	50	3 x 2 ml. Ether	3350	1	2100	2
4	10(x6)	3 x 2 ml. Ether(x6)	3400	1	2200	1

the partition coefficient of auxin between ether and water at the natural pH appeared to be about 0.85.

2. Extractions of many hours duration. Prolonged extractions of maize endosperm, and bioassays of auxin obtained thereby, were carried out next. In preliminary experiments employing no rinsing, the amounts of endosperm and solvent were varied (Table 6). At both temperatures it was apparent that the release of auxin was considerably accelerated by an increase in the ratio of solvent volume to endosperm. In series 1 the use of aqueous ether on dry tissue was similar to the extraction of dry Lemna powder performed by Thimann and Skoog (57). In both cases a burst of auxin release followed a fresh application of aqueous ether. Thus, the tissue appeared to be responding to added water, not ether.

In series 2 at 22° C. and the parallel technique at 1° C. (series 4), a larger amount of auxin was extracted the second hour than the first. During these first two hours the yield was greater at 22° C. than at 1° C. However, after that the yield was greater at 1° C. This difference in auxin yield at the two temperatures may represent a steady non-enzymatic release which is not as great at 22° C. simply because more of the source was expended the first hours of extraction. It might also indicate a rather slow enzymatic destruction of IAA at 22° C. Further results established that the latter process was at least partially responsible for the difference in yield.

In series 3 and 5 (Table 6) extraction of auxin seemed satisfactory and the procedure (twice the tissue weight in water plus a small amount of ether) was adopted as the standard technique. In these series,

TABLE 6

COMPARISON OF ETHER SOLUBLE AUXIN YIELDS IN EXTRACTIONS WITH VARYING AMOUNTS OF MAIZE ENDOSPERM AND WITH NO RINSING EMPLOYED. ACTIVITY EXPRESSED IN MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 1		Series 2		Series 3		Series 4		Series 5	
	10 gms., No Water 15 ml. Ether - 22° C.		10 gms., 10 ml. Water 15 ml. Ether - 22° C.		Standard - 22° C.		10 gms., 10 ml. Water 15 ml. Ether - 1° C.		Standard - 1° C.	
	µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA	
	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total
0- 1	125	125	800	800	2775	2775	225	225	1750	1750
1- 2	125	250	1100	1900	875	3650	475	700	725	2475
2- 3	175	425	300	2200	475	4125	400	1100	450	2925
3-24	650	1075	500	2700	625	4750	650	1750	925	3850
72-96	550	1625	625	3325			850	2600		

at both temperatures, the yield went down markedly each hour; and again there was a much larger yield in the 3-24 hour period at 1° C.

After the standard extraction procedure was selected, a standard rinsing technique was developed, and these two standard procedures were used for a number of extractions at 22° C. (Table 7) and at 1° C. (Table 8).

Series 6 and 7 (Table 7) were completely comparable except that three solvent changes were omitted from series 7. Series 6, with the more frequent solvent changes, had a slightly higher auxin yield, probably the result of decreased auxin destruction.

In series 8 the water had been adjusted to pH 2.8 with HCl and Na_2CO_3 in an effort to determine if acidification results in a more rapid release of auxin. This was not the case and the total amount was somewhat less than with the ordinary extraction procedure.

The possibility remained that the large surge of auxin which was obtained in the first few hours was the result of the presence of some detergent-like substance which was also extracted during that time. After its removal, the yield of auxin would then diminish. Therefore, an extraction (series 9) was performed in which ether of the non-acidic fraction (see Identification of Auxin) of previous extractions was used as the solvent. This ether would presumably contain the detergent, if any, from the previous extraction. Before use it was demonstrated that this non-acidic fraction possessed no activity of its own. There was found to be no advantage in using this type of ether. In fact, the yield in series 9 was slightly lower than in series 6 and 7 (Table 7). This difference, if real, was likely a result of increased emulsion formation

TABLE 7

COMPARISON OF ETHER SOLUBLE AUXIN YIELDS IN MAIZE ENDOSPERM IN STANDARD PROCEDURE EXTRACTIONS AT 22° C. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 6 µg. eq. IAA		Series 7 µg. eq. IAA		Series 8 µg. eq. IAA		Series 9 µg. eq. IAA		Series 10 µg. eq. IAA	
	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total
0- 1	3300	3300	3200	3200	3200	3200	3200	3200		
1- 2	1100	4400	1100	4300	700	3900	750	3950		
2- 3	550	4950	475	4775	300	4200	400	4350		
3- 4	350	5300	300	5075	150	4350	250	4600		
4- 6	275	5575			125	4475				
6- 8	150	5725			75	4550				
8-10	100	5825			50	4600				
10-12	50	5875	325	5400	25	4625				
12-24							575	5175	4475	4475
24-432							475	5650		

caused by using ether saturated with fatty substances. This increased emulsion formation gave poorer separation between aqueous and ether phases, thereby leaving more auxin in the extraction vessel. Therefore, the use of non-acidic fraction ether as an extractant was subsequently abandoned.

In a 24 hour period with only a single solvent change the yield was 1000-1500 µg. less than with frequent changes in 12 hours (series 10, Table 7). One must attribute this decreased yield of auxin to increased destruction or to the fact that the volume of the solvent limits extraction. Previously, a number of changes in a given one-hour period was shown to be only slightly better in extracting auxin than a single change as long as the initial quantity of solvent was sufficient (Table 5). Therefore, the loss in yield is perhaps better attributable to auxin destruction.

In series 11 to 14 (Table 8) a comparison of yields in standard technique extractions was carried out. After the third hour there was a uniform rate of auxin release in series 11. The total quantity was still less, at any given time, than that extracted under possible destruction conditions at 22° C. Series 13 and 14 showed almost the same amount of auxin, even though series 13 had five ether changes to only one in series 14. Clearly, the number of ether changes is immaterial at 1° C. One may also assume this to be true at 22° C. In that event, the losses which were manifested with only a single change of ether at 22° C. (series 10, Table 7) must have been the result of auxin destruction.

Extractions were continued in two cases for an 18 day period at 22° C. (series 9, Table 7) and at 1° C. (series 13, Table 8). During the

TABLE 8

COMPARISON OF ETHER SOLUBLE AUXIN YIELDS IN MAIZE ENDOSPERM IN STANDARD PROCEDURE EXTRACTIONS AT 1° C. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 11 µg. eq. IAA		Series 12 µg. eq. IAA		Series 13 µg. eq. IAA		Series 14 µg. eq. IAA	
	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total
0- 1	1700	1700	1950	1950	1700	1700		
1- 2	575	2275	650	2600	625	2325		
2- 3	300	2575	450	3050	350	2675		
3- 4	225	2800	250	3300	350	3025		
4- 6	225	3025			↓	↓	↓	↓
6- 8	225	3250						
8-24					775	3800	3925	3925
24-432					650	4450		

last 17 days of the extraction there was slightly more auxin released at 1° C. than at 22° C. The total amount was still considerably greater at 22° C.

Prolonged extractions were next performed at both temperatures with water as the only solvent (Table 9). In addition, series 15, 16, and 17 had 100 p.p.m. of Na-DEDT added. It was first shown that the addition of Na-DEDT in this concentration to the agar test block did not influence the resulting degrees curvature caused by synthetic IAA in the Avena curvature test (Table 1). A stronger concentration of 10,000 p.p.m. resulted in complete inhibition of curvature. Terpstra (53) had recommended a concentration of 100 p.p.m. following the suggestion by Wagenknecht and Burris (62) that Na-DEDT inactivated the IAA oxidase enzyme complex responsible for IAA destruction. Na-DEDT has recently been shown to have other interesting non-biological properties possibly of significance in Avena bioassays. A recent report from Mallinckrodt Chemical Works (1) stated that Na-DEDT possesses the ability to inhibit effectively both aldehyde and peroxide formation in diethyl ether. A concentration of only 0.05 p.p.m. is sufficient to prevent formation of these impurities. Since ether peroxides are known to interfere with the Avena test (48), this may be of importance in preventing peroxide formation in the future.

At 22° C. in water extraction (series 15-18, Table 9) there were three ranges of auxin yields. The most auxin came from the series with the most frequent water changes (series 15). One might have suspected this since it had already been shown (Table 3) that the volume of water limits auxin yield when used alone. In series 15, extraction was carried

TABLE 9

COMPARISON OF ETHER SOLUBLE AUXIN IN MAIZE ENDOSPERM EXTRACTIONS WITH 25 ML. OF WATER AS THE EXTRACTANT. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 15 - 22°C. 100 ppm Na-DEDT		Series 16 - 22°C. 100 ppm Na-DEDT		Series 17 - 22°C.* 100 ppm Na-DEDT		Series 18 - 22°C.*		Series 19 - 1°C.		Series 20 - 1°C.	
	µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA	
	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total	Amount Extracted in Indicated Time Interval	Running Total
0- 1	2300	2300							1500	1500		
1- 2	850	3150							900	2400		
2- 3	300	3450							625	3025		
3-24	500	3950	2825	2825	2800	2800	1700	1700	275	3300	1825	1825

* Extraction flasks are 100 ml. Erlenmeyer Flasks. All others are 50 cc. centrifuge tubes.

out with 100 ml. of water as compared with only 25 ml. in the later series.

The yields of series 16 and 17 were essentially equal to each other and both were lower than that of series 15. Series 16 and 17 had similar treatment except for different sizes of containers. However, in a single solvent extraction the container size played no role, at least as long as agitation was sufficient to wet the endosperm thoroughly. Omission of Na-DEDT (series 18) resulted in a much lower activity; and, thus, it appears that Na-DEDT does inhibit auxin destruction.

At 1° C. (series 19 and 20) a greater frequency of changes likewise resulted in greater auxin yields. Yields at 1° C. were lower than at 22° C., however.

Formation of Auxin. Extensive work on the auxin of maize endosperm has been done prior to the present work. Avery et al. (2, 4) while investigating the use of various solvents for extracting auxin from dormant maize endosperm found that successive water extractions of tissue removed only 10% of the available auxin from the kernel. The remainder exists in a form which requires alkaline hydrolysis to release it. They found as much as 105,000 µg. of IAA per kilogram of tissue upon alkaline hydrolysis. Berger and Avery (8, 9) continued work on the auxin of dormant maize, and obtained what they termed an auxin precursor from which IAA could be released upon alkaline hydrolysis. This precursor was thought to be protein in nature and to have a molecular weight of about 11,000.

The results of Stehsel (49) were somewhat at variance with Berger and Avery's characterization and interpretation of the auxin

precursor. Stehsel concurred in the relative amount of the substance, but preferred to call it an auxin complex, not an auxin precursor. His ontogenetic study strongly supported his contention that the auxin complex is synthesized directly from tryptophane. His experiments established the presence of an enzyme capable of converting tryptophane to auxin. Moreover, the activity of this enzyme was directly correlated with the formation of "free" auxin and auxin complex. If the auxin complex were an intermediate between tryptophane and ether soluble auxin, then it should be converted by enzymatic action to free auxin. Yet the auxin complex per se was inactive in physiological tests, and even in the presence of the proper enzymes it was apparently unaffected. Stehsel, therefore, concluded that the auxin complex was not a precursor of IAA, but a postcursor. Yamaki and Nakamura (72) reached somewhat the same conclusion, and regarded the auxin complex to be of little physiological importance in the plant.

It is unlikely that auxin yields in the present work were involved with the auxin complex, although the auxin obtained could scarcely be called "free" auxin. The auxin in maize endosperm seems to exist in three states: (a) a very small portion which is readily extractable and perhaps exists in a free state, (b) a larger portion which is bound in some way and can be freed only if enough time is allowed for extraction, and (c) the remainder, auxin complex, which can be released only upon drastic hydrolysis. Types (a) and (b) are impossible to distinguish in this study except that type (b) requires a longer extraction time. Types (a) and (b) together are readily distinguishable from type (c). Experiments at two temperatures are presented in Table 10 which exemplify

TABLE 10

ETHER SOLUBLE, HYDROLYZABLE, AND TOTAL AUXIN PRESENT IN ONE HOUR AQUEOUS MAIZE ENDOSPERM EXTRACT. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Fractions of Aqueous Extract	Treatment	Series 21 - 1°C. µg. eq. IAA	Series 22 - 22°C. µg. eq. IAA	Series 23 - 22°C. µg. eq. IAA
Sample A	First hydrolyzed,* then shaken with ether	6800	9400	11,300
Sample B	First shaken with ether	1350	1700	2,000
	Then hydrolyzed,* and shaken with ether	5200	7500	9,400
	Total in Sample B	6550	9200	11,400

* All hydrolyses used pH 9.6 borate buffer, 100° C. for 15 minutes.

this. In each series a one hour aqueous extract was made. The first of two samples was immediately hydrolyzed and assayed (A samples). The second sample was first partitioned with ether, then hydrolyzed, and the two portions assayed separately (B samples). It can be seen that the total auxin for sample B is approximately the same as for sample A. Extraction at 1° C. (series 21) resulted in a lower total yield of both auxin and auxin complex for the one hour period, but the per cent of ether soluble and hydrolyzable auxin remained about the same.

The fact that free auxin (type a) or loosely bound auxin (type b) was not formed from auxin complex (type c) was established (Table 11). Portions of the aqueous extract of series 23, Table 10, with the ether soluble auxin still remaining (sample A) and with ether soluble auxin removed (sample B) were shaken with twice their volume of ether for 24 hours. In sample A the initial ether soluble auxin was soon extracted and no more was produced; in sample B there was no ether soluble auxin initially present or none produced. The total auxin complex remained the same whether hydrolyzed immediately (series 23, Table 10) or hydrolyzed after repeated ether extractions (samples A and B, Table 11). This confirmed reports (9, 49) regarding the stability of the auxin complex.

Identification of Auxin. Tests for non-acidic growth regulating substances were run on three extractions at 22° C. and on one extraction at 1° C. Although these extractions were of many (12-96) hours duration no growth promoting or inhibiting activity was encountered in any of the non-acidic fractions. Furthermore, in a series of one hour extractions the same amount of activity was obtained in the acidic fraction and in

TABLE 11

THE EXTRACTION WITH ETHER OF PORTIONS OF THE AQUEOUS MAIZE ENDOSPERM EXTRACT FROM SERIES 23, TABLE 10. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hour	Sample A, a Raw Aqueous Extract	Sample B, an Aqueous Extract With Ether Soluble Auxin Removed
	µg. eq. IAA	µg. eq. IAA
0- 1	1,500	0
1- 2	100	0
2- 3	0	0
3-24	0	0
Hydrolyzed After 24 Hours — Shaken With Ether	10,000	9,600
Total	11,600	9,600

the acidic plus non-acidic fractions, as was obtained in the crude extract (Table 12). This situation prevailed at both temperatures, though the yield of acidic auxin was considerably higher at 22° C. than at 1° C. This series of experiments demonstrated that the non-acidic fraction has no effects of its own and that it does not act synergistically with the acidic fraction in either growth promotion or growth inhibition. Separation of most extracts into acidic and non-acidic components was therefore discontinued. In subsequent maize endosperm extractions, the crude extract was assumed to consist entirely of acidic auxin. Other workers (72) have reported non-acidic auxin in abundance in immature maize kernels, although criticisms could be levied against their identification techniques.

The ether extract of maize endosperm and the hydrolyzed auxin complex were chromatographed on Whatman No. 1 paper with water as a partition solvent. The R_f value of the active portion of the chromatogram of each was determined by biological tests to be 0.85-0.89. In addition a faint pink spot on the paper occurred at this same region upon spraying each strip with modified Salkowski reagent. This coincided with the color and R_f value obtained with pure IAA. Thus it appeared that the ether soluble auxin and the hydrolyzed auxin complex are both IAA. IAA had been previously isolated from maize endosperm (8, 23).

No identification of the unhydrolyzed auxin complex was attempted. Stehsel (49) reported that the auxin complex molecule was acidic and more polar than IAA, but gave the same absorption spectrum in Salkowski reagent as IAA. The auxin complex thus must contain IAA or an indole ring in its structure. The amount of IAA indicated by the color

TABLE 12

COMPARISON OF THE ACTIVITY OF THE ACIDIC AND NON-ACIDIC FRACTIONS OF AN EXTRACT, SEPARATE AND COMBINED, WITH THE CRUDE EXTRACT, AT TWO TEMPERATURES IN A ONE-HOUR EXTRACTION OF MAIZE ENDOSPERM. ACTIVITY EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Fraction	Series 1 - 22° C. µg. eq. IAA	Series 2 - 22° C. µg. eq. IAA	Series 3 - 1° C. µg. eq. IAA
Non-acidic	0	0	0
Acidic	3450	3250	1850
Acidic + Non-acidic	3450	3150	1900
Crude extract	3400	3200	1900

reaction after hydrolysis, as compared with the unhydrolyzed form was doubled. Apparently another indole ring was formed or unmasked from a complex molecular configuration such that it met the requirements for color formation. A tentative molecular weight of less than 500 units was established by diffusion experiments (49).

Pea Internodes

Extraction and Formation of Auxin.

1. Extraction Technique. It was now desired to employ the maize endosperm extraction procedure in the study of the second phase of this investigation, the formation of non-acidic auxins. Fresh pea internodes were chosen for this study, because, like maize endosperm, they possess bound auxin; yet, unlike mature maize endosperm, at times they yield a non-acidic auxin upon extraction (10, 31).

Extension of the extraction techniques acquired with maize endosperm into extraction of pea internodes brought up a new problem. Would additional water be required to perform the extraction of fresh tissue, or is the water in the plant sufficient? Since etiolated pea internodes have a high water content the assumption was made that diethyl ether alone would be as effective an extractant as ether plus water. Auxin should be partitioned into the ether from the aqueous phase of the tissue. The hypothesis proved to be correct. Agitation of the tissue had no effect upon the yield with only aqueous ether as the solvent (samples 1 and 2, Table 13). Upon agitation with added water (sample 3) practically the same yield was obtained as with samples 1 and 2; much less auxin was obtained with added water and without agitation (sample 4). Presumably some of the auxin in this sample remained in the increased aqueous

TABLE 13

EXTRACTION OF ETIOLATED PEA INTERNODES WITH VARYING AMOUNTS OF SOLVENTS AND AGITATION TREATMENT FOR ONE HOUR AT 22° C. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Sample	Water (ml.)	Ether (ml.)	Agitation Treatment	µg. eq. IAA
1	0	20	Shaken	12.5
2	0	20	Unshaken	12.0
3	4	20	Shaken	12.0
4	4	20	Unshaken	9.0

phase. As a result of this experiment 20 ml. of ether alone were employed in all ether extractions; agitation of the extraction vessel was continued even though it had no apparent beneficial effects.

Pea extractions of many hours duration were carried out with ether at 22° C. (Table 14) and at 1° C. (Table 15)and at both temperatures with water (Table 16). In all cases extracts were fractionated into acidic and non-acidic ether soluble components.

2. Acidic Auxin. With ether as the extractant at 22° C. (series 1, 2, and 3; Table 14) it was apparent that the largest portion of the acidic auxin was removed the first hour. During the second and third hours the yield dropped to about one-half that of the preceding hour. Very little auxin was obtained during the 3-24 hour period. In series 4 in which only a single change was made there was essentially no yield of acidic auxin. It thus appeared at this temperature that the auxin yield was diminished when the ether was not changed periodically (samples 1-4). The likelihood of a diminished auxin yield at this temperature was known from the literature since the IAA oxidase complex had first been isolated from peas (51, 52, 62). Apparently the reason that the acidic auxin in series 1, 2, and 3 was not destroyed by the IAA oxidase complex was that it had been removed from contact with the tissue and out of the sphere of action of the destroying enzymes, probably before very much IAA had been destroyed.

In series 5, Na-DEDT (100 p.p.m.) was added to the extracting ether, and the production of acidic auxin was quite different. About the same amount was released each of the first five hours. The 5-24 hour period showed a continued steady production resulting in a much

TABLE 14

EXTRACTION OF ETIOLATED PEA INTERNODES WITH ETHER AT 22° C. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 1		Series 2		Series 3		Series 4		Series 5		Series 6	
	µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		100 p.p.m. Na-DEDT µg. eq. IAA		100 p.p.m. Na-DEDT µg. eq. IAA	
	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic
0- 1	12.0	<0.5	8.5	<0.5	13.5	<0.5			2.0	<0.1		
1- 2	6.5	<0.5	4.0	<0.5	7.0	<0.5			1.5	<0.1		
2- 3	4.0	<0.5	3.0	<0.5	3.5	0.5			2.0	<0.1		
3- 4									2.5	<0.1		
4- 5									2.5	<0.1		
5-24	<1.0	22.0	1.5	18.0	1.5	47.5	<1.5	13.0	32.0	<0.1	30.5	<0.1
24 Hr. Total	22.5	22.0	17.0	18.0	25.5	48.0	0	13.0	42.0	0	30.5	0
24-48									2.5	<0.1	3.5	<0.1
48-96					<0.5	9.5			0.5	<0.1	1.0	<0.1

higher 24 hour yield of acid auxin. The initial burst of auxin production was gone, however. This can be explained by assuming that the Na-DEDT inhibited all enzymatic and perhaps other methods of auxin production while only a chemico-physical release mechanism continued.

In series 6, Na-DEDT was added, but there was only a single change of ether. Nevertheless, with IAA destruction stopped by Na-DEDT, the yield of series 6 was far superior to series 4 without Na-DEDT and compared favorably with the frequently changed series 5.

In extractions at 1° C. (Table 15) the same sort of stair step release of acidic auxin prevailed, except that the total amounts were less. In series 7, 8, and 9 there was a large initial burst and then, unlike comparable series at 22° C., a steady release of auxin. In series 10 with a single solvent change, the yield was much greater than in series 4 (Table 14) which had comparable treatment, but at a higher temperature. The yield in series 10 compared favorably with series 7, 8, and 9 which had frequent solvent changes. It is apparent that at 1° C. the IAA oxidase complex was non-functional.

Series 11 and 12 (Table 15) had Na-DEDT added. The 24 hour yield in series 11 was slightly less than that in series 7, 8, or 9. This seemed to indicate that even at 1° C. the Na-DEDT was inhibiting some source of acidic auxin formation. This point was borne out more graphically by the fact that the initial burst of auxin even at 1° C. was reduced to the same steady rate shown by series 11 (Table 14). This initial burst of auxin at 1° C. could not be enzymatically released. Its inhibition by Na-DEDT thus indicates that Na-DEDT was involved in some non-enzymatic inhibition. It is difficult to determine what effect

TABLE 15

EXTRACTION OF ETIOLATED PEA INTERNODES WITH ETHER AT 1° C. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hours	Series 7		Series 8		Series 9		Series 10		Series 11		Series 12	
	µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		µg. eq. IAA		100 p.p.m. Na-DEDT µg. eq. IAA		100 p.p.m. Na-DEDT µg. eq. IAA	
	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic
0- 1	10.0	<0.5	11.0	<0.5	9.0	<0.5	↓	↓	2.0	<0.1	↓	↓
1- 2	2.5	<0.5	2.5	<0.5	2.0	<0.5			2.0	<0.1		
2- 3	1.5	<0.5	1.5	<0.5	0.5	<0.5			1.5	<0.1		
3-24	5.0	<0.5	8.5	<0.5	3.5	<0.5	13.0	<0.5	4.5	<0.1	9.5	<0.1
24 Hr. Total	19.0	0	23.5	0	15.0	0	13.0	0	10.5	0	9.5	0
24-48					1.5	<0.5	2.5	<0.5	8.0	<0.1	6.5	<0.1
48-96					0.5	<0.5	0.5	<0.5	2.0	<0.1	2.5	<0.1

it might be having on the extraction process at present. Further research is now in progress to clear up this point.

The data obtained at 1° C. may shed some light on "free" and "bound" auxin. Since in extractions at 1° C. or 22° C. employing Na-DEDT a very small yield was obtained the first hour (series 5, Table 14; series 11, Table 15) one would be unjustified in calling any more than this amount "free" auxin. Other workers (58, 59, 71) have defined "free" auxin variously. Their rather arbitrary definitions have been criticized on the grounds that what they term as "free" auxin consists, at least in part, of bound auxin (53).

The extraction with water containing 100 p.p.m. Na-DEDT at 1° C. and 22° C. (Table 16) showed, in general, lower yields than comparable extractions with ether (Tables 14 and 15). This conforms with the hypothesis developed earlier that water, not ether, is responsible for auxin extraction. The volume of water was limiting the auxin yield, whereas with ether, extraction conditions were essentially equivalent to having an unlimited supply of water.

Non-acidic Auxin. The production of non-acidic auxin did not commence until about the 4th or 5th hour, at about the time the extraction of acidic auxin was virtually complete (Table 14). It should be mentioned here that while yields of non-acidic auxin are given in µg. equivalents of IAA, one is not actually justified in doing this since it certainly is not IAA and may possess a slightly different activity curve (31). Therefore, the exact amount of the non-acidic auxin may be somewhat different from the recorded values but it is hoped that the relative amounts are fairly accurate. The non-acidic fraction is more variable

TABLE 16

EXTRACTION OF ETIOLATED PEA INTERNODES WITH WATER CONTAINING 100 p.p.m. Na-DEDT. YIELDS OF ETHER SOLUBLE AUXIN EXPRESSED AS MICROGRAM EQUIVALENTS OF IAA PER KILOGRAM OF TISSUE

Hour	Series 13 - 22° C. µg. eq. IAA		Series 14 - 22° C. µg. eq. IAA		Series 15 - 1° C. µg. eq. IAA		Series 16 - 1° C. µg. eq. IAA	
	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic	Acidic	Non-Acidic
0- 1	2.5	<0.5	↓	↓	1.5	<0.5	↓	↓
1- 2	1.5	<0.5			1.5	<0.5		
2- 3	1.0	<0.5			1.5	<0.5		
3-24	2.5	<0.5	9.0	0.5	3.5	<0.5	7.0	<0.5
24 Hr. Total	7.5	0	9.0	0.5	8.0	0	7.0	0

in amount than the acidic fraction. In series 1, 2, and 3 (Table 14) the largest part of the non-acidic auxin was produced during the first 24 hours although there was still some production as long as 4 days after extraction (series 3). In series 4 the production of non-acidic auxin was strongly curtailed. Since some non-acidic auxins can be converted to acidic auxins (21, 31, 32, 33, 55) it is possible that some of the non-acidic auxin was converted to acidic auxin and thereby destroyed.

In confirmation of the investigations of other workers (10, 53) non-acidic auxin was not found at low temperature in ether extractions, or at any temperature in water extractions. In addition, in this study it was found that the presence of Na-DEDT inhibited the formation of non-acidic auxin. Therefore, only series 1-4 (Table 14) yielded non-acidic auxin.

In general, the total activity of the acidic and non-acidic fractions combined (series 1, 2, and 3) about equalled the activity of series 5 which was all acidic (Table 14). There are at least two possible explanations for the disappearance of activity in the non-acidic fraction concomitant with an increase in activity of the acidic fraction upon addition of Na-DEDT. One might assume that the non-acidic auxin in series 1, 2, and 3 was formed in some manner as a decomposition product of IAA. Therefore, upon addition of Na-DEDT, the IAA oxidase complex was inactivated, and the IAA was not broken down into non-acidic auxin. The likelihood of this is diminished by the fact that the decomposition products of IAA have been rather extensively investigated and have always been shown to be inactive or inhibitory as growth substances (51, 62). Furthermore, the data of Table 14 indicate no such trend. In

series 4 in which the acidic auxin was apparently destroyed to the extent that there was no activity left in that fraction, the activity of the non-acidic fraction was not increased; rather it was somewhat reduced. As an alternative and more satisfactory explanation, one might assume that Na-DEDT not only blocks the destruction of IAA, but also blocks the formation of non-acidic auxin. Figure 2 is a compilation from several different sources (28, 34, 37, 39, 55, 56) of the proposed routes of tryptophane to IAA, including all that is known or suspected about the pathways of synthesis and breakdown of two naturally occurring non-acidic auxins, IAc and IAN. Na-DEDT might block one or more of the following enzymes: (1) the dehydrogenase which catalyzes the transformation of tryptophane to indoleiminoacetic acid, a still hypothetical intermediate, (2) the decarboxylase which catalyzes the transformation of tryptophane to tryptamine, or (3) the dehydrogenase and/or decarboxylase which presumably catalyzes the transformation of indoleiminoacetic acid to IAN. These are not unreasonable assumptions since Na-DEDT is known to be quite effective in inhibiting copper-containing enzymes (62), and the above mentioned enzymes may very well be copper-containing enzymes.

The increase of acidic auxin over a 24 hour period upon addition of Na-DEDT may have been a result of decreased destruction of IAA by the IAA oxidase complex; it might also have been, in part at least, the result of the formation of more acidic auxin. If one assumes that the non-acidic auxin was being formed simultaneously along with IAA from a common substrate, probably free tryptophane, then with the blocking of the non-acidic formation system by Na-DEDT more substrate was available for conversion to IAA; hence, the greater activity of this fraction.

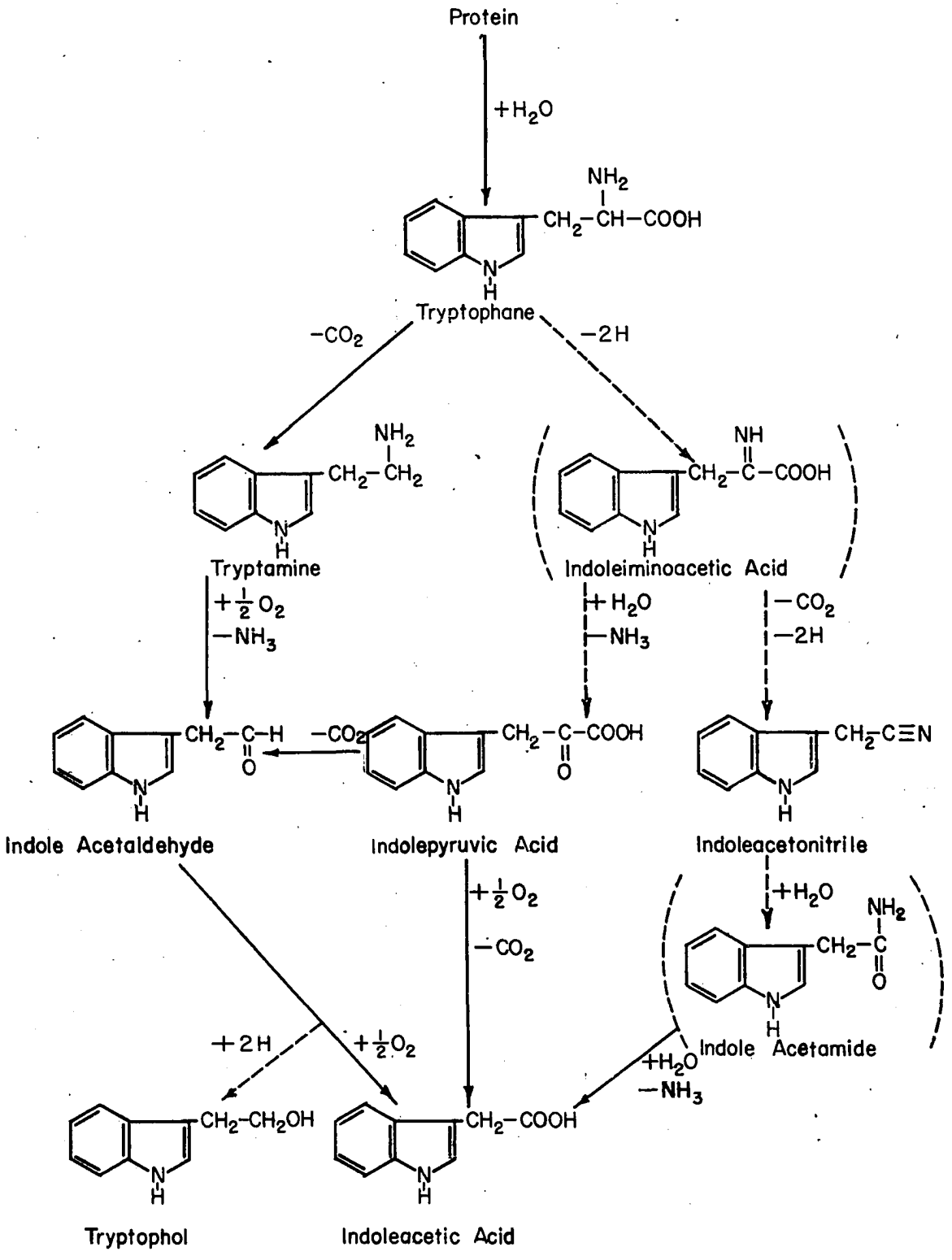

Figure 2. BIOGENESIS OF INDOLEACETIC ACID

If the non-acidic substance is IAN or some non-acidic substance other than IAc, then upon the addition of Na-DEDT the continued formation of the acidic auxin might pass either through IAc or indole-3-pyruvic acid (IPA) on its way to IAA. If, on the other hand, the non-acidic auxin is the intermediate IAc which is merely being trapped in the ether, then with the addition of Na-DEDT, IAA must be formed directly through IPA and never pass through IAc.

Several facts lead one to conclude that most of the non-acidic substance is not IAN. Jones and coworkers (28) were able to isolate crystalline IAN from cabbage rapidly extracted at freezing temperature. However, in peas the non-acidic auxin appears to be formed only during the extraction, only when organic solvents are used, and only at a temperature suitable for enzymatic activity. Thus the same situation certainly does not prevail in peas as in other demonstrated IAN-containing plants. Whatever substance the non-acidic auxin may be, it is apparent that in this variety of peas at least, it is not of great importance in the plant itself and is apparently formed only under special extraction conditions.

Identification of Auxin. No attempt was made to identify the acidic auxin; it was assumed to be IAA. An attempt was made to identify the non-acidic auxin by paper chromatography. Non-acidic auxin from pea internodes, solutions of pure IAA, solutions of pure IAN, and synthetic non-acidic preparations were run on paper chromatograms with various partitioning solvents (Table 17). The paper strips were then sectioned and tested biologically or examined under ultraviolet light and tested chemically for auxin activity.

TABLE 17

RESPONSE OF AUXINS RUN ON PAPER CHROMATOGRAMS TO MODIFIED SALKOWSKI REAGENT

Substance Tested	Solvent System	Approximate R_f	Color of Spot
IAA	Water	0.89	Pink
IAA	Isopropanol - Ammonia - Water	0.49	Pink
IAA	70% Ethanol	0.78	Pink
IAN	Water	0.43*	Blue-Gray
IAN	70% Ethanol	0.84	Blue-Gray
Non-Acidic Fraction From Peas	70% Ethanol		None

* Tailed badly.

Ultraviolet light, at least in the presence of flavo-proteins, is known to inactivate auxin (13). After a few preliminary tests in which activity appeared diminished after exposure to ultraviolet light, duplicate strips were always run, the same strip never being subjected to both treatments. The results with ultraviolet light were inconclusive. The fluorescence of other non-active substances and the relatively large amounts of auxins required for fluorescence (about 1 µg.) limit the use of this technique, especially with naturally occurring auxins. However, striking coincidence with biologically active areas was obtained with the non-acidic isatin preparations. At R_f 0.80 to 0.85 with 70% ethanol there was a dark greenish-brown fluorescence. Some ninhydrin preparations showed a similar, almost black fluorescence at this same location. This is approximately the same region in which the bulk of the biological activity was found in these preparations.

Since the non-acidic auxin was not concentrated enough to yield a spot upon spraying with Salkowski reagent, biological tests alone were used to locate active areas. In Figures 3a, b, and c the results of biological tests are given. The cross-lines represent the sizes of segments eluted for the bioassays. The non-acidic extract from peas was run with different solvents and a suggestion of the R_f values was obtained. In water the R_f was no more than 0.60; in 70% ethanol about 0.85. These values could be the value for IAN. However, in isopropanol-ammonia-water prepared according to Bennett-Clark, Tambiah, and Kefford (6) the non-acidic auxin did not apparently travel at the front while IAN has been reported to do this (47).

The R_f values of IAc, the other outstanding possibility for the

non-acidic auxin, are incompletely known. The only persons who have synthesized IAc with certainty were Brown, Henbest, and Jones (14, 15). Unfortunately, subsequent studies (7) with this pure compound did not include chromatographic analysis. The R_f value of 0.45 for IAc with 70% alcohol as a partition solvent which is commonly quoted (47) is that of Yamaki and Nakamura (72). Their value was supposedly obtained by chemical tests using a benzidine-glacial acetic acid mixture, Van Eck's reagent. Several attempts by the writer to duplicate these results with Van Eck's or Salkowski reagent were futile. Furthermore, Yamaki and Nakamura claimed that, using the procedure of Larsen (31) for the preparation of a non-acidic substance, presumably IAc, they obtained a red spot at a low R_f value coincident with the spot obtained with the native extract. Many attempts by the writer and others (16) to duplicate this procedure have failed. Partitioning with 70% ethanol gave only a brown spot at around R_f 0.85, a value which seems to hold true for many indole compounds (47). It is not possible then to determine the discrepancy between these results and the published report of Yamaki and Nakamura. It should be pointed out, however, that since pure IAc has been shown to be extremely labile (14, 15) and since the method of preparation used by the Japanese workers results in an extremely low yield of uncertainly identified material it seems unlikely that the rather harsh treatment given it by these workers (i.e., drying and redissolving) would result in the microgram quantities necessary for chemical detection. Furthermore, their biological tests were not nearly as conclusive as the chemical tests. Since the biological tests are much more sensitive, they should have been more conclusive if the

substance had actually been IAc.

If one then questions the validity of the low R_f value obtained for IAc by Yamaki and Nakamura, it still remains possible that the native non-acidic auxin is IAc. It is altogether possible that more than one non-acidic auxin is present in the synthetic preparation of non-acidic auxin. Strips 9, 10, 12, and 13 (Figures 3b and c) suggest this. The bisulfite bound and regenerated portion (strips 9 and 12) of the artificial non-acidic auxin had an R_f value quite close to that of other indole compounds. However, strips 10 and 13 containing the portion not bound by bisulfite showed activity also. Hence, this portion of the non-acidic preparation could not be the aldehyde, IAc, but could still possibly be the nitrile, IAN.

One factor, especially in those chromatograms using water as the partition solvent, which decreased the effectiveness of the separation on paper was the fact that tailing occurred to such an extent as to make many strips unusable.

Thus, unfortunately, the identity of both the native non-acidic auxin and the synthetic non-acidic preparation remain a matter for speculation until further investigations are made.

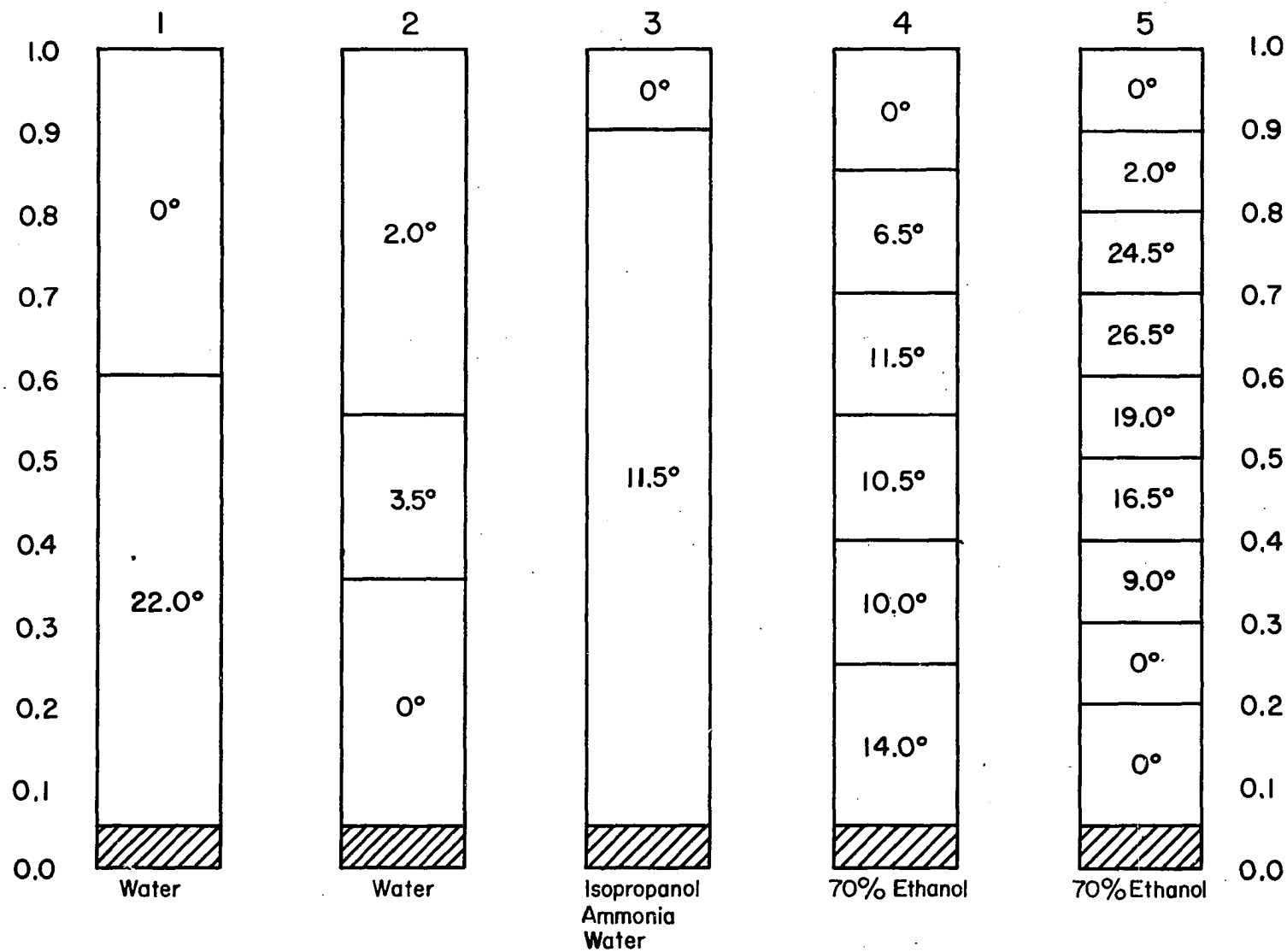

Figure 3a. Activity in the avena curvature test of substances separated by paper chromatography. Non-acidic extract from peas.

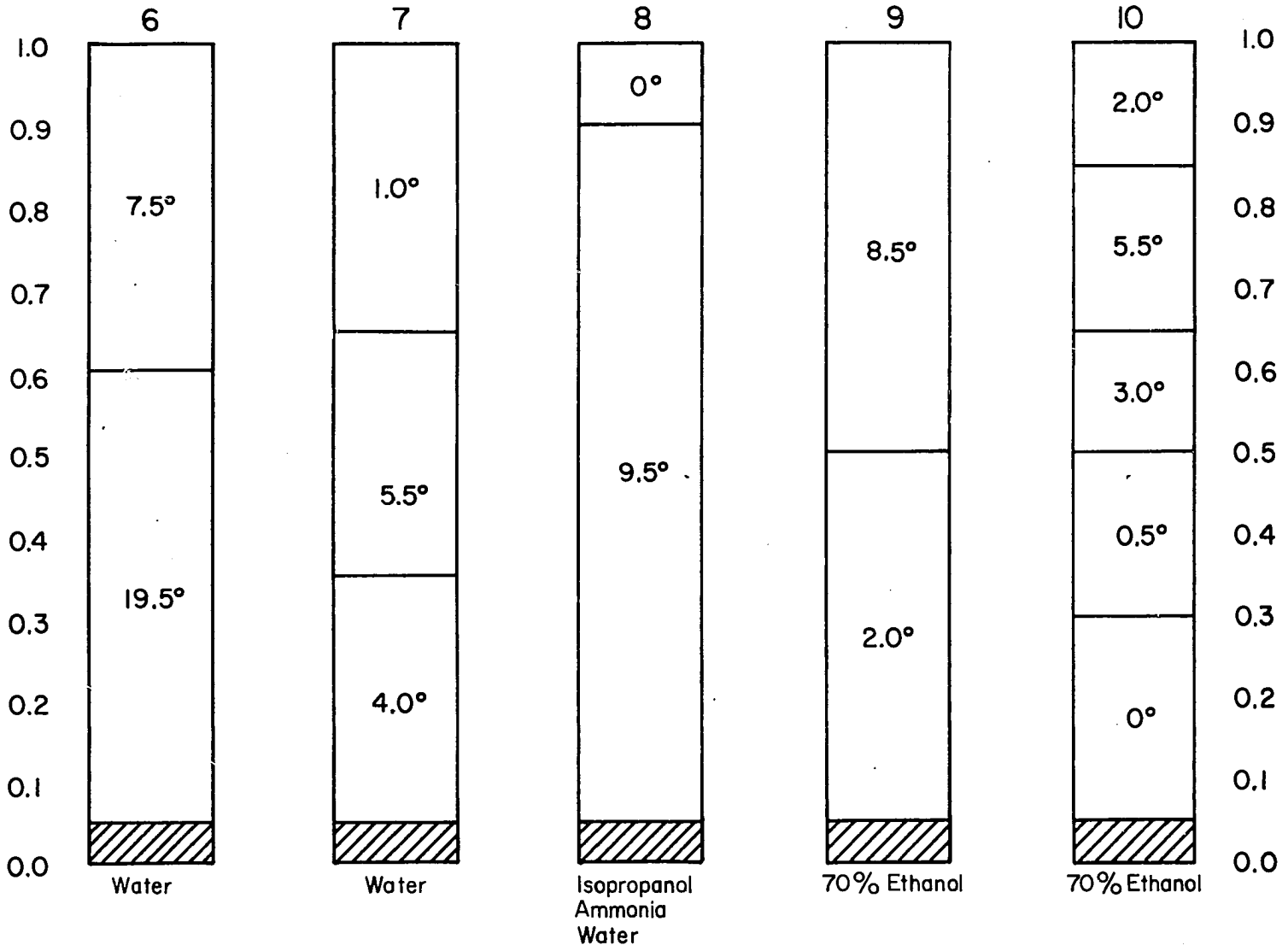

Figure 3b. Activity in the avena curvature test of substances separated by paper chromatography. Non-acidic preparation (I Ac ?) from ninhydrin and tryptophane.

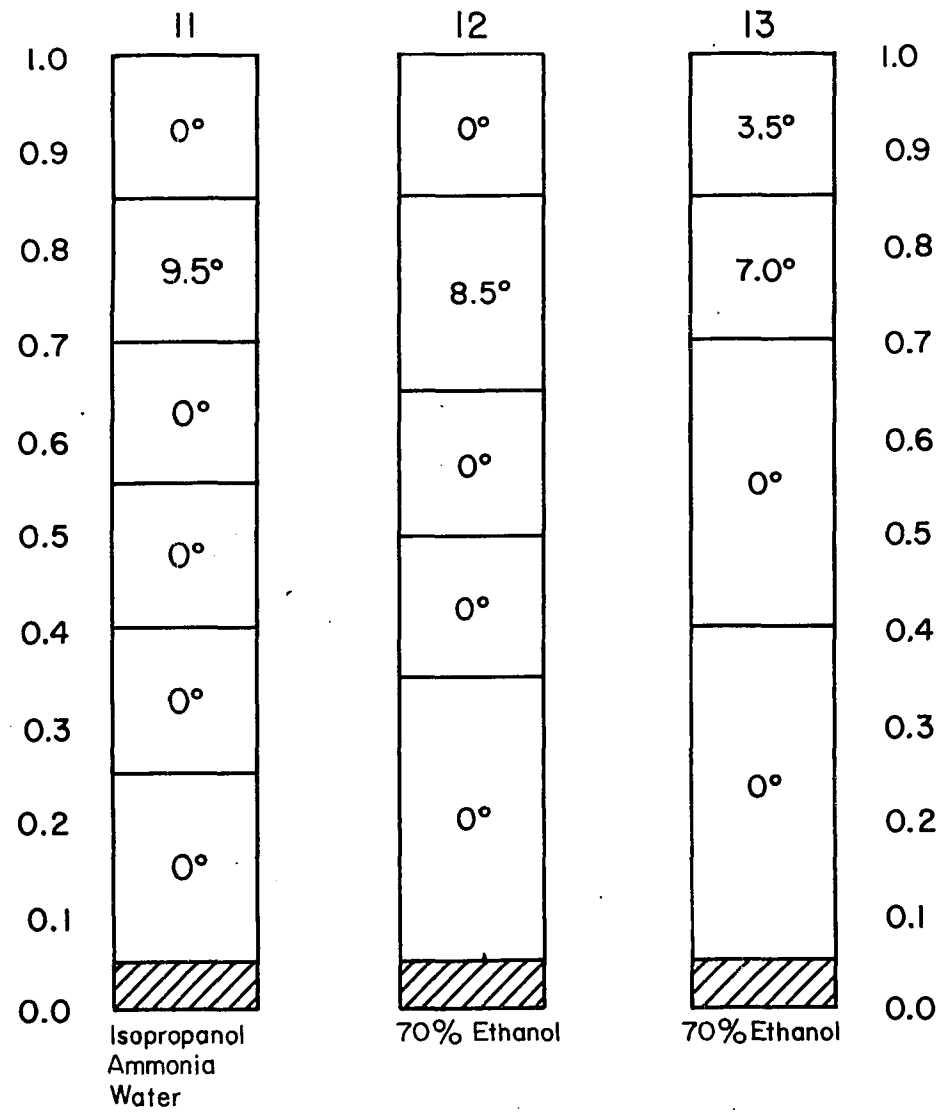

Figure 3c. Activity in the avena curvature test of substances separated by paper chromatography. Nan–acidic preparation (I Ac ?) from isatin and tryptophane.

SUMMARY

1. Attempts were made to devise a satisfactory procedure for extracting auxin from plant tissues. A standard extraction procedure employing an ether-water mixture under constant agitation was adopted.

2. The water volume was found to limit auxin extraction. Apparently auxin was extracted by the water and then partitioned into the ether. Partitioning was improved by continuous agitation of the ether-water mixture.

3. Auxin yield was not increased by any of the following treatments: (1) the addition of acid to the water, (2) the use of inactive non-acidic ether, (3) the addition of fresh ether, or (4) repeated rinsing with fresh ether.

4. Auxin yield from maize endosperm was greater during the first few hours at 22° C. than at 1° C. even though destruction of auxin was demonstrated at the higher temperature. Sodium diethyldithiocarbamate (Na-DEDT) inhibited this destruction.

5. Maize endosperm auxin apparently exists in three states: (1) a small ether-soluble portion readily extractable with ether, (2) a larger loosely-bound ether-soluble portion extractable only with water, and (3) an auxin complex which could be extracted with water but was soluble in ether only after drastic alkaline hydrolysis.

6. Non-acidic auxin was not found. The acidic auxin, both the

ether-soluble and the auxin complex hydrolysate, gave chemical and biological tests identical with indoleacetic acid (IAA).

7. In further investigations fresh etiolated pea internodes were extracted and the formation and significance of the auxins thus obtained were studied.

8. It was not necessary to add water or agitate fresh pea tissue to obtain maximum auxin yields with ether as an extractant; however, if water was added agitation became essential for maximum yields.

9. With pea internodes, as in maize, more acidic auxin was obtained at 22° C. than at 1° C. during the first three hours of extraction. After three hours the yield of auxin was greater at 1° C. owing to the inactivation of the IAA oxidase complex at this temperature.

10. Na-DEDT (100 p.p.m.) inhibited auxin destruction and the formation of non-acidic auxin. It also appeared to have other inhibitory effects upon extraction thus reducing the amount of auxin which could be called "free" auxin.

11. Non-acidic auxin production occurred only during extraction with organic solvents at temperatures favorable for enzymatic activity. Thus, it appeared to be an extraction artifact of little significance *in vivo*.

12. Acidic auxin increase with Na-DEDT over a long extraction period probably resulted from decreased auxin destruction and increased acidic auxin formation. The latter event presumably occurred because the non-acidic formation system was blocked and there was subsequently a greater availability of the common substrate.

13. Identification of non-acidic auxin by ultraviolet fluores-

cence and chemical tests was unsatisfactory. Biological tests in conjunction with paper chromatography were more satisfactory, but inconclusive. Possibly the non-acidic auxin was indole acetaldehyde or indole acetonitrile or perhaps a mixture of the two.

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