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AN ARTIFACT IN CHROMATOGRAPHY OF INDOLIC AUXINS[1, 2, 3]

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During investigations on the chromatographic separation of natural auxins an artifact was discovered which could be a cause of many errors. In extracts of grapes, peaks of biological activity appeared on paper chromatograms at the positions of 3-indolylacetonitrile (IAN) and the ethylester of 3-indoleacetic acid (IAE) (2, 3). In new experiments with Concord grapes (5) peaks of biological activity were found again at the same Rf's as IAN and IAE. These regions were cut out, eluted from the paper, and the eluates re-chromatographed separately. Each of the two new chromatograms always showed at least two peaks of activity located at the IAN and IAE positions, which suggested an interconversion between the two active auxins. A similar apparent interconversion was reported for the water-soluble growth substances extracted from tomato roots (1). The apparent interconversion occurring between the auxins of the grape extracts, however, existed only when a control paper strip, spotted with synthetic IAN and IAE, was present together with the strip spotted with the extract in the same chromatographic tube. A thorough investigation of this situation was undertaken.

METHODS

Ascending chromatography in the dark was used throughout. Two-centimeter wide strips of Whatman No. 3 MM paper were hung in large tubes according to a technique described previously (2, 3). One hundred ml of solvent was poured into each 65 × 500 mm chromatographic tube. It has been shown (2, 3) that hexane and other water-insoluble hydrocarbons can separate neutral auxins such as IAN and IAE, provided enough water impregnates the fibers of the paper. To increase the amount of water present in the solvent, a mixture of petroleum ether (bp 35–70° C), chloroform and water was used. It gave reproducible Rf's which could be modified at will by changing the ratio of petroleum ether to chloroform, the water added being always 10 % (v:v). Thus, when this ratio was 85: 5, the Rf's of IAN and IAE were 0.15 and 0.55, respectively, as compared with 0.30 and 0.70 when the ratio was 80: 10, and 0.45 and 0.85 when it was 75: 15. Unless indicated otherwise all the paper strips were equilibrated overnight (about 15 hrs) over the solvent (at 25° C) before actually running the chromatograms.

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In order to determine the location of the auxins on the paper, the oat first-internode test was used (4). The paper strips were cut into 20 equal segments each of which was then incubated with 10 first-internode sections of Brighton oats in test tubes containing 1 ml of pH 5.0 citrate-phosphate buffer (4). The tubes were rotated at 1 rpm at 25° C in the dark for 20 to 24 hours. At the end of this period, the first-internode sections were measured to the nearest 0.1 mm. The average elongation over the initial 4 mm length was plotted against Rf to give the histograms of figures 1 to 6. In these histograms any deviation from the controls (dotted lines) which is equal to or larger than 0.2 mm is statistically significant.

Results

I. Effect of Control Strip in Chromatographic Tubes: Two blank paper strips were hung in clean tubes over fresh solvent. One tube contained the blank strip alone, the other contained, in addition, a control strip which had been spotted with a mixture of 10 μg each of 3-indolylacetic acid (IAA), 3-indolylbutyric acid (IBA), IAN, and IAE. After equilibration and chromatography in the petroleum ether solvent, the blank paper strips were dried in an air current in the hood and cut into 20 1-cm pieces for bioassay. The bioassay showed at least three auxin peaks on the blank paper which had been chromatographed with the control strip. These peaks were located at the Rf's where the synthetic auxins had moved on the control paper (fig 1, top). The highest auxin activity occurred at the IAN position. The blank paper which had been chromatographed without a control strip showed no biological activity (fig 1, bottom). These results were obtained over and over again. With the petroleum-ether solvent or a hexane: water (90: 10 v/v) solvent, peaks of biological activity always appeared at the IAN and IAE positions when a control strip spotted with synthetic IAA, IAN, and IAE was chromatographed together with another strip. At the IAA position, however, biological activity was detected only rarely and remained very low.

The transfer of biological activity from one paper to another occurred also with solvents such as isopropanol: ammonia: water (80: 10: 10 v/v), but to a smaller degree. Thus, biological activity appeared on blank paper strips when they were chromatographed in the isopropanol solvent together with control strips spotted with a mixture of IAA (2 μg), IBA (1 μg), IAN (10 μg), and IAE (5 μg). The biological activity appeared at the IAN and IAE position. No activity could be detected on blank strips when controls were absent (fig 2). The *n*-butanol: ammonia: water solvent used by Britton et al (1) in their studies of the interconversion of auxins X, Y, and Z gave also indications of a movement of biological activity from one paper strip to another, but the amounts of auxins found on blank papers chromatographed with control strips were very low.

The experiments clearly indicate that each time a control strip spotted with synthetic indolic auxins was added to a tube containing a blank paper, some biological activity could be found on the blank chromatogram. This effect was generally most pronounced with IAN and with solvents containing hexane or petroleum ether.

II. Effect of Solvent During Equilibration: A possibility that suggested itself was that some of the auxins spotted on the control strips were in fact distilling over to the solvent in the tube, and that this solvent was then carrying the auxins to the blank strip. In order to check this possibility, a blank strip (A) (fig 3, top left) was first equilibrated over a petroleum ether: chloroform: water (80: 10: 10) solvent together with a control strip (C_1) spotted with IAA (2 μg) + IBA (1 μg) + IAN (8 μg) + IAE (4 μg). After about 15 hours the blank paper (which never touched the solvent) was taken out and rapidly transferred to another tube without a control strip. After 4 hours of re-equilibration in the second tube, the blank strip A was lowered into the solvent and chromatographed. Biological activity showed up on blank strip A, indicating that a transfer of auxins had taken place from the control strip C_1 to A during the first equilibration period and without the intermediary of the liquid phase of the solvent contained in the first tube.

To investigate this paper-to-paper transfer further, another experiment was set up in which a blank strip B was equilibrated with a control C_2 similar to C_1 in a tube without any solvent. After about 15 hours, strip B was transferred to a new tube containing the petroleum ether solvent, re-equilibrated for

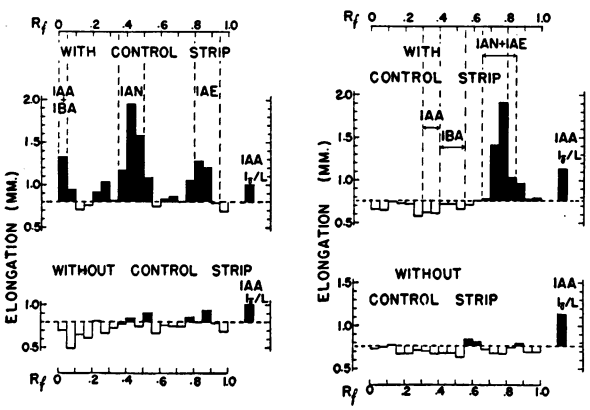

Fig. 1 (*left*). Histograms of two blank paper strips chromatographed in petroleum ether: chloroform: water (75: 15: 10) in the presence of a control strip spotted with synthetic auxins (*top*), or with no control strip (*bottom*).

Fig. 2 (*right*). Histograms of two blank paper strips chromatographed in isopropanol: ammonia: water (80: 10: 10). (*Top*): control strip with synthetic auxins present in the same tube. (*Bottom*): no control strip.

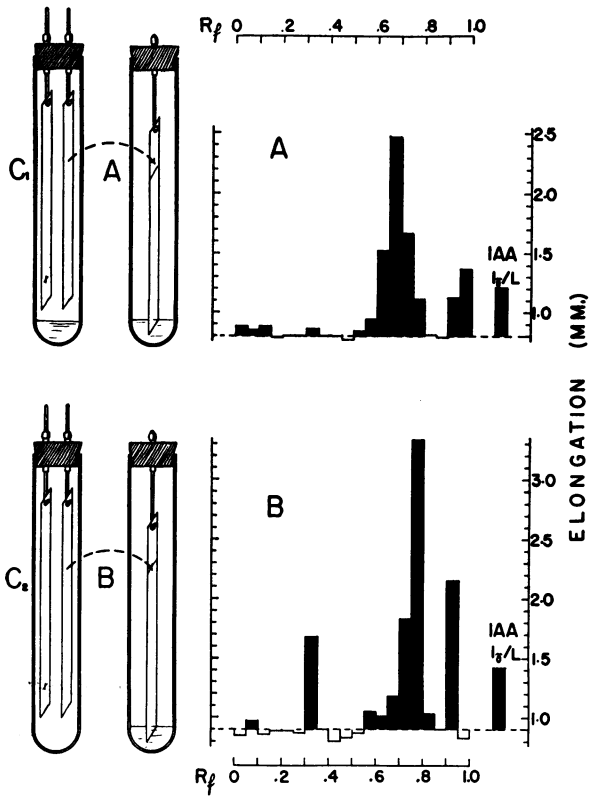

FIG. 3. Transfer of synthetic auxins from one paper to another during equilibration with (*top*) and without (*bottom*) a solvent in the equilibrating tube. C_1 and C_2: control papers spotted with synthetic IAA, IBA, IAN, and IAE. A and B: blank papers first equilibrated with the controls for 15 hours, then moved to a different tube for chromatography in petroleum ether: chloroform: water (80: 10: 10). The histograms show the biological activity found on the blank papers after chromatography.

4 hours, and chromatographed. This blank strip also was found to have clear-cut biological activity (fig 3, bottom).

From these two experiments one can conclude that transfer of biological activity from a control paper strip spotted with indolic auxins to a blank paper occurs through the air, without an intermediary solvent. It seems to be a sort of distillation or sublimation from the spot of the control strip to the blank strip.

A point which remained a puzzle for a long time was the location of the biological activity on the blank strips: it occurred generally at the same Rf's as on spotted chromatograms. This effect was found to result from our habit of hanging the two strips parallel to each other. Thus the indolic compounds could move horizontally across the 3 to 4 cm-wide vapor phase during the equilibration period and impregnate the blank paper at the same position as that of the spot on the spotted strip. An experiment in which the blank strip was equilibrated with its lower end lower than that of the spotted strip resulted in a correspondingly higher Rf of the biological activity on this blank strip.

III. EFFECT OF SHIELDING: If there is a horizontal transfer of auxin from one paper strip to another, then placing a shield between the two strips should prevent it. This possibility[5] was tested in the following way: A 2-cm wide paper strip was spotted with 8 μg of IAN and hung in a chromatographic tube over hexane: chloroform: H_2O (75: 15: 10) parallel with a blank strip. Between the two, a 4-cm wide paper strip (Whatman 3 No. MM) was interposed. After equilibration for about 15 hours at room temperature, the blank strip and the shield were bioassayed. The shield showed a definite growth-promoting area corresponding to the IAN which had moved horizontally from the spotted chromatogram. On the contrary, the blank strip behind the screen, had not picked up any biological activity. These results confirm the existence of a horizontal transfer of substance through a vapor phase. The moving auxin is picked up by the first surface it hits. Protection from contamination by this process can be achieved by the use of a paper shield.

IV. EFFECT OF LENGTH OF EQUILIBRATION PERIOD: Since the transfer of auxins from one pa-

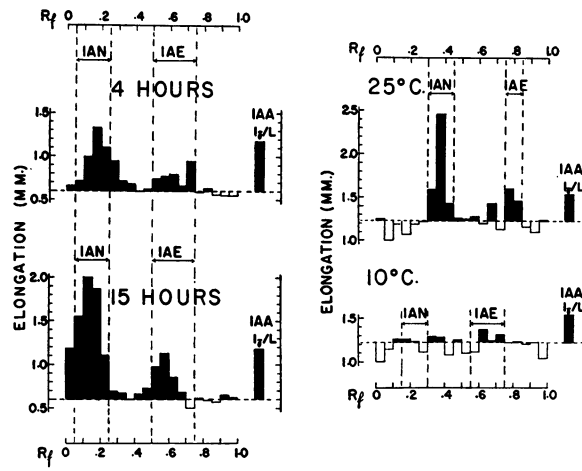

FIG. 4 (*left*). Effect of the duration of the equilibration period. Histograms of two blank paper strips chromatographed in petroleum ether: chloroform: water (85: 5: 10) together with a control strip spotted with IAA, IBA, IAN, and IAE. Equilibration times over the solvent: 4 hours (*top*) and 15 hours (*bottom*).

FIG. 5 (*right*). Effect of temperature. Histograms of two blank paper strips chromatographed in the presence of control strips spotted with IAA, IBA, IAN, and IAE in petroleum ether: chloroform: water (75: 15: 10) at 25° C (*top*) and 10° C (*bottom*).

[5] Suggested by Dr. E. M. Shantz.

per to another occurs mainly during the equilibration period, an experiment was performed to find if the duration of the equilibration period had any effect upon the amount of auxins transferred. Two blank paper strips were equilibrated over petroleum : ether : chloroform : water (85 : 5 : 10) in two different tubes, each together with a control strip spotted with IAA (2 μg) + IBA (1 μg) + IAN (10 μg) + IAE (5 μg). The first strip was equilibrated for 4 hours, the second for 15 hours before chromatography. The results (fig 4) showed that transfer of IAN and IAE had occurred already after 4 hours of equilibration; the amounts of auxins transferred increased with time. A quantitative estimation indicated that there was ten times more IAN and IAE transferred after 15 hours than after 4 hours.

V. Effect of Temperature: If the process by which indolic auxins are transferred from one paper strip to another resembles a distillation, it must be affected by temperature. Two blank paper strips were chromatographed, each in a different tube and each with a control strip spotted with 10 μg each of IAA, IBA, IAN, and IAE. The solvent was petroleum ether + chloroform + H_2O (75 : 15 : 10). The equilibration time was approximately 15 hours in both cases, but one tube was maintained at 25° C and the other at 10° C. The results are shown in figure 5. At 25° C, there was a clear-cut transfer of IAN and IAE, but not at 10° C. Similar results were obtained with isopropanol : ammonia : water (80 : 10 : 10). A fortiori, no transfer of IAN or IAE occurred when equilibration and chromatography were performed at the even lower temperature of 1° C.

VI. Effect of Used Solvent: Although it has been shown that most of the transfer of indolic auxins from one paper to another could occur in the absence of any solvent, one expects that if indole compounds can move from one paper to another, they can also distill from the paper to the solvent. To check this point, a blank strip was chromatographed in a petroleum ether-chloroform-water (80 : 10 : 10) mixture which had been used once previously to chromatograph three strips, each spotted with IAA (2 μg) + IBA (1 μg) + IAN (8 μg) + IAE (4 μg). There was no control strip in the tube during the chromatography of the blank strip. Biological activity was present on the blank strip (fig 6), whereas no such activity could be detected on similar blank chromatograms run in fresh solvent.

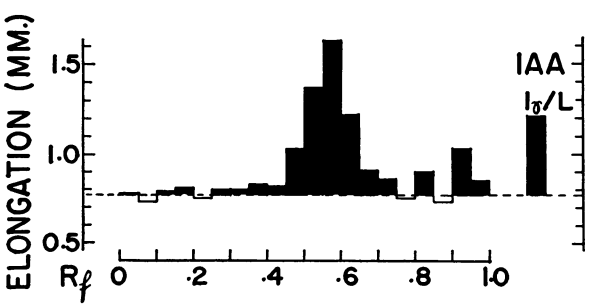

Fig. 6. Histograms of a blank paper strip chromatographed in petroleum ether : chloroform : water (80 : 10 : 10) without a control but in a solvent which had been used once before to run three chromatograms spotted with IAA, IBA, IAN, and IAE.

Conclusions

These experiments show that misleading results may be obtained by running control strips spotted with synthetic indolic auxins together with other chromatograms (the control strips may lose some of the auxins to other chromatograms in the same tube). Among the auxins tested, IAN seemed to transfer with the greatest ease; IAE was second easiest, and IAA generally did not appear to be transferred. It is expected that other indolic compounds such as indole itself, could be transferred with even greater ease. Re-using solvents in which chromatograms have been run before (which is commonly done in many laboratories) is also a source of these artifacts.

In view of these results, we have re-examined some of our previously published data. The results of Nitsch and Nitsch (3) and Nitsch (2) in which plant extracts have been chromatographed in the presence of control strips spotted with IAA, IAN, and IAE have to be interpreted in the light of the present findings. Thus it is possible that the IAN and IAE claimed to have been found in Tokay grapes may actually be artifacts since these auxins were not detected in Concord grapes (5) when no control strips were present. The IAN and IAE peaks found in chromatograms of tissue cultures of *Parthenocissus tricuspidata* (2, fig 22) are most likely to be artifacts also. The consideration of these peaks as artifacts would actually make our previous results more logical, in that the difference between normal, habituated, and crown-gall tissues would lie in differences in the amounts of auxins located at Rf's 0.3 and 0.5 which are not artifacts.

To be certain to obtain chromatograms free of artifacts of the kind reported here, one should: A. Use one chromatogram per tube with the control strip in a separate tube, and B. Use fresh solvent in a clean tube each time. However, low temperatures (1 to 10° C), at which little transfer occurs, provide a means of running several chromatographic strips in the same tube, including a control with synthetic IAN and IAE, without the apparent transfer of compounds.

Summary

Indolic auxins, especially 3-indolylacetonitrile and the ethyl ester of 3-indolylacetic acid, have been shown to move from one chromatographic paper strip to another. This transfer of active substances occurs during equilibration and is not dependent upon the presence of a solvent.

The transfer phenomenon is dependent upon temperature and the length of the equilibration period; it is particularly marked with solvents containing hexane or petroleum ether. Use of the same solvent repeatedly for several chromatographic runs also produces artifacts and should be avoided. Methods of circumventing these difficulties are described.

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PHYSIOLOGICAL STUDIES ON NODULE-NITRATE REDUCTASE[1, 2]

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A previous publication (9) reported the occurrence and some of the properties of a reduced diphosphopyridine nucleotide (DPNH) nitrate reductase associated with Rhizobium cells from nodules of several different legumes. Further studies on properties of the nitrate reductase system from soybean nodules (7) have shown that succinate, leuco-methylene blue, and reduced benzylviologen also will serve as electron donors. In addition, evidence has been obtained indicating that either menadione (2-methyl-1, 4-naphthoquionone), or a cofactor from Rhizobium cells, is necessary in this system for the transfer of electrons from DPNH to nitrate (5, 6, 7). When succinate is used as the electron donor, the cofactor is not required.

Physiological experiments (5) have indicated that the nitrate reductase activities of Rhizobium cells from nodules of soybean plants inoculated with different strains of *Rhizobium japonicum* are correlated positively with nitrogen-fixing efficiencies of the various strains. When soybean plants were inoculated with pure cultures of Rhizobia and grown aseptically in the absence of a source of combined nitrogen, they produced nodules with high nitrate reductase activity. As yet, the possible relationship, if any, of nodule-nitrate reductase to the process of nitrogen-fixation remains obscure. The possibility has been considered (5, 7) that the nodule-nitrate reductase is non-specific for oxidant, and that nitrate is not the natural substrate for the enzyme.

This manuscript describes some results of additional physiological experiments in which the effects of various factors on the specific activity of the succinate-nitrate reductase in nodules have been studied. In general, experiments have been designed to determine whether those factors reported to influence the fixation of nitrogen also influence the activity of the nodule enzyme.

Materials and Methods

Culture Methods: Sand-culture was used in growing the leguminous plants for the experiments reported in tables I, II, III, and IV, and water culture for experiments 5 and 6 where deficiencies of soybean plants were investigated. Soybean seeds (*Glycine max* (L.) Merr. var. Lee) or seeds of other legumes indicated in table I were inoculated with commercial nitrogen-fixing inoculum and sown in flats of sand for producing seedlings for experiments 1, 2, 3, 4, and 5. Flats were supplied daily with half strength nitrogen-free nutrient solution (14). After 5 to 8 days, the seedlings were transferred to the culture vessels used in the various experiments. Soybean seedlings used for Mo deficiency experiments

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