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On the question of the integration of exogenous bacterial DNA into plant DNA

(eukaryotic transformation/foreign DNA integration/bacterial contamination)

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ABSTRACT **Extensive studies with pea, tomato, and barley failed to confirm the evidence presented by previous investigators for integration or replication of exogenously applied bacterial DNA in these plants. Labeled DNA of buoyant density in CsCl intermediate between that of high density donor bacterial DNA and of plant DNA was never observed with axenic plants. Intermediate peaks, similar to those used as evidence for recombination by earlier investigators, were observed only when the plants were contaminated with bacteria. Plant DNA prepared by a published procedure [Ledoux, L. & Huart, R. (1969) *J. Mol. Biol.* 43, 243–262] was found to be contaminated with unidentified impurities. Such DNA was partially protected from the action of DNase and produced aberrant banding patterns in CsCl after shearing. Much of the published evidence for integration of foreign DNA in plants is based upon experiments with plant DNA prepared by this procedure. We conclude that contamination is the likely explanation for what has been interpreted as evidence for integration.**

Foreign DNA uptake and its integration and replication in plants have been reported for several plant systems by Ledoux and his collaborators (1–3). Double stranded segments of several types of donor bacterial DNA are said to be covalently joined with recipient plant DNA in an alkali-stable linkage, and then preferentially replicated during subsequent growth of the plants (1–3). This phenomenon is of extreme theoretical interest and could serve as the basis for an entirely new approach to crop plant improvement.

The evidence supporting the integration model is indirect. DNA species were identified solely on the basis of buoyant density in CsCl, and not by a base-sequence-dependent technique such as nucleic acid hybridization. The indirect evidence can be summarized as follows. Donor bacterial DNA of high density is administered to plant seeds or shoots, which are then exposed to [^{3}H]thymidine. A crude DNA extract is prepared from various plant tissues and subjected to CsCl density gradient centrifugation. A prominent peak, often over half of the radioactivity, is reported to occur at a density intermediate between that of donor bacterial DNA and recipient plant DNA. This "intermediate peak" has been taken to represent recombinant molecules. Upon denaturation, the radioactivity in the intermediate peak shifted as a single peak toward higher density. After sonication, radioactivity in the intermediate peak splits into two components with densities corresponding approximately to donor and recipient DNA. In some experiments, radiolabeled bacterial DNA is used without subsequent thymidine addition, and a major intermediate peak is observed. Thus, in both types of experiments, density in CsCl was the only evidence for the occurrence of bacterial–plant DNA recombinant molecules.

It was our plan to isolate the intermediate peak and assay it for donor base sequences by DNA·DNA hybridization. If its intermediate density were a consequence of linkage between light plant and heavy bacterial DNA, then it should hybridize to donor as well as recipient DNA. In the experiments reported here using axenic plants, we did not detect any intermediate peaks similar to those reported by Ledoux. However, when precautions against bacterial contamination failed, we observed prominent intermediate peaks. Such peaks, ascribable to bacteria growing on the roots, were the only results we ever obtained which resembled those taken as an indication of linkage of donor and recipient DNA.

Abbreviations: SSC, 0.15 M sodium chloride–0.015 M trisodium citrate; X SSC means multiples of SSC concentration; TCA, trichloroacetic acid.

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METHODS

Treatment of Pea Seeds and Seedlings with Donor DNA. Donor DNA was prepared in three ways from *Pseudomonas aeruginosa* grown in nutrient broth. (*i*) For experiments with intact seedlings and excised shoots, DNA was extracted by a standard procedure (4) involving chloroform deproteinization. (*ii*) A stationary phase culture, frozen and thawed four times or untreated, was centrifuged and the supernatant filtered sterilely. These sterile filtrates were used with intact seedlings and with excised shoots because of the interpretation of Stroun *et al.* (5) that an intermediate peak in tomato seedlings could be caused by the uptake and integration of DNA from bacterial autolysates. (*iii*) Preparative CsCl gradient centrifugation was used, after ribonuclease and pronase treatments (6). DNA prepared this way was used with pea seeds on the recommendation of L. Ledoux.

Pea (*Pisum sativum*, L. cv. Alaska) seeds were surface sterilized by sequential 5 min exposure to 1% Turgicide G (a cationic, quaternary ammonium disinfectant, 1% Turgicide K (an anionic, phenolic disinfectant), and 1% sodium hypochlorite. *Experiment A.* To 20 five-day-old seedlings was added either 4 ml of donor DNA (100 μg/ml) in 0.03 × SSC (SSC is 0.15 M NaCl–0.015 M sodium citrate) or 3 ml of culture filtrate. After 51 hr, the roots were washed, 75 μCi of [^{3}H]thymidine (40–60 Ci/mmol, New England Nuclear) in 4 ml water added, and tissue harvested 39 hr later. Roots and shoots were separated and DNA was extracted from each. *Experiment B.* Shoots from 20 nine-day-old seedlings were excised just above the cotyledons and placed cut end

down in 2 ml of donor DNA (100 μg/ml) in quarter-strength Hoagland's mineral solution no. 2 (7) containing 500 units/ml of penicillin G and 5×10^{-4} M of chloramphenicol. The plants were exposed to air to facilitate transpiration. After 17 hr, all the liquid had been taken up by the shoots (less than 0.5 ml evaporated from an adjacent vessel without shoots) and 100 μCi of [^{3}H]thymidine was added for labeling periods of 24 and 72 hr before harvest. A similar experiment was done with fifteen 12-day-old excised shoots by administering culture filtrate for 43 hr followed by 26 hr of labeling with 17 μCi of [^{3}H]thymidine. *Experiment C.* Groups of 20 seeds were given 2 or 3 ml of donor DNA (0–760 μg/ml). After an overnight imbibition, all the liquid was absorbed by the swollen seeds; water was added minimally thereafter. After 8, 9, or 12 days, [^{3}H]thymidine (160 or 250 μCi) was added for various times before harvest. DNA was extracted from roots.

Treatment of Tomato Shoots with Donor DNA. Donor DNA was isolated (8) from *Agrobacterium tumefaciens* ATCC 15955. Six-week-old tomato plants (*Lycopersicon esculentum* Mill., cv. Smith's Special) were surface sterilized in the lower stem region by scrubbing with 1% sodium hypochlorite and then with 70% ethanol. The cut stem was placed in 1 ml of sterile donor DNA (200 μg/ml) or sterile 0.1 × SSC (1). After 48 hr, the plants were given 100 μCi of [^{3}H]thymidine for 28 hr before harvesting. Plating of the labeling solution on nutrient agar yielded no colonies. DNA was isolated from plant tissue as described (1) except that ethanol-precipitated DNA was collected by spooling rather than centrifugation and the Pronase treatment was omitted (the phenol treatment was included).

Treatment of Barley Seeds with Donor DNA. Donor DNA used in Seattle was prepared (8) from *Micrococcus lysodeikticus*. *Sarcina flava* donor DNA used in Mol, Belgium was a gift of L. Ledoux. Barley (*Hordeum vulgare* L., cv. Himalaya and Proctor) seeds were surface sterilized in Seattle as described for pea seeds. Barley seeds (cv. Himalaya, Proctor, Trait d'Union) were surface sterilized in Mol by stirring for 2 hr in 50% (v/v) H_2SO_4 and then in 5% calcium hypochlorite for 30 min. DNA was administered to 30–60 sectioned seeds as described earlier (2).

Extraction of DNA from Plant Tissues. Pea roots or shoots were ground in a mortar with about 1 ml of 1 M NaCl–0.1 M NaEDTA at pH 8.0–1% sodium dodecyl sulfate per g of tissue. The extract was heated for 5 min at 70–72°, cooled, made 1 M in $NaClO_4$, and subjected to deproteinization with chloroform, to spooling of ethanol-precipitated fibers (hereafter termed "spooling"), and to ribonuclease and Pronase (1 mg/ml, 37°, 1–3 hr) digestions as detailed elsewhere (4). DNA was extracted from barley roots by a Pronase procedure (2). In some cases, phenol deproteinization and spooling were substituted for the Pronase treatment and the collecting of ethanol-precipitated fibers by centrifugation.

Density Gradient Centrifugation. Gradients were formed in a Spinco 65 rotor in 4.6 ml of CsCl solution containing pea DNA by centrifuging at 40,000 rpm for 48 hr in 0.01 M Tris (pH 8.6) at 25°. Before centrifugation, the DNA in CsCl–Tris solution was lightly sheared by forcing it three times through a 26-gauge needle on a 5 ml syringe at maximum thumb pressure. This permits fractionation of large amounts of DNA without artifactual skewing of the peak (4, 9). DNA for barley experiments was centrifuged in 5 ml of CsCl solution in a Spinco 40 rotor at 35,000 rpm for 60–64 hr. Fractions were collected from the tube bottom.

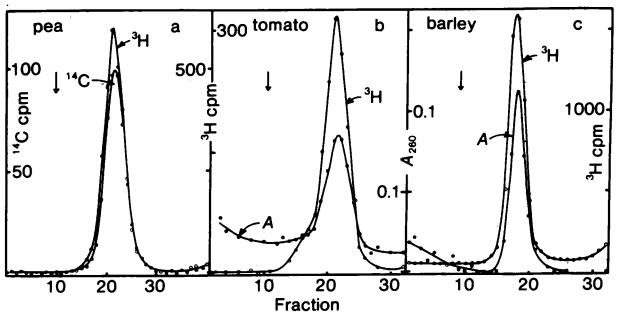

FIG. 1. CsCl gradients of DNA from pea, tomato, and barley treated with bacterial DNA. (a) Pea seeds were germinated in 522 μg of *P. aeruginosa* DNA per ml for 8 days followed by incubation for 6 hr with [^{3}H]thymidine. Root DNA (79 μg, 220 cpm of ^{3}H per μg) was mixed with 5 μg of DNA (700 cpm of ^{14}C per μg) from peas labeled for 7 days with [^{14}C]thymidine. Of each fraction, 26% [13% in (c)] was dried on paper discs for counting. Arrow shows position of donor DNA determined in a parallel gradient of mixed donor and ^{14}C-labeled pea DNAs. Results are essentially identical for 0, 33, 65, 130, 261, and 522 μg of donor DNA per ml. (b) DNA (21 μg, 2500 cpm/μg) from tomato shoots which were given 200 μg of *A. tumefaciens* DNA per ml for 48 hr and were then incubated for 28 hr with [^{3}H]thymidine; 13% of the radioactivity (insoluble in 10% TCA) of each fraction was counted. Absorbance was measured after dilution to 57% [44% in (c)] with water. Arrow shows position of donor DNA, as in (c), determined from refractive index measurements. (c) DNA (29 μg, 2900 cpm/μg) from Himalaya barley roots was centrifuged after the following procedure: seeds were treated for 24 hr with water, 7 hr with *M. lysodeikticus* DNA (270 μg/ml), 3 days with water, and 3 hr with [^{3}H]thymidine (70 μCi/ml). Gradient profiles were essentially identical to these depicted here for pea, tomato, and barley control (no donor DNA) experiments.

DNase sensitivity was tested at 100 μg of enzyme/ml in 0.04 × SSC–2 mM sodium phosphate buffer at pH 7.0–4 mM $MgCl_2$. After 45 μl samples were incubated for 30 min at 37°, 5 μl of 1 M NaOH was added and the material layered onto sucrose gradients (4.8 ml of 5–20% sucrose in 0.9 M NaCl–0.1 M NaOH). Centrifugation was at 25° in a Spinco SW 50.1 rotor for 5 hr at 39,000 rpm. To each fraction, 100 μg of bovine serum albumin was added and trichloroacetic acid (TCA) insoluble radioactivity assayed in toluene-Liquifluor (New England Nuclear).

RESULTS

Experiments with Peas. After pea seeds were allowed to germinate in unlabeled bacterial DNA, we added [^{3}H]thymidine to monitor subsequent DNA synthesis. No DNA peak of intermediate density was ever observed. Typical results are shown in Fig. 1a. The slight, symmetrical shift of ^{3}H to a higher density was seen in all experiments in this series (33–522 μg of donor DNA per ml as well as no donor DNA). Since this slight shift was observed without donor DNA, it could not be due to joining of dense donor DNA to light plant DNA. With two additional methods for administering DNA to pea seedlings (see *Methods*), we obtained results essentially identical to that in Fig. 1a. In one case, where unlabeled donor DNA (760 μg/ml added to seeds) slowed and then apparently stopped growth, a slight shoulder was seen on the dense side of the pea DNA peak in CsCl after labeling with [^{3}H]thymidine. This shoulder was not reproducible, did not resemble the distinct peaks earlier (1–3), and perhaps represented organelle or satellite DNA synthesis in the nongrowing plants.

Experiments with Tomato. When unlabeled *A. tumefaciens* donor DNA was administered to tomato plants, fol-

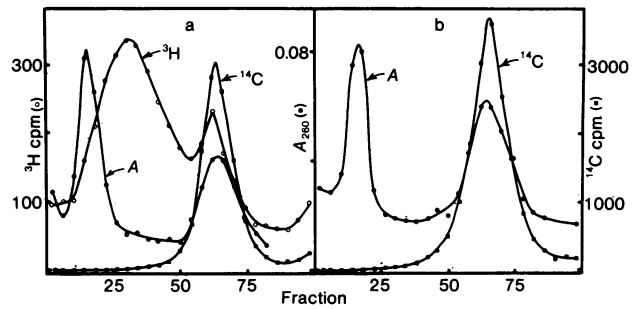

FIG. 2. CsCl gradient of root DNA from Proctor barley treated with bacterial DNA. Seeds were treated for 11 hr with water, 12 hr with [^{3}H]DNA from *Sarcina flava* (600 μg/ml, 1.5 × 10^5 cpm/μg), 72 hr with water and 6 hr with [^{14}C]thymidine (1 μCi/ml). (a) 34 μg of treated root DNA (^{14}C, 350 cpm/μg; ^{3}H, 120 cpm/μg) plus bacteriophage 2C DNA marker (1.742 g/cm^3). (b) 55 μg of control (no bacterial DNA) root DNA (^{14}C, 340 cpm/μg) plus 2C DNA marker.

lowed by incubation with [^{3}H]thymidine, we did not find a peak of the radioactivity at intermediate density (Fig. 1b) as reported earlier (1). The data from control plants were essentially identical to those in Fig. 1b (data not shown). The slight trailing of radioactivity toward higher density (Fig. 1b) and for control is ascribable to the nuclear satellite DNA present in tomato (ref. 10, and M. D. Chilton, unpublished). Bacterial contamination was rigorously excluded in our experiments, while in the earlier work (1) "no or very few bacteria are observed . . . in the final solution. They could not be held responsible for such high quantities of H^3 DNA (larger than the plant's incorporation)." Bacterial contamination can, however, cause prominent false satellite peaks (refs. 9 and 11, and Fig. 3).

Experiments with Barley. No intermediate density DNA peak was ever observed when *Micrococcus lysodeikticus* DNA was administered to axenic barley seeds followed by incubation with [^{3}H]thymidine (Fig. 1c). The data (not shown) for control plants were essentially identical to those in Fig. 1c. Failure to detect an intermediate peak of radioactivity could be due to high molecular weight of plant DNA, such that the interspersed donor DNA sequences contribute little to the density of the isolated fragments. Accordingly, despite the lack of a density satellite in CsCl gradients, labeled barley DNA was assayed for the presence of donor bacterial DNA sequences by DNA·DNA hybridization. Both treated and control DNA bound extensively to barley DNA-filters, but neither bound appreciably to donor bacterial DNA-filters (Table 1). Thus, DNA from treated plants has no more base sequence homology with *M. lysodeikticus* DNA than does control plant DNA. In such DNA-filter reactions, approximately 10–20% of labeled bacterial DNA added binds to bacterial DNA-filters. If 10% of the label in our treated seedling DNA had been in donor bacterial DNA sequences, we should have seen significant binding in this experiment. Over 50% of label in treated plants of earlier investigators was reported to occur at intermediate density, of which over 25% should be donor bacterial sequences, according to their interpretation (2, 3). Our hybridization results thus rule out the occurrence of gross preferential labeling of integrated donor DNA, as claimed by earlier investigators.

The foregoing experiments with unlabeled donor DNA could detect integration only indirectly, through subsequent replication of recombinant DNA molecules after [^{3}H]thymidine addition. A direct detection of the recombinant molecule is reportedly possible when highly labeled donor DNA is employed (2, 3). The bacterial component of the recombinant can represent 0.3–0.7% of recipient plant DNA (calculated from data in refs. 2 and 3). An attempt to repeat this observation, performed in Mol, is shown in Fig. 2. Tritium-labeled *Sarcina flava* DNA was used as donor and [^{14}C]thymidine added to monitor synthesis. This experiment thus assesses both integration and replication of exogenous DNA. The dense band of ^{3}H in Fig. 2a represents polymerized donor DNA because the peak of ^{3}H is at donor density (1.730 g/cm^3). This band is broad, suggesting that the donor molecules have been partially degraded *en route* through the endosperm to the roots used for DNA extraction. There is no peak in the intermediate density region. We observed a similar broadened band at donor density without an intermediate peak in earlier work (4) using donor [^{32}P]DNA from *P. aeruginosa* with pea seedlings, and donor [^{32}P]DNA from *P. aeruginosa* or donor [^{32}P, ^{3}H, ^{2}H, ^{15}N]DNA from tobacco with cultured tobacco callus cells. We thus failed in the present work and in earlier work (4) to obtain results which could be interpreted as evidence for integration of exogenous DNA into plant DNA. The ^{14}C peak in Fig. 2a resembles that in the control (Fig. 2b), again providing no evidence for replication of putative "recombinant" molecules.

Table 1. DNA·DNA hybridization

[^{3}H]DNA (μg)	Treatment: DNA (% ^{3}H-bound)	Treatment: H_2O (% ^{3}H-bound)	Filterbound DNA
7.4	0.7	0.8	*M. lysodeikticus*
1.5	1.2	1.0	*M. lysodeikticus*
7.4	17.1 (76.5°)	23.4 (76.5°)	barley
1.5	26.6 (78°)	25.0 (76.5°)	barley

[^{3}H]DNA from DNA treated (1840 cpm/μg) or H_2O treated (1170 cpm/μg) barley + 10 μg of filterbound DNA was incubated 18 hr at 60° in 0.2 ml of SSC. Thermal stabilities in SSC are shown in parentheses. Hybridization procedures have been described (17).

The above experiments were conducted with axenic plants. However, when precautions against bacterial contamination failed, prominent intermediate peaks were found. An example is shown in Fig. 3, where the majority of radioactivity is present in an intermediate peak. Material from a similar intermediate peak for an experiment done in Mol, Belgium was shown to hybridize extensively with DNA extracted from a culture of the predominant contaminating bacterium isolated from the barley roots (11). The ^{3}H-labeled material from this intermediate peak did not hybridize with donor *M. lysodeikticus* DNA (11). These intermediate peaks, ascribable to bacteria growing on the roots, are similar to those presented (1–3) as evidence for linkage of donor and recipient DNA.

Using the Pronase DNA extraction procedure which does not include chloroform or phenol treatments or spooling, Ledoux and Huart (2) reported that upon sonication, their intermediate peak separated into components of buoyant densities approximating that of donor and recipient DNAs. In our work with material prepared by the Pronase method, the radioactivity in control plant DNA, which appeared as a symmetrical peak before shearing (essentially identical to Fig. 1c), sedimented as roughly two peaks after shearing by sonication (Fig. 4a) or shearing with a French pressure cell (Fig. 4b). DNA prepared with phenol and spooling (see

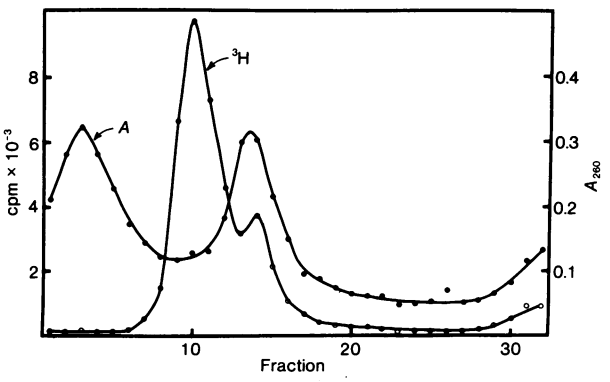

FIG. 3. CsCl gradient of DNA from Himalaya barley roots contaminated with bacteria. Root DNA (42 μg, 200 cpm/μg) after 3 hr labeling with [^{3}H]thymidine was mixed with 25 μg of denatured *M. lysodeikticus* DNA. Absorbance (●) was measured after dilution to 44% with water. ^{3}H (○) insoluble in 10% TCA was counted.

Methods) showed a single, symmetrical peak in CsCl before (data not shown) and after shearing (Fig. 4c). When treated plant DNA was subjected to sonication and to pressure cell shearing, peak splitting somewhat less pronounced than in Fig. 4a and b was observed; treated plant DNA prepared with phenol and spooling appeared essentially identical to Fig. 4c. It should be noted that the peak splitting we observed was not as distinct as that reported earlier (2).

A typical DNA preparation isolated by the Pronase method had an A_{260}/A_{230} ratio of 0.67 and A_{260}/A_{280} of 1.47. The best ratios obtained were 1.18 and 1.78, respectively. By comparison, these ratios were at least 1.71 and 1.78, respectively, for DNA prepared using phenol and spooling. The absorption spectrum of DNA prepared by the Pronase method varied with different preparations but was always characterized by high absorbance at wavelengths below 260 nm. DNA isolated by the Pronase method was further characterized by its susceptibility to DNase. To [^{3}H]thymidine-labeled barley DNA prepared by the Pronase method was added [^{14}C]DNA from *Bacillus subtilis*, and the mixture incubated with DNase. The rate of sedimentation in alkaline sucrose gradients of the marker bacterial DNA was markedly reduced by the action of the enzyme. The sedimentation velocity of labeled plant DNA was much less affected by DNase. Labeled barley DNA obtained after phenol extractions and spooling was as susceptible to DNase as the bacterial marker DNA. Thus, resistance to DNase digestion is not a property of more extensively purified barley DNA. We conclude that impurities not removed in the Pronase extraction procedure are responsible for partial resistance to DNase, for spectral alterations, and possibly for aberrant banding in CsCl after shearing.

DISCUSSION

We offer the following general criticism of the use of density in CsCl as the only physical-chemical criterion on which to identify a DNA. Radioactivity peaks of unanticipated density could result in several ways, none of which involve integration with foreign DNA. (*i*) DNA from contaminating bacteria, viruses, or mycoplasmas may be present. The test agar used may not allow the growth of some contaminating organisms. (*ii*) Satellite and/or cytoplasmic DNAs may be preferentially labeled. We reported a rapidly labeled dense satellite DNA in cultured tobacco cells (4). A dense satellite has been reported in tobacco pith tissue (12) which is said to

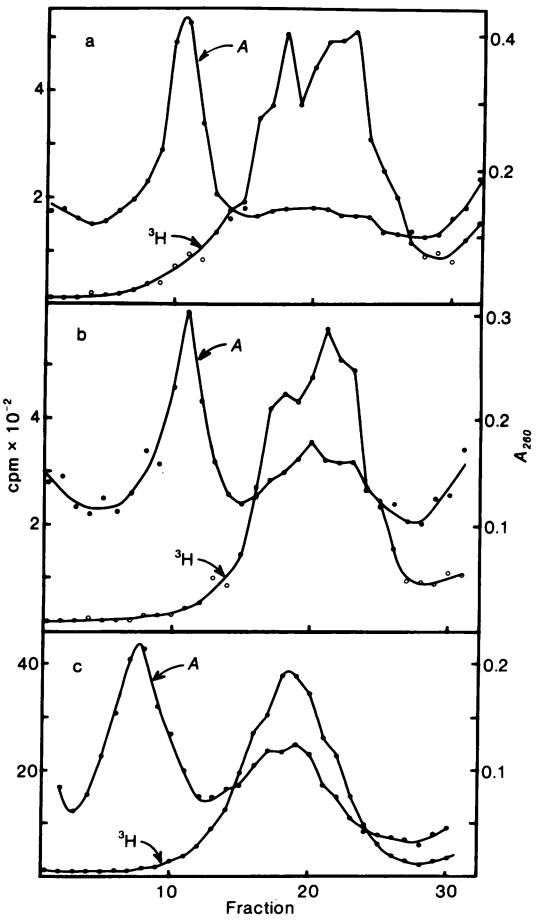

FIG. 4. CsCl gradients of sheared Himalaya barley root DNA. DNA labeled for 3 hr with [^{3}H]thymidine was prepared by the Pronase method (a, b) or with phenol and spooling (c). Each gradient has 25 μg of denatured *M. lysodeikticus* DNA. (a) About 50 μg of DNA (2600 cpm/μg) sheared in SSC by three 10-sec treatments with an MSE sonicator. Of each fraction, 13% was counted in Aquasol. Absorbance was measured after dilution to 40% [60% in (c)] with water. (b) Same DNA (74 μg) as in (a) sheared at 12,000 pounds per square inch (82.7 MPa) in a French pressure cell. Assays were as in (a). (c) 35 μg of DNA (8400 cpm/μg) sheared as in (b); radioactivity insoluble in 10% TCA from entire fractions was counted.

be preferentially synthesized under certain stress conditions. Barley seedlings in the published experiments (2) are likely to be under stress from water deprivation since the roots do not contact liquid; water may reach the roots only via the sectioned endosperm which does contact liquid. An intermediate peak of cytoplasmic origin was observed by Hotta and Stern using donor [^{32}P]DNA from M. lysodeikticus and barley seeds that were subsequently exposed either to x-irradiation or to low humidity such that "cellular multiplication was largely arrested" (13). They observed no such peak in the absence of these stress treatments. Their data could be interpreted as stress-stimulated synthesis of a cytoplasmic DNA in which ^{32}P from donor DNA breakdown was used. We noted above a dense shoulder in CsCl of DNA from pea seedlings whose growth was stopped by high concentration of donor DNA. (*iii*) Impurities associated with DNA may alter its density. Ledoux and Charles demonstrated a satellite band in partially purified labeled ascites cell DNA which was absent in more extensively purified DNA. They wrote, "The presence of proteins can produce artifacts . . ."

(14). (*iv*) Unusual bases may alter the density of DNA. From the foregoing considerations it is apparent that sequence dependent criteria such as DNA·DNA hybridization or nearest neighbor analysis are required for more accurate identification of a DNA.

We have been unable to obtain any evidence for integration or replication of exogenously added bacterial DNA in plants. Some of the experiments reported here were performed in the laboratory of Dr. Ledoux using donor DNA provided there. In an effort to reconcile the discrepancies between our results and those of Ledoux and his collaborators, it is necessary to consider alternative interpretations of their data.

The first evidence for integration comes from work with labeled donor DNA leading to an intermediate peak whose density is said to vary with that of the donor DNA in experiments with tomato (1), barley (2) and *Arabidopsis* plants (3). With axenic pea seedlings and bacterial donor DNA we detected no intermediate density DNA peaks (4). In addition to the negative results in Fig. 2, we also obtained negative results using bacterial or density labeled tobacco DNA with tobacco cells in culture (4). We now suggest that the intermediate peaks observed by Ledoux are not the result of integration, but may be DNA from bacteria contaminating the roots that was synthesized from breakdown products of the labeled donor DNA. Alternatively, the peaks may represent satellite and/or cytoplasmic DNAs preferentially labeled with breakdown products. It is difficult to reconcile this interpretation with claims (1–3) that the density of the intermediate peak varies with that of the donor. However, the combination of coincidence with small variations and scatter in data points make these claims less than convincing.

The second line of evidence for integration is the appearance of an intermediate peak resulting from preferential replication of supposed "recombinant" molecules formed after uptake of unlabeled donor DNA. Several bacterial DNAs are said to serve successfully as donors, and several plants, including tomato, pea, and barley, are said to be suitable recipients. Using axenic plants, we observed no intermediate peak despite many attempts and the use of several procedures for administering donor DNA. We suggest that the intermediate peaks reported (1–3) are not the result of replication of "recombinant" molecules, but may be due to either of the alternative causes discussed above, for example, DNA from contaminating bacteria. A bacterial-contributed peak is shown in Fig. 3 and was also reported for work done in Mol (11). We (9) and others (15, 16) have extensively investigated the problem of bacteria contributing false satellite peaks in DNA extracted from several plants.

The third evidence for integration is the splitting of the intermediate peak upon sonication into peaks with densities approximating those of donor and recipient DNAs. A recombinant molecule might behave this way, whereas bacterial DNA does not. The peak splitting observed (Fig. 4) was not as dramatic as that reported earlier (1–3), but was observed only with DNA prepared with the Pronase method and not with the phenol method. Barley DNA prepared by the Pronase procedure partially resists DNase action, whereas, that prepared by the phenol procedure does not. An impurity is apparently bound to DNA prepared by the Pronase method. It is possible that aberrant banding in CsCl could be due to such impurity. In the work with *Arabidopsis* (3) peak splitting was also observed and the Pronase method was used.

In conclusion, we believe that the published physical-chemical data (1–3), which we have been unable to confirm despite extensive efforts, are insufficient evidence to support the claim of integration between bacterial and plant DNA. We, therefore, suggest that the idea of foreign DNA integration and replication in plants should be viewed with skepticism until it can be demonstrated that the experiments are free of artifacts.

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