Penetration and Distribution Studies in Bean, Cotton, and Barley from Foliar and Root Applications of Tween 20-C^{14}, Fatty Acid and Oxyethylene Labeled

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Evidence from column, paper, and thin-layer chromatography indicates that Tween 20 is a mixture of numerous compounds. Carbon-14 distribution patterns in bean, barley, and cotton were determined following foliar and root applications of Tween 20-C^{14} (oxyethylene and fatty acid labeled), and of fractions of both materials eluted from a CM Sephadex column. Marked differences existed between the oxyethylene and fatty acid labeled surfactants with respect to the distribution of C^{14} in the plants from either root or foliar application. The fatty acid label was principally phloem mobile, whereas the oxyethylene label was mainly xylem mobile. The penetration and subsequent movement of label from either sample of Tween 20-C^{14} in all species studied were very low. No Tween 20-C^{14} was detected in the plant outside the treated area following either foliar or root application of fatty acid labeled surfactant; results with oxyethylene labeled surfactant were less conclusive.

The use of radiolabeled surfactants for the study of cuticular penetration and the nature of the role of the surfactant in herbicide penetration has received little attention, mainly because the availability of labeled surfactants has been limited.

Work carried out to date with labeled materials (*3*, *4*, *7*) indicates that, of the surfactants tested, little or no movement of original surfactant material occurs in the plant, and any noticeable movement from leaf applications of labeled surfactant has been in the apoplast in an acropetal direction. No surfactant has been demonstrated in labeled parts of the plant that are outside the treated leaf. It would thus appear from the limited experiments carried out to date that the enhancement of herbicidal action by surfactants is principally concerned with the penetration and absorption processes at the site of herbicide entry.

The surfactant Tween 20 (Atlas Chemical Industries, Inc., Wilmington, Del., formerly listed as polyoxyethylene sorbitan monolaurate) has been widely used in many biological investigations (*8*). When Tween 20-C^{14} labeled in both the oxyethylene chain and fatty acid portion of the mixture became available, it was decided to conduct experiments concerning the penetration, distribution, and fate of Tween 20 in plants from both foliar and root applications.

Proprietary Tween 20 is actually a complex mixture, essentially an ethoxylated sorbitol and its anhydrides esterified with lauric acid. By the nature of its commercial or "batch" synthesis, Tween 20 conceivably could include free polyols, polyethylene polyols, polyoxyethylated sorbitol, polyoxyethylated sorbitan, polyoxyethylated sorbide and the mono- and diesters of the above polyols.

The radiolabeled samples used in this study were special laboratory syntheses which might differ somewhat from the quality controlled commercial product.

Knowledge of the purity and molecular structures of these foliarly applied materials is important to gain basic information on the penetration process. When initial investigations of the distribution of the two Tween 20-C^{14} materials in plant tissues from foliar applications revealed opposing distribution patterns, the purity of these two samples of Tween 20-C^{14}, as well as some T-1947-C^{14} (polyoxyethylene polyoxypropylene polyol) was checked to see if impurities were causing these distribution differences.

The hydrophilic character of the surface active agent Tween 20 is supplied by the oxyethylene chains and the anhydrosorbitol moieties, whereas the lipophilic (hydrophobic) character is found in the long hydrocarbon chain of the fatty acid (*1*).

This paper describes the methods used to characterize partially the radiolabeled portions of the Tween 20-C^{14} samples and to study the penetration and distribution patterns of C^{14} in several plants from foliar and root applications of these materials.

Methods and Materials

In this paper Tween 20-C^{14} randomly labeled in the oxyethylene chain (0.332 μc. per mg.) will be designated as Tween 20-C^{14} (Oxy.), whereas Tween 20-C^{14} labeled at the one (1) position in the fatty acid moiety (0.355 μc. per mg.) will be designated Tween 20-C^{14} (F.A.). The T-1947-C^{14} included in certain studies has a specific activity of 0.373 μc. per mg. and was presumed to be randomly labeled.

Fractionation of Tween 20-C^{14} and T-1947-C^{14} by Column Chromatography. Columns of a gel filtration material Sephadex G-25 (a modified dextran) and a cation exchanger material GM-Sephadex C-50 (a carboxymethyl ether derivative of Sephadex G) were prepared according to the manufacturer's instructions. A measured amount of Tween 20-C^{14} (F.A. or Oxy.) or T-1947-C^{14} was added to each column and the column eluted with distilled water. The droplets of effluent that emerged from the column were collected in nickel-coated steel planchets (10 drops per planchet). The planchets were dried and counted with a gas flow GM counter, and the corrected activity of each planchet was plotted against planchet number of milliliters of effluent. After establishing the pattern of radioactivity present in the effluent, various fractions consisting of either 5 or 10 ml. were collected from the columns, dried by a filtered air stream and made to 0.5 ml. with distilled water for use in further experiments.

Paper and Thin-Layer Chromatography. The Tween 20-C^{14} samples, both original and Sephadex fractionated material, as well as the T-1947-C^{14}, were chromatographed by one-dimensional ascending chromatography on Whatman No. 1 paper with the following systems: 1-butanol saturated with water, 5% acetone in petroleum ether (b.p. 30° to 60° C.), and 1% methanol in petroleum naphtha (b.p. 60° to 70° C.).

Before or after the chromatograms were autoradiographed they were sprayed with Dragendorff's reagent (*9*)

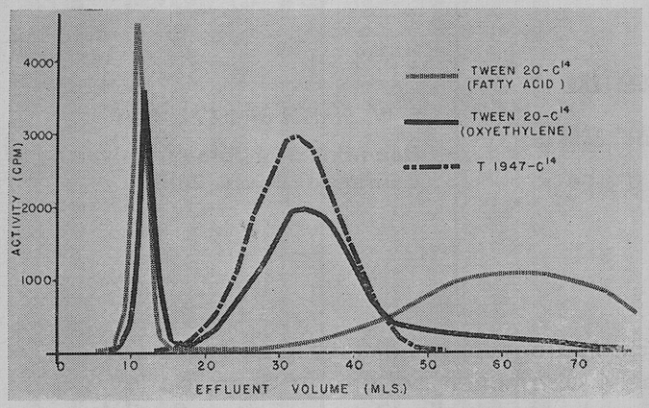

Figure 1. Graph of activity and effluent volume for the two samples of Tween 20-C^{14} and T-1947 C^{14} from a CM-Sephadex column

Column length, 14 cm.; flow rate, 75 ml. per hour; void volume, 12 ml.

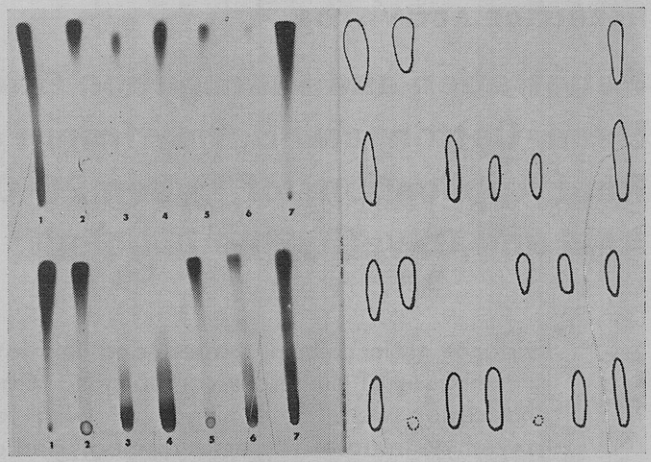

Figure 2. Autoradiographs and original chromatograms of the original Tween 20-C^{14} samples and various fractions collected from a CM Sephadex column

Chromatograms developed in 1-butanol saturated with water.
Top row: 1, Tween 20-C^{84} (oxyethylene); 2, 2nd fraction Tween 20-C^{14} (fatty acid); 3, 3rd fraction; 4, 4th fraction; 5, 5th fraction; 6, 6th fraction; 7, original material, Tween 20-C^{14} (fatty acid).
Bottom row: 1, Tween 20-C^{14} (fatty acid); 2, T-1947-C^{14}; 3, 4th fraction from oxyethylene label; 4, 3rd fraction from oxyethylene label; 5, 2nd fraction from oxyethylene label; 6 and 7, original material Tween 20-C^{14} (oxyethylene label)

to establish the position of the surfactant materials on them.

The Tween 20-C^{14} samples were also chromatographed on mannitol and a powdered sugar thin-layer plate developed in this laboratory (*10*). The petroleum ether and petroleum naphtha systems as used for paper chromatography were employed for the thin-layer work.

Distribution Pattern in Barley, Cotton, and Bean Plants of Tween 20-C^{14} (Fatty Acid and Oxyethylene Labeled) and the Various Fractions Separated on the Sephadex Column. The original Tween 20-C^{14} solutions and the various fractions separated on the Sephadex column were applied in lanolin rings to leaves of barley, bean, and cotton plants growing in culture solution. Each plant received the same amount of radioactivity in 100 μl. of solution as measured by the gas flow GM counter. (This dose was equivalent to 100 μl. of 0.1% Tween 20-C^{14} original solution—i.e., 0.03 μc.) Doses of 0.003 and 0.3 μc. of original Tween 20-C^{14} were also applied.

The methods used for autoradiographing the plant materials were essentially the same as described by Crafts and Yamaguchi (*3*). The two original solutions of Tween 20-C^{14} were applied as root applications of 0.3 μc. in 100 ml. of nutrient solution to 9-day-old barley plants as well as bean and cotton plants.

Quantitative Penetration and Distribution Study of Foliar Applications of Tween 20-C^{14} to Bean Plants. Tween 20-C^{14} (F.A. and Oxy.) was applied in a lanolin ring to the primary leaf of the bean. At zero time and after four days the treated spots were cut from the leaves and washed with a jet of distilled water (25 ml.) and the plants divided into treated leaf distal to the treated spot, treated leaf remaining and petiole, stem below treated leaf plus roots, and opposite primary leaf and trifoliate leaves. The plant parts were placed in 80% ethanol and ground in a blender to an even particle size and made to 50 ml. total volume. A 1-ml. aliquot of these blends was taken, dried in a planchet and counted by gas flow GM counter. Correction for self absorption in the plant material was made by constructing a standard curve in the region 0 to 15 mg. of plant material that had been handled in the same way as the treated plant except that known amounts of Tween 20-C^{14} had been added.

The plant material outside the treated spot showing the highest activity was extracted with 80% ethanol, separated into various fractions by ion exchange columns, and the neutral fraction which contained most of the radioactive material was chromatographed in two dimensions with solvents phenol-water (4:1, v./v.) and 1-butanol–propionic acid–water (47:22:31, v./v./v.).

Extracts were also made from plants that had Tween 20-C^{14} applied to the roots. These extracts were treated as described above for foliar materials.

Results

Fractionation by Sephadex Column. The cation exchange material CM Sephadex C-50 proved the most satisfactory of the two Sephadex materials tested for fractionation of the Tween 20-C^{14} samples. Figure 1 shows the results of collecting 56 samples (10 drops each) from a CM Sephadex column and determining the activity of each sample. Both types of Tween 20 material show two major peaks of activity. The first peak is the same in both cases and occurs at the front of the column eluant. The second peak occurs at different positions in the eluent, tending to indicate different materials.

Table I. Total Amount of Radioactivity in Various Fractions Collected from a CM Sephadex Column Eluted with Distilled Water

(Column length 8 cm.)

Tween 20-C^{14}, Fatty Acid Labeled		Tween 20-C^{14}, Oxyethylene Labeled	
Volume of fraction, ml.	Total activity, c.p.m.	Volume of fraction, ml.	Total activity, c.p.m.
5	970	5	100
10	293,515	10	207,880
10	66,065	10	184,635
10	108,095	10	93,455
10	90,305	25	25,490
10	19,480	..	...
10	3,395	..	...
65	581,825	60	511,560

In each case, 0.6 ml. of 1.0% Tween 20-C^{14} was applied to each column. 10 μl. samples from the original 1% Tween 20-C^{14} material gave a total count in 0.6 ml. of 570,000 for the fatty acid labeled and 510,000 for the oxyethylene labeled materials.

There was no release of radioactivity from the column when elution was carried out with 0.01*N* NaOH or 0.01*N* HCl after elution with distilled water. All the applied radioactivity was recovered in the eluate and no radioactive material was left on the column. When T-1947-C^{14} was applied to the column and eluted with distilled water, one single peak appeared that corresponded to the second peak of the Tween 20-C^{14} (Oxy.) as shown (Figure 1).

Table I shows the total number of counts in the various fractions collected from an 8-cm. column when both the

Tween 20-C^{14} samples were eluted separately. These same fractions were dried, made to 0.5 ml. total volume, and used in the chromatography and distribution studies.

The apparent disparity between the results in Figure 1 and the data in Table I is explained by the different column lengths employed—i.e., Figure 1, 14 cm. and Table I, 8 cm.

Chromatography. The autoradiographs of some of the paper chromatograms of the Tween 20 materials are shown in Figure 2; also shown are the original chromatograms on which the areas colored by Dragendorff's reagent have been marked. The Tween 20-C^{14} (Oxy.) has separated into two main components, both on the Sephadex column and when chromatographed with *n*-butanol saturated with water. The work by Shinoda and coworkers (*9*) suggests that the material moving at the front of the paper chromatograms under this solvent system is the surfactant proper—i.e., Tween 20—whereas the materials lagging behind are polyoxyethylene polyol materials. This same pattern holds true for the Sephadex column because when the fractions are chromatographed and sprayed with Dragendorff's reagent, the typical color reactions correspond to the (two) areas of radioactivity. An interesting anomaly is shown with T-1947-C^{14} (Oxy.); paper chromatography shows all of the T-1947-C^{14} moving at the front in the 1-butanol system, but on the Sephadex column the T-1947-C^{14} material comes off in the same fraction as the second peak.

When Tween 20-C^{14} (F.A.) is chromatographed in 1-butanol virtually all of the labeled material moves at the front. The same pattern is exhibited also by the label in the fractions separated on the Sephadex column but Dragendorff's reagent shows the usual presence of polyoxyethylene polyols in the same fractions as the oxyethylene labeled material. When Tween 20-C^{14} (F.A.) is chromatographed on paper and thin-layer media in both the acetone–petroleum ether and methanol–petroleum naphtha systems, a large number of spots appear both in the original material and in each fraction from the Sephadex column. As many as 13 spots have been observed on a single chromatogram and an exactly similar separation occurs on a mannitol or powdered sugar thin-layer plate developed with the same solvents (Figures 3 and 4). Tween 20-C^{14} (Oxy.) does not move in these systems. The numerous spots observed on these chromatograms are not colored by Dragendorff's reagent and, thus, do not appear to be of a polyoxyethylene polyol nature. However, the greater part of the radioactivity appears to stay at the origin for the Tween 20-C^{14} (F.A.), similar to that of the oxyethylene label. The spots may be either impurities associated with the laboratory synthesis or the result of breakdown (possibly microbial) within the prepared sample.

In Tween 20 aqueous samples (10 and 100 grams per liter) left standing on the shelf for several months, a slime-like

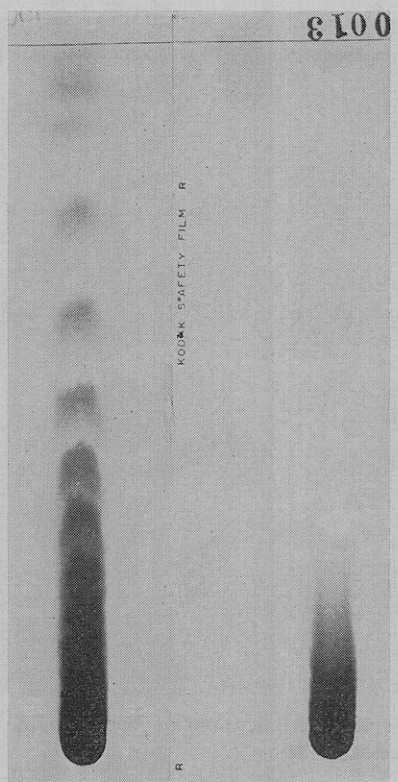

Figure 3. Radiochromatogram on paper of original Tween 20-C^{14} fatty acid labeled material (left) and original Tween 20-C^{14} oxyethylene labeled material (right)

Chromatogram developed in 5% acetone in petroleum ether (b.p. 30° to 60° C.)

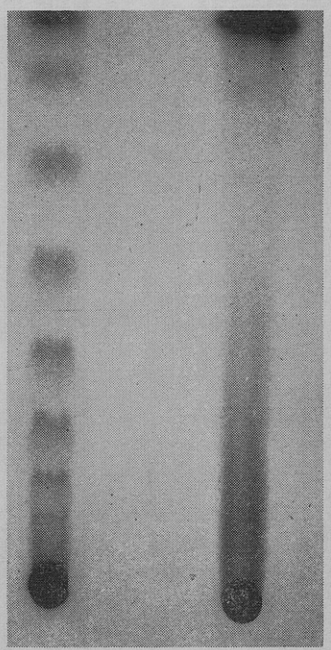

Figure 4. Thin-layer radiochromatograms on sucrose media of first main peak from fraction collected from Sephadex column when Tween 20-C^{14} (fatty acid) was eluted

Chromatograms developed in (left) 5% acetone in petroleum ether and (right) 20% acetone in petroleum ether

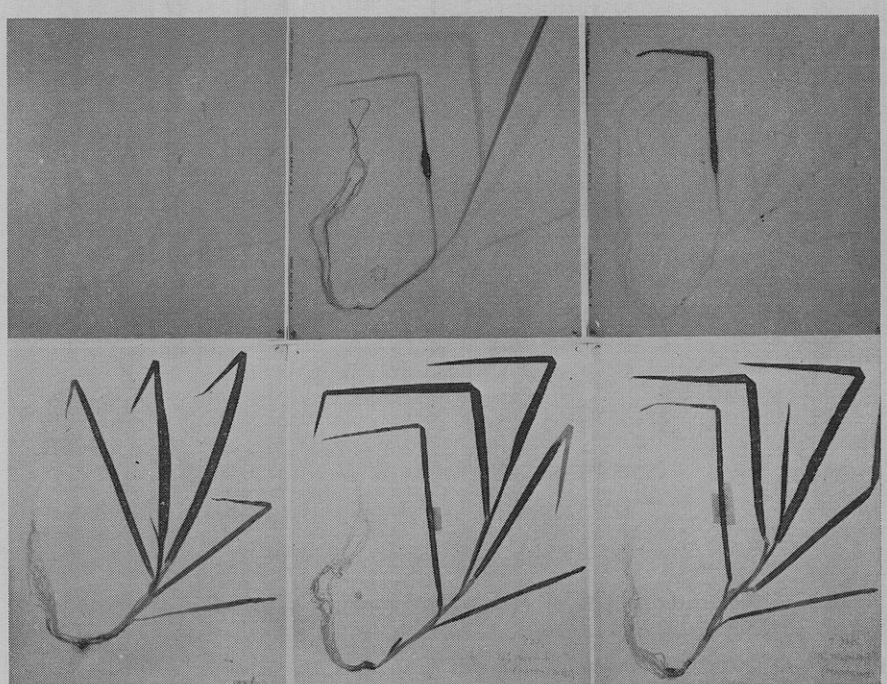

Figure 5. Autoradiographs of barley 7 days after treatment with Tween 20-C^{14} (0.3 μc. per plant)

Left, untreated; middle, original Tween 20-C^{14} fatty acid labeled material; right, original Tween 20-C^{14} oxyethylene labeled material

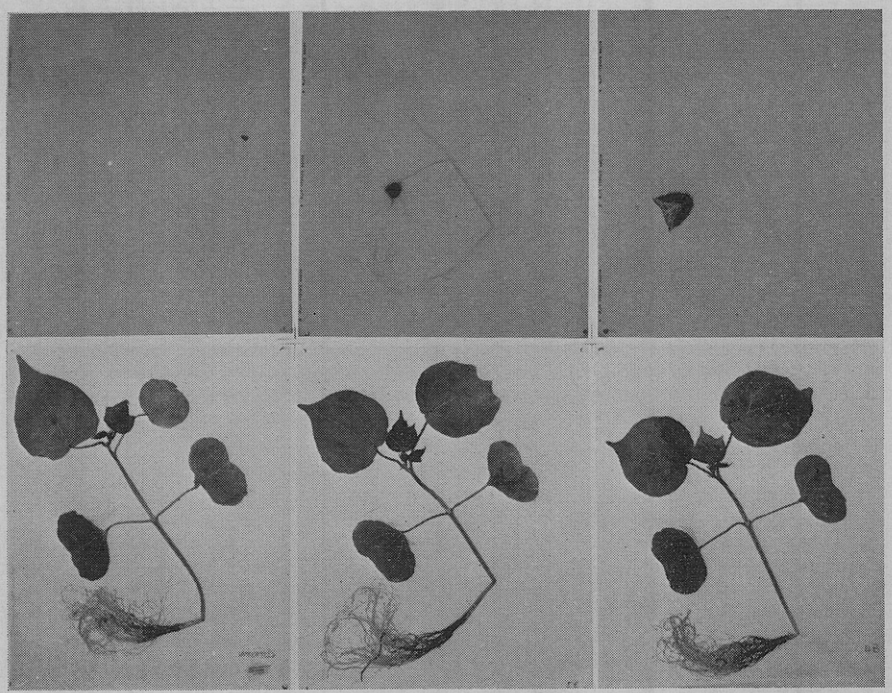

Figure 6. Autoradiographs of cotton 4 days after applications of Tween 20-C^{14} and fractions thereof collected from a Sephadex column

Left, untreated; middle, 2nd peak from fatty acid labeled material; and right, 2nd peak from oxyethylene labeled material

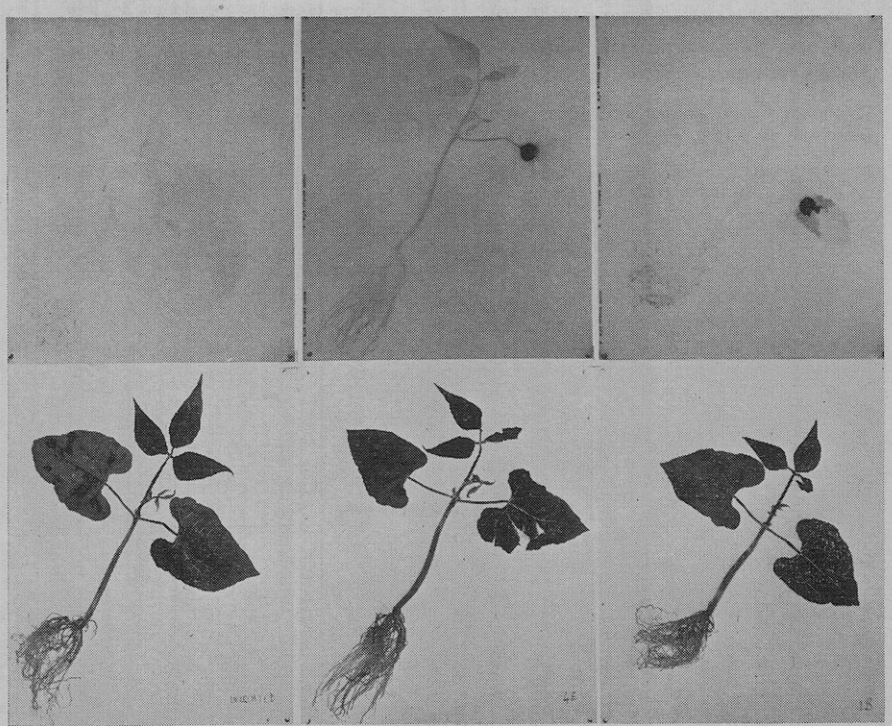

Figure 7. Autoradiographs of red kidney bean 4 days after applications of Tween 20-C^{14}

Left, untreated; middle, fatty acid labeled; and right, oxyethylene labeled. Both these fractions were from the main peak of the Sephadex column elutions

deposit occurs in the solution. The phenomenon was observed in both of the radiolabeled samples; however, counts made of the clear supernatant liquid showed that the level of radioactivity in the fatty acid labeled material had dropped considerably whereas the oxyethylene labeled material stayed the same. This indicates some possible microbial breakdown, or polymerization and possible separation of the less soluble fatty acid esters resulting in the slime-like material.

Distribution in Plants of C^{14} from Leaf and Root Applications of Tween 20-C^{14}. A major difference is noticed between the distribution of C^{14} in plants from foliar applications of Tween 20-C^{14} (F.A.) and Tween 20-C^{14} (Oxy.). This difference is shown in Figures 5, 6, and 7 and appears to be true for all three species of plants studied. There was no difference in distribution of C^{14} in cotton, bean, and barley among the various fractions separated on a Sephadex column from the same original material.

The C^{14}-labeled oxyethylene material moved only in the apoplast—i.e., only acropetally from the treated spot. The distribution pattern was exactly the same as that obtained for T-1947-C^{14} (*4*), and even after 7 days, only fractional amounts of C^{14} were moved out of the treated leaf.

Applications of the fatty acid labeled material, on the other hand, appeared to result in movement of the label via phloem, into the roots and young growing parts of the plant.

Visual observations of the treated spot areas on the autoradiographs for all applications of the various fractions collected from the Sephadex column as well as the original materials would tend to indicate that the materials are not penetrating the leaf surface of the species studied, or, if absorbed, they are not readily mobile in the plant. This is shown by the high density of the autoradiograph over the actual treated spot compared with the light nature of the autograph corresponding to the rest of the plant (Figures 5, 6, and 7).

Root applications of these materials showed similar distribution patterns to leaf applications—i.e., the fatty acid labeled material gave autographs with a higher density of C^{14} in young growing points than did the oxyethylene material that was concentrated at leaf tips and edges. The autoradiographs of the root-treated 9-day-old barley plants confirm the two contrasting patterns of distribution (Figure 8), as described above, and the poor penetration of these materials through living membranes.

Quantitative Studies. Table II shows the quantitative results of the penetration and translocation study in beans. A very small quantity of the total radioactivity applied was found outside the treated spot. After 4 days, only 1.3% of the fatty acid label and 3.1% of the oxyethylene label had moved from the point of application. The applied Tween 20 (10% w./v.) in both cases caused some burn on the treated spot after 24 hours and may have influenced these results, but because the results agree with previous observations to such a high degree, this localized scorching can probably be neglected. In plant extracts, over 90% of the activity stayed in the neutral fraction. When chromatographed in two dimensions, with the solvents indicated, the activity of the oxyethylene labeled extracts corresponded to the position of the original

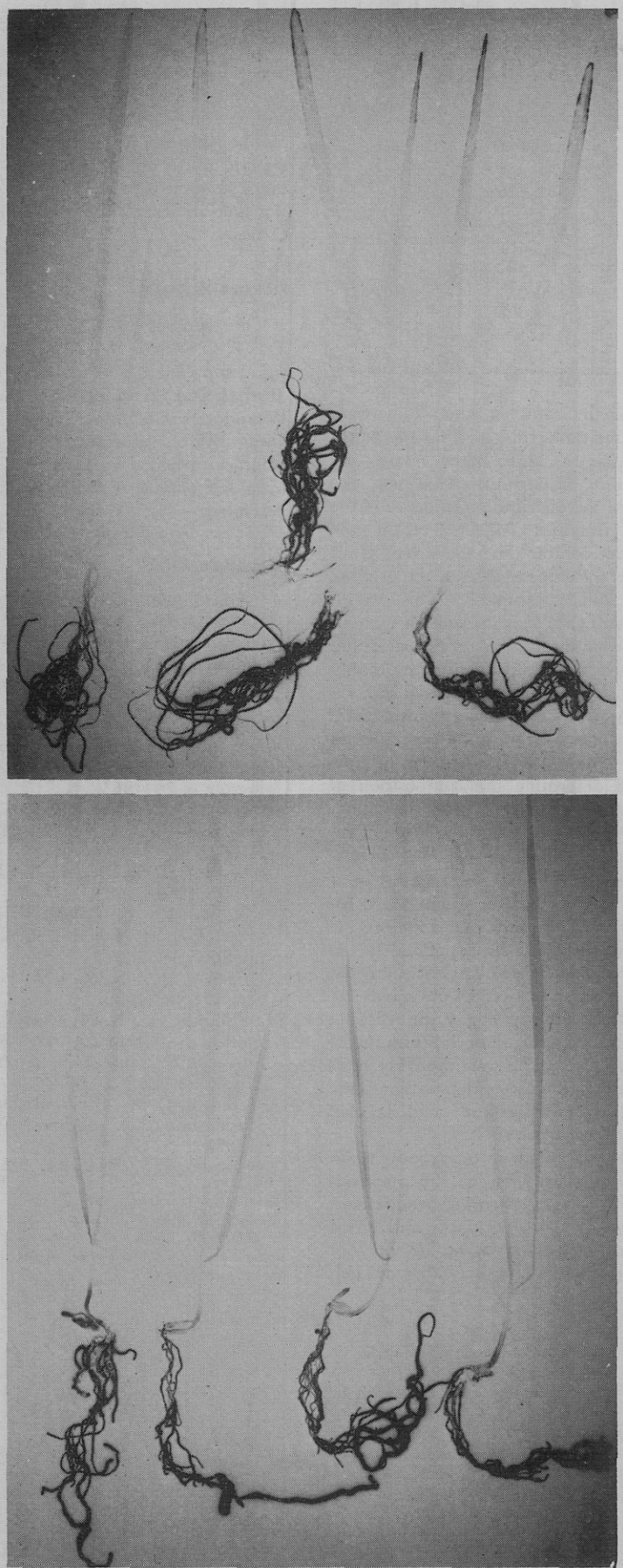

Figure 8. Tween 20-C^{14} applied to 9-day-old barley plants, root treatment, 0.30 μc./10μl./100 ml. solution

Harvested, 6, 24 and 48 hours, and 4 days after treatment, respectively, as shown from left to right on the autoradiograph. Bottom: fatty acid label. Top: oxyethylene label

material, which was chromatographed in the same system. However, the activity in the extracts from Tween 20-C^{14} (F.A.) treated plants showed two main regions of activity that were quite distinct from the original Tween 20-C^{14} (F.A.).

Discussion

Surfactant materials containing an ethylene oxide chain by nature of their manufacture are usually a mixture of a homogeneous series of surfactant compounds with a Poisson distribution around a certain mean number of ethylene oxide moles per molecule. This is particularly true of the nonionic ethylene oxide condensate-type materials like Tween 20 and, in this case where a special laboratory synthesis of the radiolabeled materials was carried out, numerous other possibilities exist for contaminants.

When Tween 20 (Oxy.) was applied to the Sephadex column, the original material became separated into two fractions, possibly the actual surfactant-like materials and polyoxyethylene polyol-like materials. This is supported by the evidence obtained when these fractions are chromatographed on both paper and thin-layer media (Figure 2). Shinoda and coworkers (*9*) obtained a similar separation with Tween 60 and Tween 80, the monostearate and monooleate counterparts of Tween 20, respectively. In each case, the material staying near the origin was considered to be polyoxyethylene polyols. Information supplied by the manufacturers of the Tween compounds (private communication) suggests that the conclusions of Shinoda and coworkers were erroneous and the nature of these compounds is unknown.

When fatty acid labeled Tween 20 is eluted through the Sephadex column, a portion of the label appears in the same fraction as the oxyethylene labeled material, but other fractions collected contain more or less radioactivity than similar fractions collected for the polyoxyethylene labeled material. Each of these fatty acid labeled fractions showed the same 10–13 different compounds when chromatographed on Whatman No. 1 paper and thin-layer medium (Figures 3 and 4).

Thus, these Tween 20-C^{14} samples, by virtue of their manufacture, contain many compounds and together with the differences in chain length of the three polyoxyethylene side chains numerous homologs will exist. Chromatographic work carried out indicates that this is probably true although the true nature of these compounds is not known.

In studies with the three plant species materials of a Tween 20 origin that were labeled in either the fatty acid or oxyethylene portions of the molecule showed two different distribution patterns of C^{14}. The results may be interpreted to mean that breakdown or metabolism

Table II. Total Activity (C.P.M.)[a] Found in Various Parts of the Bean Plant 4 Days after Foliar Treatment with Tween 20-C^{14}

Plant Part	Tween 20		Untreated
	Fatty acid labeled	Oxyethylene labeled	
Washings	21,290	37,025	...
Treated spot	48,920	31,118	14
Treated leaf (distal portion)	122	1,729	24
Petiole and treated leaf (basal portion)	150	220	42
Roots and stem below treated leaf	208	102	16
Opposite primary leaf and trifoliate leaf	474	148	11
Total	71,164	70,342	107
Total counts applied (average of four)	70,942	70,378	...

[a] Average of two replications.

of the substance occurs. The phenomenon occurred whether the materials were applied to the roots or leaves of the plants.

The hydrolysis of 2,4-dichlorophenoxyacetic acid (2,4-D) esters has been shown to occur both on the leaf surface and within plant tissues (*2*, *5*, *6*, *11*). Hagen, Clagett, and Helgeson (*5*) have shown with the butyl ester of 2,4-D and castor bean lipase that hydrolysis of these esters can be catalyzed by plant enzymes. Such reactions could also occur with Tween 20. The ester, whether formed between the lauric acid portion of the molecule and the sorbitan ring or a polyoxyethylene chain, would be cleaved and the resulting fatty acid portion further metabolized by β-oxidation. Numerous esterase enzymes have been demonstrated in plants, although perhaps not specifically at the leaf surface.

The evidence presented in Table II shows that the movement of radiolabeled carbon from foliarly-applied Tween 20-C^{14} occurs to a very small extent. Only a very small percentage of the C^{14} from foliarly-applied Tween 20-C^{14} is translocated from the treated spot, and approximately 50% of the total activity could be washed from this spot after 4 days' treatment. The amount moved out of the treated spot (1 to 2%) is so small that it need not necessarily be from Tween 20 because many compounds exist in the original samples. No free fatty acid was found in either of the samples when they were methylated and subjected to gas chromatography. Thus, they were not involved as impurities. However, the density of the distribution patterns of the various fractions collected from the Sephadex column in all three plant species is the same as that of the original Tween 20-C^{14} samples. This would tend to indicate that in each case about the same amount of material was being metabolized or translocated (presumably C^{14}-labeled products of metabolism).

Because extremely low amounts of C^{14} were translocated from these treated areas of leaves, it was difficult to quantitize and determine the composition of these materials by chromatography. However, extracts from the Tween 20-C^{14} (Oxy.) treated leaf that were chromatographed in two dimensions in a typical sugar system (neutral compounds) showed only one spot on the chromatogram that corresponded to the original material. The fatty acid labeled extracts showed two main areas of radioactivity that were quite distinct from the original material chromatographed in the same system. This tends to support previous evidence that some metabolism of the fatty acid labeled material has occurred.

Although difficult to interpret without a thorough knowledge of the materials present in Tween 20, these results confirm previous work by Norris and Freed (*7*) that surfactant materials do not penetrate plant surfaces readily as such and are very poorly translocated in plants, if at all.

Because such relatively low amounts of C^{14} from Tween 20-C^{14} are translocated and no intact Tween 20 was found in plants outside the treated leaves, it would appear that the site of action of the surfactant is at the point of application and in the immediately underlying tissues.

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