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A Technique for Collection of Exudate from Pea Seedlings[1]

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ABSTRACT

Ethylenediaminetetraacetic acid (EDTA), at concentrations higher than 1.0 millimolar, is phytotoxic to etiolated seedlings of *Pisum sativum*. Substantial vascular exudation from pea epicotyls could be obtained without tissue damage at 0.5 millimolar EDTA if the solution was buffered at pH 7.5 with sodium *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid. Treated seedlings exuded 950 micrograms (leucine equivalents) of ninhydrin-positive material per day and 870 micrograms (glucose equivalents) of anthrone-positive material per day. Amino acid analysis showed the exudate to have glutamine as the major amido nitrogen containing compound and sucrose was shown to be the major sugar. Radiolabeled tryptophan and sucrose applied to cotyledons were transferred through the epicotyl and into the collection medium. The pH profile for exudation shows half maximal exudation at pH 7.2, indicating the promotion of exudation by EDTA is probably not due simply to Ca^{2+} chelation.

Vascular exudates from seedling plants should contain the compounds transported between the seed and the growing meristematic region. Agar blocks have been employed to collect hormone exudates of decapitated seedlings (19) but this procedure is limited in the amounts collectable, provides no assurance against vascular blockage, and yields a residue difficult to use for chemical characterization. Published techniques for exudate collection from mature plants have utilized chelating agents (7) and reducing agents (25) to enhance exudation. However, application of such techniques to seedling plants and plant epicotyls caused tissue damage, as evidenced by translucency and loss of ability to regenerate a shoot. We have studied the effect of various solutions on vascular exudation from young pea seedlings and found that a solution containing 0.5 mM EDTA buffered to pH 7.5 with 10 mM Hepes will promote exudation while preserving tissue integrity. We have partially characterized this exudate with respect to amount and composition and find that the material exuded into the collection solution is comparable in amount to that which would normally flow from seed to shoot. A previous brief report of this work has appeared (6).

MATERIALS AND METHODS

Plant Material. Pea seeds (*Pisum sativum* L. cv Alaska, Vaughan-Jacklin Corp.,[3] Ovid, MI and Meyer Seed Co., Baltimore, MD) were soaked in running tap water for 2 h, sterilized in either 0.1% $HgCl_2$ or 1.0% NaOCl for 20 to 30 min, and grown on germination paper in the dark for 7 to 8 d at 25°C and 80% RH. Epicotyls were 14 to 19 cm at the time of use and were cut to leave 7 cm of epicotyl above the seed.

Chemicals. ^{14}C(U)-Sucrose (4.9 mCi/mmol and 673 mCi/mmol) was obtained from New England Nuclear and ^{3}H-(ring 5)-L-tryptophan (26.3 Ci/mmol) and ^{14}C-(methylene)-DL-tryptophan (57 mCi/mmol) were from Amersham. Disodium EDTA was from Sigma or Mallinckrodt, heparin was from Sigma, thioglycerol from Aldrich, polyvinyl pyrrolidone from Oxford Laboratories, and Hepes, Mes, and MOPS,[4] all zwitterionic buffers, were gifts from Dr. N. E. Good or were obtained from Research Organics. Glucose, galactose and sucrose were from Calbiochem, raffinose from Difco, and stachyose was from Sigma. Final pH adjustments were made with 2 N NaOH or HCl, as necessary. Counting was in ACS solution (Amersham) in a Packard 3003 (^{14}C) or a Beckman CPM-100 (^{3}H) liquid scintillation counter. The counting efficiency was 77.2% for ^{14}C and 56.0% for ^{3}H. Later experiments utilized a Beckman 9000 counter with computer corrections to obtain DPM.

Exudation Technique. All seedling manipulations were at 25°C and 80% RH with illumination provided by a phototropically inactive green safelight. Ten plants were bundled together with laboratory tape (Shamrock, Bellwood, IL) and cut while submerged under the test solution. The seedlings were then transferred to 20 ml of fresh solution contained in an 18 × 150 mm test tube so that the seedlings were incubated upside down with 1 cm of the epicotyl submerged in the solution. The roots were covered with wet paper toweling and the entire plant and collection assembly then placed in a closed humid chamber to prevent drying. Sampling was accomplished by removing the seedlings, mixing the test solution, and then removing 200 μl aliquots of this solution for analysis. In some experiments, 1 ml of test solution was sampled and plated on nutrient agar. After 72 h of growth at 37°C, microbial populations in excess of 3 colonies/ml were never detected. Labeled compounds were applied in a 1.0-μl drop of 50% aqueous ethanol placed between the cotyledons with a syringe. Following isotope application, the seedlings were incubated for 1.5 h prior to cutting. In initial experiments with ^{14}C-sucrose, the application amounted to 12,200 dpm (0.5 nmol) on each of 36 seedlings. For HPLC analysis, 0.1 μCi (0.15 nmol) of high specific activity ^{14}C-sucrose was applied to each of

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[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

[4] Abbreviation: MOPS, 3-[*N*-morpholino]propanesulfonic acid.

10 seedlings. For experiments utilizing ^{3}H-tryptophan, 2×10^5 dpm (3.42 pmol) were applied to each of 20 seedlings.

In experiments without buffer, the pH was maintained with a Radiometer titrator type TTTl equipped with a Corning model 476050 combination electrode and used in the pH stat mode with 10 mM NaOH for titration. Ten seedlings were inverted in a beaker containing 200 ml of 0.5 mM EDTA, the pH electrode, and a small glass capillary used for addition of base. The solution was stirred slowly and the beaker was insulated from the stirring motor by a 2-cm thick block of styrofoam. During a 24-h titration period, 10.0 ml of base solution was used to maintain the pH at 7.5. In control beakers without plant material, 7.1 ml was required for pH control, indicating that the seedlings produced 29 μeq of acid during the titration period. Aliquots of 10 ml were collected from the beakers and reduced to 1 ml *in vacuo*. From this 1-ml sample, 0.5 ml was used for the ninhydrin reaction and 0.2 ml for the anthrone test.

Analytical Procedures. Neutral sugars were determined by means of the anthrone reaction (20) with A_{620} determined on a Gilford 240 spectrophotometer. The 200 μl samples were collected in 10 ml teflon-lined screw cap vials, frozen until use, and then analyzed without transfer. Neither EDTA nor Hepes interfered with the anthrone assay and other compounds were tested for interference and minor corrections made where necessary. Values are reported in glucose equivalents utilizing glucose standards assayed at the same time as the unknowns.

The identities of the sugars in the exudate were determined by two methods. First, ^{14}C-sucrose was used to label the sugar pool of the seedling (see above). The exudate solution was concentrated to 1.5 ml and 100 μl samples analyzed on a 4 × 250 mm column of Bio-Sil Amino 5S (Bio-Rad) using 1 ml/min of 70% acetonitrile/H_2O (v/v) as mobile phase. The HPLC unit consisted of a Rheodyne 7125 injection valve, a Waters 6000A pump, Waters R401 differential refractometer, and a Gilson 201 fraction collector programmed to collect 0.5 min fractions directly into 7 ml liquid scintillation mini-vials. Preliminary experiments showed that passage of the exudate through a combination of a Baker-10 aromatic sulfonic acid column followed by a Baker-10 quaternary amine column for removal of ionic compounds, a procedure we initially used, was unnecessary and thus this step was omitted in later analyses.

In addition to analysis of the radiolabeled sugars, analyses of the sugar content of normal, unlabeled exudate was also performed. This analysis was similar to that followed for the ^{14}C-sucrose labeling except that the collected fractions from HPLC were concentrated and applied to a silica gel 60 TLC plate. After development (4×) with *n*-butanol-2,6-lutidine-water (60:30:15), the plate was examined by dipping in Stahl's anisaldehyde-sulfuric acid reagent (21) and incubating at 105°C for 25 min. Examination of the product color and intensity allowed for tentative identification and semiquantitative evaluation.

Amino nitrogen was determined by means of the ninhydrin reaction (18) with leucine as a standard. Amino acid analyses were performed by Dr. D. T. A. Lamport on an autoanalyzer using the hydrolysate system with ninhydrin detection and automated peak integration. Half of the sample used for amino acid analysis was hydrolyzed in 6 N HCl at 110°C for 10 min *in vacuo*. Longer hydrolysis times (2 and 20 h) resulted only in decreases in some peak areas. Thin layer electrophoresis was done as previously described (22).

Tryptophan in cotyledons was determined by isotope dilution analysis using an internal standard of 1 μCi ^{14}C-tryptophan added to 172 g (from 365 seedlings) of cotyledons. Extraction in aqueous acetone; chromatography on Dowex 50 × 8-100, Sephadex LH-20, and silica gel TLC; and quantitation using UV adsorption were as described (5). Analysis of tryptophan in the exudate was also by the isotope dilution method (5) using ^{14}C-tryptophan (6.1×10^5 dpm) as the internal standard. Recovery of transported ^{3}H-tryptophan was determined by adding 500 μg of cold carrier tryptophan, followed by reisolation and then quantitation using the reverse isotope dilution method. A full discussion of these isotope methods can be found in Hall and Bandurski (5). Purification of tryptophan from the exudate was accomplished after the addition of ^{14}C labeled or cold carrier tryptophan by reducing the sample to 1 ml *in vacuo* at 45°C. The sample was placed on a 1 × 35 cm column of Sephadex LH-20 and developed with 50% aqueous ethanol. Fractions containing tryptophan were pooled and reduced to about 30 μl. This residue was streaked on a silica gel 60 thin layer plate (No. 5763, E. Merck) and developed in ethyl acetate, methyl ethyl ketone, ethanol, water (5:3:1:1). After development, the tryptophan was located as a UV absorbing area. In addition, a 1-cm strip was cut from the edge of the plate and sprayed with Ehmann's reagent (4) to detect indoles. The tryptophan area was scraped from the plate, placed in a funnel with Whatman No. 44 filter paper, and eluted with 2 to 3 ml of 50% aqueous ethanol. The filtrate was then taken to dryness, resuspended in 200 μl of water, and used for assay and counting.

Tryptophan isolated from the exudate was assayed with Ehmann's reagent (4). An aliquot of the sample (20 μl) was mixed with 200 μl of the reagent and allowed to react for 1 h at 45°C. The reaction was terminated by the addition of 600 μl of distilled water and the absorption at 590 determined on a Cary 15 spectrophotometer by scanning from 800 to 350 nm. The absorption spectrum of the Ehmann product from the reisolated carrier tryptophan was identical to that obtained with authentic tryptophan standards.

Total protein in the exudate was investigated using the microscale Coomassie blue dye binding assay (Bio-Rad). No protein could be detected in the exudate using this assay, thus indicating that the protein content of the exudate was below 1 μg/ml. This amounts to less than 2 μg of protein per plant, per day. Protein, therefore, represented less than 1% of the total amino nitrogen present in the exudate solution.

The increase in dry weight in the growing zone was used as a measure of the approximate amount of material supplied by the vascular fluid. This was estimated by marking the epicotyls of 7-d-old pea seedlings 4 cm below the hook and, after 24 h, excising that portion which was 4 cm above the mark and below the hook. Excised segments were then dried at 100°C until a constant weight was obtained (72 h).

RESULTS

During the initial studies leading to these investigations a variety of substances were tested, both singularly and in combination, for their ability to enhance exudation. The results of these preliminary studies is best illustrated by Table I which shows the effect of Hepes-buffered EDTA compared with a mixture of the other reagents tested. The complex mixture provided no significant improvement over only EDTA and buffer and even the slight increase noted could be accounted for by the

Table I. *Comparison of Two Incubation Solutions on the Amount of Exudation from 10 Pea Seedlings into 20 ml of Solution in 24 h*

Treatment	Glucose Equiv.
	μg/seedlings
0.5 mM EDTA + 10 mM Hepes (pH 7.5)	7183 ± 1105
Complex mixture[a]	7508 ± 1723
H_2O control	221 ± 21

[a] Complex mixture consisted of 1% soluble polyvinylpyrrolidone, 0.01% heparin, 5 mM sodium pyrophosphate, 10 mM thioglycerol, 0.5 mM EDTA, and 10 mM Hepes (pH 7.5).

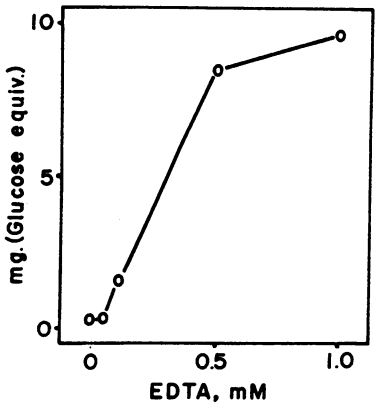

FIG. 1. Effect of EDTA concentration on exudation of anthrone-positive material from 10 pea seedlings during a 24-h incubation. Concentrations higher than 1.0 mM resulted in damage to the tissue.

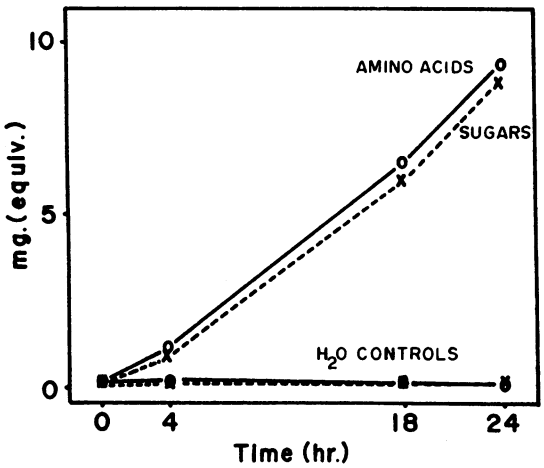

FIG. 2. Time course for exudation of anthrone-positive (sugars) and ninhydrin-reactive (amino acids) material from 10 pea seedlings. Upper lines are for 0.5 mM EDTA and 10 mM Hepes (pH 7.5) treated seedlings, lower values are for the H_2O controls. The ordinate indicates the amount of ninhydrin- and anthrone-reactive material relative to leucine and glucose standards.

increased buffering and chelating capacity of the additional reagents. The choice of 0.5 mM EDTA was based on the data shown in Figure 1, as well as visual observation of the seedlings following a 24-h incubation. At EDTA concentrations of 1.0 mM or greater, severe disruption was noted in the tissue exposed to the test solution. Typically, the terminal 1 cm of the segments would be translucent and lack turgor. However, at 0.5 mM EDTA the segments showed only slight damage after 24 h, and more than 80% of the seedlings regenerated a shoot at the cut ends when incubated in the light following the experiments. Thus, since 0.5 mM EDTA provides near optimal exudation (Fig. 1) and does not appear to significantly damage the tissue, this concentration was chosen for additional study.

Figure 2 shows that seedlings incubated in EDTA and Hepes exude amino acids and sugars at a fairly linear rate during a 24-h period. Seedlings incubated in water alone did not exude significant amounts of material during this time period. Exudation in Hepes/EDTA was reduced to the level of water control by 1 mM $CaCl_2$. The effect of Hepes was primarily due to its buffering capacity, since titration of unbuffered EDTA solutions produced similar results (data not shown). Hepes alone produced no more exudation than water controls. Amine buffers, such as Hepes, are known to inhibit transglycosylation enzymes (13); however, this does not appear to be its primary effect in this system. Quantitatively, the amount of sugars exuded over a 24-h period ranged from 0.4 to 1.0 mg/shoot; however, this difference appears to be a function of slight changes in growth conditions, since seedlings grown at the same time showed nearly identical rates of exudation. The dry weight increase in the growing zone of intact seedlings averaged 5.3 mg/24 h. The sugars, amino acids, and organic acids exuded into the collection media accounted for approximately half this mass. With consideration of the uncertainties inherent in these measurements, the results indicate that the material collected represents a significant proportion of the dry weight increase of intact seedlings.

At least some of the material collected in the EDTA solution was transported from the cotyledons to the shoot, as shown in experiments using radiolabeled sucrose and tryptophan (Fig. 3 and see below). ^{14}C-Sucrose applied to the cotyledons resulted in significant amounts of radioactivity in the collection solution, whereas water controls did not release measureable ^{14}C. The rate of ^{14}C release was approximately linear with time (Fig. 3) and paralleled the release of anthrone-positive material seen in the earlier experiments. HPLC analysis of the ^{14}C-labeled sugar content of the exudate showed peaks of radioactivity at the retention volumes of fructose, glucose, sucrose, and raffinose (Fig. 4). When total sugar content in unlabeled exudate was examined by HPLC and TLC, the levels of fructose and glucose relative to sucrose were much lower than in the labeled sample, and raffinose was absent. This result was noted consistently throughout several experiments.

^{3}H-Tryptophan movement from cotyledon to shoot was measured by collecting and analyzing labeled compounds in the EDTA solution. Application of 4.7×10^{-6} dpm of ^{3}H-tryptophan to 20 seedlings resulted in 2100 dpm in the exudate after 24 h. Most of the labeled tryptophan recovered was in the form of the free amino acid, with only 38% as other compounds. Isotope dilution analysis of the tryptophan content of the cotyledons

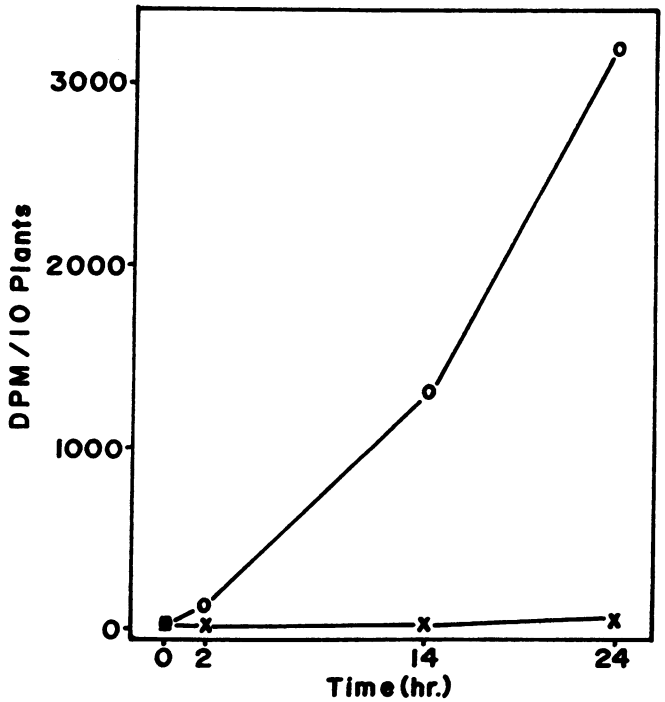

FIG. 3. Exudation of ^{14}C from pea seedlings after pretreating the cotyledons with ^{14}C-sucrose (12,200 dpm/plant, 4.9 mCi/mmol). Exudate collected in 0.5 mM EDTA plus 10 mM Hepes (pH 7.5) (O) or in water (×).

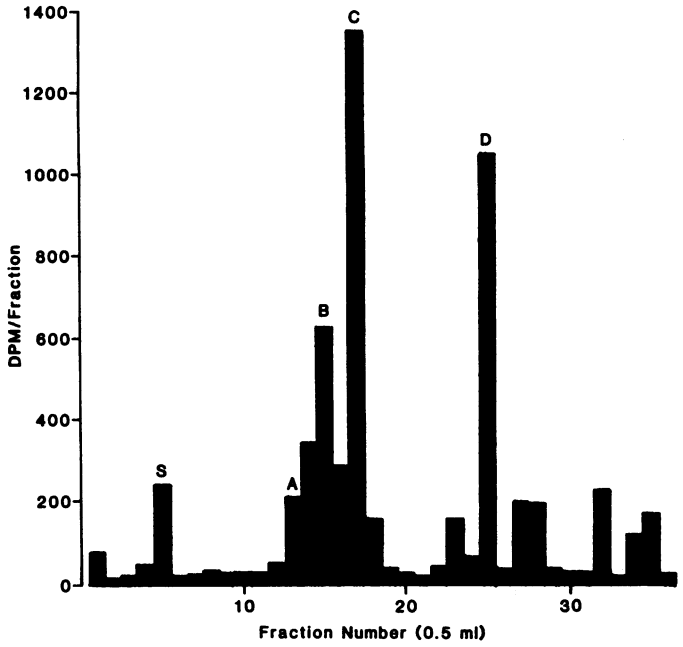

FIG. 4. Radioactivity eluting from Bio-Sil Amino-5S HPLC column after injection of a concentrated fraction of the 24-h exudate obtained from treatment of 10 seedlings with ^{14}C-sucrose (1 μCi, 673 mCi/mmol). Letters indicate solvent front (S) and the retention of authentic standards of fructose (A), glucose (B), sucrose (C), and raffinose (D). Stachyose, if present, would elute in fractions 30 to 32 (15–16 ml).

Table II. *Amino Acid Analysis of Pea Exudate*

Assignment	Amount
	nmol/plant
Ala	56[a]
Asp	404[b]
Glu	Trace
$GluNH_2$	1811[c]
Gly	173[a]
His (basics)	264[a]
Homoserine[d]	Trace
Leu, Ile	29
Lys	53[a]
NH_3	1997[a]
Phe	84
Ser[d]	98
Thr	146[a]
Val	142

[a] Peak area increased after hydrolysis; value given is from hydrolysate. [b] Includes traces (<10%) of asparagine. [c] As glutamate after hydrolysis. [d] Homoserine could not be determined in the hydrolyzed fraction because it was obscured by the Asp and Thr peaks. Serine was only determined in the hydrolyzed fraction due to overlap with the $GluNH_2$ peak.

showed that 500 nmol/g fresh weight or 230 nmol/seedling was present. After correction for dilution by the pool of free tryptophan in the seed, but not correcting for tryptophan turnover or tryptophan in the shoot, the amount of tryptophan that moved from seed to shoot and was then collected was 1.5 nmol·20 seedlings^{-1}·d^{-1} as free tryptophan or 2.4 nmol·20 seedlings^{-1}·d^{-1} based on total counts. Thus, the cotyledon appears to be a major source of the tryptophan in the exudate since it was also found by isotope dilution analysis that the 24-h exudate from 20 seedlings contained 3.5 nmol of tryptophan.

The major amino acid found in the exudate was glutamine, with substantial amounts of aspartate also present (Table II). Prior to hydrolysis, several ninhydrin-positive peaks were observed in the region expected for small peptides. These disappeared after a short acid hydrolysis and several amino acids were detected in greater amounts. Appreciable amounts of threonine, serine, and glycine appeared in the hydrolyzed fraction as well as a large increase in the amounts of basic amino acids. Thin layer electrophoresis confirmed the presence of high levels of glutamine in the nonhydrolyzed exudate and smaller amounts of aspartate, glutamate (trace), asparagine, and a basic amino acid.

The pH dependence of the exudation process is shown in Figure 5. Also shown in Figure 5 is the chelation of Ca^{2+} by EDTA as a function of pH calculated from the data of Martell and Calvin (10). It is apparent that the exudation curve is displaced from the Ca^{2+} chelation curve by about 1.5 pH units, indicating that the effect of EDTA is probably more complex than simply chelation of free Ca^{2+}. Identical results were obtained when Hepes buffer was adjusted to the indicated pH or if buffers with the more appropriate pK_a were used (*i.e.* Mes [pH 5.5–6.0]; MOPS [pH 6.5–7.0]; Hepes [pH 7.5–8.0]).

DISCUSSION

When plant stems are severed, only small amounts of exudate will usually be released. Such exudation has been collected in capillaries and used for studies of vascular contents (8, 12, 16, 17); however, such methods have limitations due to the small quantities that can be obtained. Some plants, for example Cucurbitaceae, *Phoenix*, *Ricinus*, and *Yucca* (3, 12), will exude more significant amounts of material. However, the number of such plants is limited and, even in these species, young seedlings do not usually exude for prolonged periods. We have shown that the use of EDTA to enhance exudation can be extended to seedling stage plants provided care is taken to limit the phytotoxic effects of this compound. Seedling peas will exude for 24 h or longer under these conditions with little or no visible damage and, if placed under lights, will regenerate a shoot at the cut surface.

The predominance of sucrose in the exudate and of ^{14}C-sucrose and ^{14}C-raffinose after labeling are consistent with what has been reported for vascular exudates obtained using other techniques (3, 16). The presence of monosaccharides in the exudate could

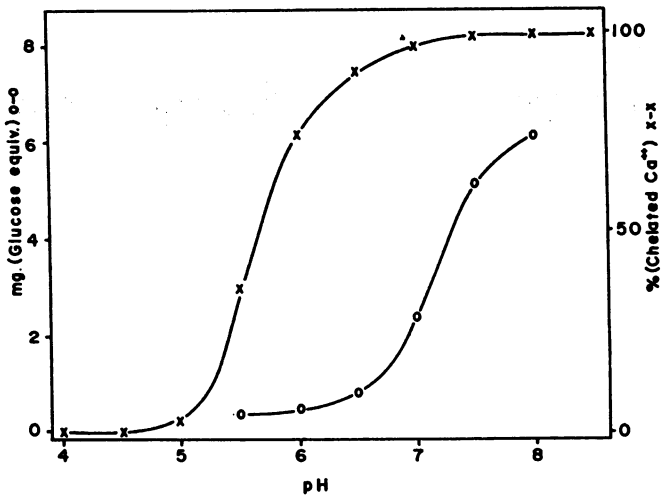

FIG. 5. Exudation of anthrone-positive material as a function of initial pH (O). Solutions contained 0.5 mM EDTA and 10 mM Hepes. Also shown (×) is the effect of pH on per cent Ca^{2+} chelated by EDTA calculated from data of Martell and Calvin (10).

indicate that they are a normal constituent of vascular material of pea or are due to invertase activity at the cut surface (26). However, only small amounts of monosaccharides were present in the exudate obtained without ^{14}C-sucrose labeling (glucose:sucrose ratio of approximately 1:10). The presence of ^{14}C-raffinose and slightly higher relative amounts of monosaccharides after application of ^{14}C-sucrose could indicate a difference in sugars transported based on a different route of entry into the vascular system. The amino acid composition of the collection media following a 24-h exudation was also similar to that reported from vascular exudate obtained from numerous sources (1, 2, 8, 11, 22). However, the amino acids found in exudate from seedling tissue differed from that found in exudate from mature pea (cf. Urquhart and Joy [23], [24]), the most notable difference being the abundance of glutamine in seedling exudate, while in mature plants asparagine is more prevalent. The total amount of material exuded was similar to the dry weight increase observed in intact growing shoots. The exudate was low in protein and this is consistent with what is known for the composition of vascular exudates (3). Bacterial contamination was minimal within the time period (1 d) studied.

An important finding resulting from this work is the pH dependency of exudation. Although free Ca^{2+} should be essentially fully chelated at pH 7.0, this gives less than half maximal exudation (Fig. 5). This result was not expected, since it has been postulated that the effect of EDTA on exudation was primarily due to calcium chelation (7). The values for the pH dependency of calcium chelation in Figure 5 were obtained by recalculating data in Martell and Calvin (10), but the chelation of Ca^{2+} by EDTA at pH 7.0 is more clearly shown by direct calculation using known stability constants (9, 15). Thus, the log $K_{1(apparent)}$ at pH 7.0 is the log of the stability constant (K_1) minus log α, which, for EDTA at pH 7.0 and for calcium, is $10.6 - 3.3 = 7.3$ (9, 15). The fraction of free metal should be less than $1/K_{1(apparent)}$ which is 1/antilog 7.3 or 5.01×10^{-8}. The change, therefore, in amount of Ca^{2+} chelated in going from pH 7.0 to 8.0 would be very small indeed, yet such a change more than doubles the amount of exudation. It could be argued that the intercellular pH at the site where EDTA is working could be quite different from that in the external solution. This argument, however, is unlikely since exudation is actually quite sensitive to small changes in the pH of the external solution and conditions that should favor EDTA uptake (acidic media) actually inhibit exudation (Fig. 5). It is possible that EDTA is competing for calcium already bound to some constituent within the plant tissue.

The present finding should prove useful in investigations aimed at understanding movement of nutrients and hormones from seed to developing shoot (14), and should provide greater insight into the need for careful pH control during prolonged EDTA-enhanced exudation. We feel that the disparity between amount of Ca^{2+} chelated at various pH values and the curve for exudation may provide a useful physiological tool for studying those factors which normally prevent vascular exudation from cut surfaces.

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