ber of phagosomes (*9*) or blue residual bodies spread throughout the cytoplasm, and the cell begins to send out pseudopods (Fig. 1h). Within 6 to 8 hours, most of the cells are again actively streaming (Fig. 1i), still with a large number of blue residual bodies spread throughout the cytoplasm. Some of these bodies are still present 3 weeks later, gradually being eliminated by being pinched off at the posterior portion in the more usual manner described for the elimination of indigestible residue in the amoeba (*6, 7*).

If amoebas are treated in an identical fashion with 125 m*M* NaCl or 0.01 percent gelatin, they undergo an intense cycle of pinocytosis and then return to their normal condition within 1 or 2 hours after the induction of pinocytosis—without going through the extrusion process noted previously with Alcian blue. In amoebas, then, the fate of pinocytotically ingested material seems to vary based on the chemical nature of the solute (for example, how tightly it binds to the cell surface) and perhaps its nutritional value. In the case of gelatin, which is ingested by pinocytosis, extrusion is not necessary because this protein can be broken down and utilized by the cell. Induction of pinocytosis by a high level of Na^+ in the external medium also does not elicit the extrusion response noted for Alcian blue, even though a relatively large amount of Na^+ enters the cell under these conditions (*10*). *Amoeba proteus* is capable of regulating the cytoplasmic level of Na^+, and this may be associated with the activity of the contractile vacuole (*11*). Assuming that Na^+ does enter the cell during pinocytosis, it could be removed by activity of the contractile vacuole, restoring cytoplasmic Na^+ to the previous level.

When pinocytosis is induced with Alcian blue in *A. proteus*, the dye is taken up by the cell together with any solute that may be present in the external medium. For example, it was shown (*2*) that labeled sucrose in the external medium is taken into the amoeba when pinocytosis is induced with Alcian blue. Sucrose taken up in this manner reaches a maximum concentration 10 to 20 minutes after the induction of pinocytosis, and this concentration remains constant for the next hour or so. During this period, though, the cell begins to expel a portion of the accumulated dye through extrusion channels. This suggests that the cell is capable of discriminating between beneficial solute and potentially harmful substances taken up by pinocytosis, which are then treated in entirely different manners.

The only condition imposed on a successful inducer of pinocytosis in the amoeba is that it be a cation (*4*). Presumably these substances induce pinocytosis by binding to negative sites on the amoeba's surface. Thus the binding phase is nonselective. Any selectivity that may be associated with this mechanism of solute uptake must reside in the cytoplasm. Through a variety of extrusion mechanisms, including the quick elimination of a portion of a potentially harmful solute by bulk extrusion, the cell seems to be capable of coping with any type of solute it may encounter. This suggests that the bulk extrusion mechanism observed in the amoeba may reflect a primitive secretory capability shared by many other cell types.

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Sensitive and Rapid Diagnosis of Potato Spindle Tuber Viroid Disease by Nucleic Acid Hybridization

Abstract. *A sensitive and reliable new method for the detection of potato spindle tuber viroid in potato tubers has been developed. The method is based on hybridization of highly radioactive recombinant DNA to viroid RNA that has been attached to a solid support. The method can be automated and permits the rapid testing of large numbers of tubers.*

The potential of the potato for producing more well-balanced protein and calories per unit area, time, and water than any other major food crop is recognized in countries with low income, high population, and scarce food (*1*). Major efforts are in progress to adapt the potato to growth in subtropical and tropical climates, and already a lowland tropical potato cultivar has been developed (*1*).

Because potatoes are vegetatively propagated, virus diseases are a major problem in potato production, and practical means for the exclusion of viruses from "seed" potatoes are a necessity. A sensitive automated method for the simultaneous detection in potato tubers of several important viruses has been developed (*2*). This method, based on an enzyme-linked immunosorbent assay (ELISA), permits sampling of 300 tubers per hour.

The potato spindle tuber disease poses

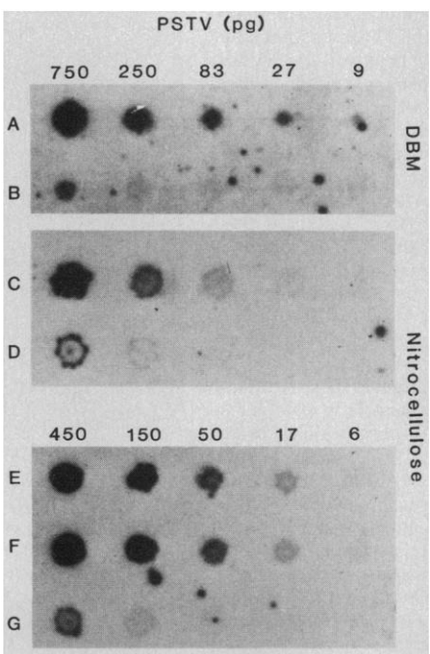

Fig. 1. Sensitivity of PSTV detection by hybridization and autoradiography after binding to DBM paper and nitrocellulose membranes. Low molecular weight RNA containing 0.4 to 0.5 percent PSTV was isolated from PSTV-infected tomato seedlings (*8*) and diluted with a mixture of 0.15*M* sodium acetate and 0.85*M* acetic acid (rows A and C), sap prepared from healthy Katahdin sprouts (rows B, D, and G), water (row E), or homogenization buffer (row F). Portions of successive threefold dilutions were then transferred to DBM paper (rows A and B) or nitrocellulose membranes (rows C to G).

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a potentially serious threat in seed potato production, germ plasm collections, and potato-breeding programs. The disease is caused by a low molecular weight (1.3×10^5) RNA, the potato spindle tuber viroid (PSTV) (*3*). The PSTV is transmitted through vegetative propagation, foliar contact, and true seed and pollen (*4*); diagnosis is difficult because, at moderate temperatures, foliage symptoms often are indistinct or lacking. In plants grown at high temperatures, however, PSTV causes severe damage, and total crop loss may ensue (*4*). Thus, exclusion of PSTV from seed potatoes to be planted in subtropical or tropical climates is essential.

Because PSTV lacks the antigenic protein coat characteristic of viruses, an assay based on the ELISA technique has not been reported. Bioassay on suitable tomato cultivars (*5*) and polyacrylamide gel electrophoresis of extracted nucleic acids (*6*) have been used to detect PSTV. The former assay is slow, requires extensive greenhouse space, and is unreliable (*7*); the latter is laborious and expensive. Neither method is suitable for the rapid screening of thousands of seed potato tubers. One possible alternative is hybridization of highly radioactive DNA complementary to PSTV (PSTV cDNA) with PSTV bound to a solid support, and autoradiographic detection of the resulting DNA-RNA hybrids. Hybridization in solution has already been used to detect the presence of viroids in purified RNA preparations (*8*).

We have cloned PSTV cDNA sequences using recombinant DNA techniques; one clone (pDC-29) contains almost the entire 359-nucleotide sequence of PSTV (*9*). It is available in large quantities and at high specific radioactivity. RNA covalently binds to diazobenzyloxymethyl (DBM) paper (*10*), but this support is expensive and requires a number of carefully controlled steps to prepare and use. RNA also stably binds to a nitrocellulose membrane (*11*); this method is simple, inexpensive, and reproducible. To simplify sample preparation, clarified plant sap (*12*) rather than purified nucleic acid was chosen as the source of PSTV.

The PSTV stably binds to either DBM paper or nitrocellulose membrane (Fig. 1, rows A to D) (*13*). Comparison of relative autoradiographic intensities shows that the presence of sap from uninfected tuber sprouts reduces the binding approximately tenfold, but 83 to 250 pg of PSTV are still easily detected after hybridization with radioactive recombinant DNA. This amount is equivalent to a concentration of 0.04 to 0.125 μg of PSTV per gram of tuber sprouts. Actively growing potato tissue contains ≥ 0.5 μg of PSTV per gram of tissue (*6*). Our hybridization method is therefore adequate to detect PSTV in potato tissue. The relatively high ionic strength and diethyldithiocarbamate concentration of the extraction buffer required to release PSTV from nuclei and inhibit enzymatic polyphenol oxidation do not interfere with PSTV binding to nitrocellulose (Fig. 1, rows E to G).

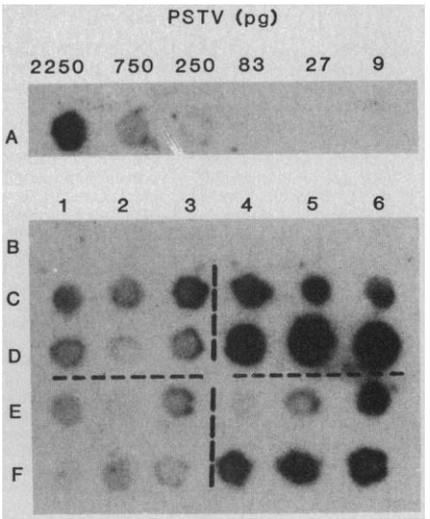

Fig. 2. Reliability of PSTV detection by hybridization and autoradiography after binding to nitrocellulose membrane. (Row A) Successive threefold dilutions of low molecular weight RNA containing PSTV that was diluted with sap prepared from healthy Katahdin sprouts; the sample in the first column did not contain sap. (Row B, left to right) Composite sap samples prepared from sprouts taken from several healthy Chieftain, Irish Cobbler, Green Mountain, Katahdin, Kennebec, and Saco tubers. (Rows C and D) Sap samples prepared from sprouts taken from individual PSTV-infected tubers: row C, columns 1 to 3 from Katahdin, columns 4 to 6 from Kennebec; row D, columns 1 to 3 from Saco, columns 4 to 6 from Katahdin line 235. (Rows E and F) Composite sap samples, each prepared from tissue taken from three individual PSTV-infected tubers: columns 1 and 4, epidermis from between eyes; columns 2 and 5, barely sprouted eyes (≤ 2 mm); columns 3 and 6, sprouts (≥ 5 mm). Row E, columns 1 to 3 from Katahdin, columns 4 to 6 from Kennebec; row F, columns 1 to 3 from Saco, columns 4 to 6 from Katahdin line 235.

No reaction was detected with sap prepared from healthy tubers of six commercial varieties (Fig. 2, row B). Sap samples prepared from sprouts of individual PSTV-infected tubers of a given variety contain similar concentrations of PSTV (Fig. 2, rows C and D). Finally, the three portions of tuber tested—sprouts, axillary buds ("eyes"), and the epidermis between the axillary buds—all contain detectable concentrations of PSTV (≥ 0.08 μg of PSTV per gram of tissue) (Fig. 2, rows E and F).

The data presented demonstrate the feasibility of PSTV detection by our method. Although the entire screening procedure requires 4 days for completion, a large number of samples can be applied to each nitrocellulose membrane, and several membranes can be subjected to hybridization simultaneously. Preparation of sap from tuber tissues is simple; automation as described by Gugerli (*2*) appears feasible. The sensitivity of our nucleic acid hybridization assay is equal to or greater than that of the ELISA tests used to detect potato viruses. Thus, potato tubers can be tested simultaneously for the presence of important viruses and for PSTV. The method is approximately ten times more sensitive than polyacrylamide gel electrophoresis (*6*).

Although different in principle, ELISA and our nucleic acid hybridization method each contain an amplification step. The ELISA method derives its sensitivity from the conjugation of an enzyme to the virus-specific antibody. The test measures not an antigen-antibody complex as in other immunological procedures but the reaction product of the antibody-conjugated enzyme. Similarly, the RNA-DNA hybrids formed in our procedure contain not only PSTV cDNA but also up to a tenfold excess of unhybridized pBR322 vector DNA. The PSTV-specific insert of pDC-29 contains 460 base pairs, while the pBR322 vector contains 4362 base pairs. The use of dextran sulfate to accelerate the rate of nucleic acid hybridization causes additional amplification with double-stranded probes. The randomly cleaved, partially complementary probe fragments can form extensive networks both before and after the DNA sequences complementary to PSTV hybridize to the immobilized PSTV (*14*). We would expect a similar assay for PSTV that used single-stranded cDNA to be significantly less sensitive.

As far as we know, this is the first application of a membrane filter hybridization technique to the detection of an RNA pathogen. Recently a conceptually similar "nucleic acid spot hybridization" procedure has been described (*15*) to screen eukaryotic cell lines for their Epstein-Barr virus DNA content.

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12. Potato tubers were allowed to produce etiolated sprouts at 20° to 25°C. Sprout samples (0.1 to 0.2 g) were homogenized at room temperature in a small conical ground-glass homogenizer with 200 m*M* K_2HPO_4, 10 m*M* sodium diethyldithiocarbamate, and 0.1 percent Triton X-100 (1.5 ml per gram of tissue). Tissue debris was removed by brief low-speed centrifugation, and the sap was used immediately.
13. Portions (3 to 5 μl) of the sap samples to be tested were pipetted either onto treated nitrocellulose membrane (*11*) and then baked for 2 hours at 80°C in a vacuum oven or onto DBM paper backed by two sheets of Whatman 3 MM paper and transferred with 150 m*M* sodium acetate and 850 m*M* acetic acid at 4°C (*14*). The same buffer [40 percent formamide, 0.18*M* NaCl, 10 m*M* sodium cacodylate, 1 m*M* EDTA, 0.1 percent sodium dodecyl sulfate, and yeast transfer RNA (400 μg/ml) at *p*H 7.0] was used before and during hybridization. The reaction before hybridization (16 hours at 42°C in the presence of 1 percent glycine) and the hybridization reaction [24 hours at 55°C in the presence of 10 percent dextran sulfate and ^{32}P-labeled nick-translated pDC-29 recombinant DNA (1 to 2.5 × 10^6 cpm/ml)] were performed essentially as described (*14*). Nick translation in the presence of deoxycytidine [α-^{32}P]triphosphate followed a protocol supplied by the Amersham Corporation and yielded DNA with an initial specific activity of ~ 2 × 10^8 cpm/μg. The ratio of buffer volume to membrane (or paper) area was at least 1 ml per 35 cm^2. Nick-translated DNA was denatured by heating for 2 minutes at 100°C in the presence of 50 percent formamide before addition to the hybridization reaction. Nitrocellulose membranes and DBM paper were washed at room temperature with five changes of 0.36*M* NaCl, 5 m*M* tris-HCl (*p*H 7.5), and 0.1 percent sodium dodecyl sulfate and then with two changes of the same buffer diluted tenfold before autoradiography, which was carried out for 24 to 48 hours at −70°C with Kodak X-Omat film and DuPont Cronex Lightning-Plus intensifying screens.
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Circadian Rhythms of Blood Minerals in Humans

Abstract. *Circadian rhythms of ionized calcium and phosphate concentrations have been demonstrated in human blood. A computer-derived model curve representing the 24-hour fluctuations in ionized calcium cannot be correlated consistently with curves for total calcium or phosphate. Knowledge of these circadian rhythms provides a physiological basis for further understanding the interactions between blood minerals and calcium-regulating hormones.*

Several hormones and minerals undergo daily variations in concentration in human blood (*1–5*). Analysis of deviations from normal circadian patterns may provide a sensitive basis for diagnostic evaluation and therapeutic intervention in many disease states (*2, 3*).

Attempts to define the biological rhythms of calcium and inorganic phosphate (P_i) in mammalian blood have yielded disparate results (*4, 5*). To our knowledge, no one has examined, in any species, 24-hour patterns of blood Ca^{2+}, the physiologically active fraction of total calcium (Ca_T) (*6*). This report establishes the presence of 24-hour patterns for blood Ca^{2+} and serum P_i in humans.

Seven 20- to 30-year-old males in good health and without a history of drug or vitamin use were studied during a 24-hour period. After their informed consent was obtained, they were adapted to study rooms for 12 hours overnight. In the morning an indwelling venous catheter was placed in the antecubital fossa of each subject. To maintain catheter patency for blood sampling every 30 minutes, 650 ml of a solution containing dextrose (0.05 g/ml), sodium chloride (0.04 mEq/ml), and heparin (5 U/ml) was slowly infused throughout the 24-hour period. Meals were offered at 0900, 1200, and 1730 hours; the 24-hour intake of calcium and phosphorus was estimated to be at least 500 and 900 mg, respectively. Activity was limited to movement within the study rooms during the daytime.

Observations at each of the 48 time points were averaged for all subjects to produce the curve shown in Fig. 1A. The U-shaped curve shows that the concentration of Ca^{2+} fell during the day after an initial maximum at 1000 hours, began rising late in the evening, and continued to rise until the concentration measured at 1000 hours was again reached in the early morning. This pattern is seen more clearly after the data are smoothed by using running medians to filter out small fluctuations (Fig. 1B) (*7*). The smoothed data were fitted by a third-order polynomial regression of the variable (Ca^{2+}) against time (*8*). The resulting parameters were used to construct a simple mathematical representation of the curve which preserves all but minor variations in the raw data. This representation, designated the Ca^{2+} model, is shown in Fig. 2A. Pearson correlation coefficients (*9*) between the Ca^{2+} model and the smoothed and raw data are .97 and .92, respectively. The model, therefore, provides an excellent fit to both the raw and smoothed data.

Although the maximal changes in the model appear relatively small (0.30 mg/dl), they are based on intersample variations that are many times greater than the error of measurement for individual mineral concentrations. Changes in Ca_T similar in magnitude to those reported here for the Ca^{2+} have been associated with significant alterations in the concentration of circulating parathyroid hormone (*5*). The oscillations in Ca^{2+} cannot be attributed to fluctuations in the *p*H of the blood samples, since the range of blood *p*H was extremely narrow. Moreover, the constancy of the Ca^{2+} circadian rhythm, as well as the one for serum phosphate described below, was evident in four studies carried out for as long as 40 hours. We conclude that these circadian oscillations in Ca^{2+} are of physiological significance.

The data obtained for serum P_i appear less "noisy" than those for Ca^{2+}; the shape of the P_i curve is readily apparent without median smoothing (Fig. 1C). The circadian pattern of serum P_i concentration consists of a minimum at about 1100 hours followed by two peaks, the largest occurring between 0200 and 0400 hours. We did not observe oscillations in serum P_i associated with meals. An eighth-order polynomial of time was fitted to these circadian variations; the resulting curve is shown in Fig. 2B. The correlation between the raw data and the model is .98 (*10*).

Previous studies have reported a single peak in P_i concentration during the night, when the concentration of Ca_T is at its daily low (*5*). Our data reveal two clearly separable peaks with maximum concentrations in the evening and early morning. Changes in serum P_i of this magnitude have been associated with physiological alterations in the biosynthesis of parathyroid hormone (*11*) and 1,25-dihydroxyvitamin D_3 (*12*).

Total calcium (Fig. 1, D and E) follows a circadian pattern quite different from that of Ca^{2+}; the correlation between

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