subject of several recent investigations (Ayanaba et al., 1973; Tate and Alexander, 1974). Nitrite may be formed in soil via the nitrogen cycle or from ammonium or nitrate fertilizers. In certain soils under fairly high ammonia and pH conditions resulting from heavy fertilization with urea, concentrations of 90 ppm nitrite nitrogen have been reported to persist for several months (Chapman and Liebig, 1952). Thus in instances when nitrite accumulates temporarily in high concentration, particularly in soils low in organic matter and clay content, and exceptionally high level of the herbicide glyphosate in soil due to accidental spillage or over application may therefore represent a potential starting material for the synthesis of *N*-nitrosoglyphosate. The latter is persistent in soil as it was observed that Fox soil treated at the highest levels contained about 7 ppm of II even after 140 days. It was also observed that a standard solution of II was stable for at least 6 months. At the present time we are not aware of any reported study concerning the carcinogenic properties of compound II, although it is known to be weakly mutagenic (Seiler, 1977). It may be noted that *N*-nitrososarcosine, an *N*-nitrosoamino acid structurally related to *N*-nitrosoglyphosate, has been shown to be carcinogenic (Druckrey et al., 1963).

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Electrophoretic Detection of Protein in Highly Pigmented Tobacco

Measurement of protein content of tobacco leaves during and after curing, using the Lowry method, gives falsely high values due to the reaction of the reagent with pigmented components that develop during the curing. The presence of protein in the curing and cured leaf can be shown by the presence of enzymatic activity. Peroxidase can be detected in leaf extracts by disc electrophoresis, but it is impossible to use this technique for protein in general, due to the interference of the highly pigmented components with the protein stain. A preparative electrophoretic technique, using Sephadex as a support medium, has been developed for the removal of these interfering pigments from the protein. The protein fraction is then resolved by standard disc electrophoresis, and individual proteins are visualized by staining with amido black.

Recent interest in tobacco protein as a by-product of certain processing techniques and its possible utilization as a food supplement have stimulated a reexamination of methods of detection and analysis of protein that are applicable to tobacco. The Lowry (1951) method of protein analysis, which involves the use of the Folin-Phenol reagent, can usually be applied without difficulty to green tobacco leaves but not to cured tobacco. Aqueous or buffer extracts of cured tobacco contain small amounts of soluble protein. Such extracts also contain appreciable amounts of high molecular weight, brown pigmented complexes composed, at least in part, of polyphenolic, protein, and carbohydrate moieties (Bailey and Schepartz, 1974). These pigments react with the Folin reagent to give falsely high protein values (Bailey et al., 1970). Protein content is known to decrease during curing (Frankenburg, 1946; Vickery and Meiss, 1953). We have observed this decrease during the first half of the curing period, followed by an

apparent increase in the latter half (Bailey et al., 1970). Obviously this observed increase could not have been due to protein, indicating the inability of this assay method to give a true picture of the protein content of the cured leaf. The Folin-Phenol reagent, which reacts with tyrosine and tryptophan in proteins, can also react with phenolic structures (Layne, 1957). Therefore, the polyphenolic components of the brown pigments that develop during curing are probably responsible for the apparent increase in protein. Any other method based on absorbance in the UV or visible range is subject to similar difficulty due to the high degree of pigmentation and its absorbance in the same range. Thus, the well-known method of Warburg and Christian (1941), which is based on absorbances at 280 and 260 nm, cannot be used under these circumstances. Although not subject to pigment interference, early methods of protein analysis (Frankenburg, 1946; Vickery and Meiss, 1953) were all indirect procedures, with Kjeldahl digestion and protein calculated from the insoluble nitrogen content.

The presence of protein in the curing and cured leaf can be shown indirectly by the presence of enzymatic activity. Thus, in cigar tobacco we have monitored catalase activity throughout the curing process, using an oxygen electrode to determine H_2O_2 decomposition (Schepartz and Bailey, 1974). We have also used peroxidase activity as an indicator of protein presence. The decrease in peroxidase activity during curing was determined colorimetrically with guaiacol as the color reagent (Bailey et al., 1970). The high sensitivity of this procedure permitted it to be used on samples so dilute that the pigment color did not obscure the desired color reaction with the guaiacol.

Using the disc electrophoresis procedures of Racusen (1967) and Racusen and Foote (1965), we have visualized tobacco peroxidase activity on polyacrylamide gels (Bailey et al., 1970), but the quantity of protein on the gel was too small to be detected by the usual protein stain, amido black. Under the conditions of electrophoresis employed, the brown pigments traveled with the front as a single discrete band. When the sample size was increased to provide additional protein for visualization, the increased amount of pigment interfered with the electrophoresis and the pigment no longer moved with the front. As a result, the entire gel blackened when the protein stain was applied.

Using disc electrophoresis, Sheen and Townes (1970) studied the soluble proteins in leaves of several types of cigarette tobaccos at maturity and under simulated air-curing conditions in the dark, thus artificially minimizing brown pigment formation. Although these authors reported severe interference because of the pigmented background, they were able to resolve several protein bands in the cured samples and noted a sharp decrease in protein during the cure.

Until now, we have been unable to achieve satisfactory separation of pigments from proteins in normally cured tobaccos by conventional methods, such as ammonium sulfate precipitation, various types of column chromatography or electrophoresis. In the present report, separation of the brown pigment from the protein was accomplished by a preparative electrophoretic technique that involved the use of Sephadex G-10 rather than acrylamide as a support medium. By this system protein was separated from the pigment in sufficient quantity to be visualized after standard electrophoresis on acrylamide gels.

EXPERIMENTAL SECTION

The apparatus used (Figure 1) was a sandwich-type electrophoresis unit designed by D. Racusen and constructed by L. White (University of Vermont). Buffers and

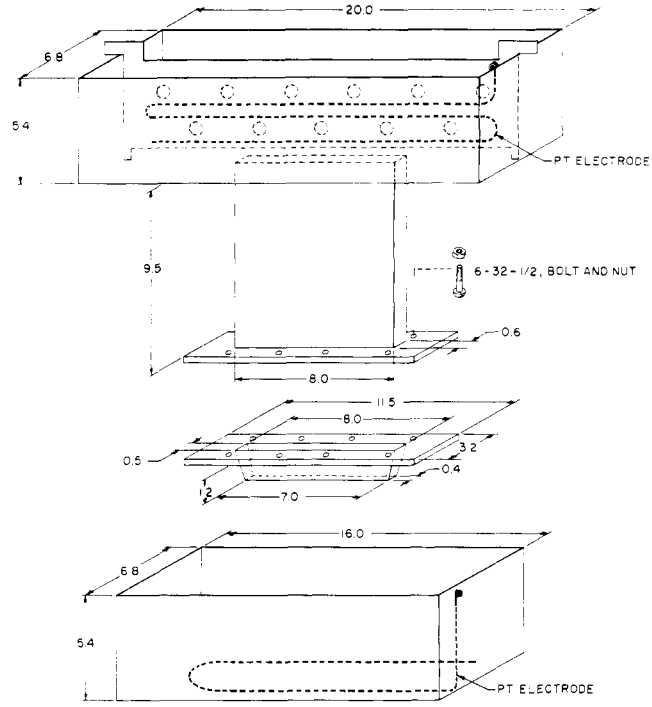

Figure 1. Exploded view of sandwich-type electrophoresis unit, giving structural details, dimensions in centimeters. Ends of upper and lower reservoirs constructed of 6 mm (0.25 in.) plexiglas; all other plastic parts of 3 mm (0.125 in.) plexiglas.

gels were prepared according to Davis (1964). Samples of cigar-filler tobaccos were donated by the General Cigar Company, Lancaster, Pa. Protein-containing tobacco fractions were prepared as previously described by Bailey et al. (1970).

A 1.5-cm layer of 7.5% acrylamide gel prepared with running buffer (0.05 M phosphate, pH 7.0) was placed in the base of the unit. Above this was placed 7.5 cm (about 40 ml) of Sephadex G-10, equilibrated with the running buffer. Ten milligrams of tobacco extract dissolved in 2 mL of the running buffer (containing 10% sucrose) was layered over the Sephadex. The upper buffer (0.05 M Tris–glycine, pH 8.3) was then layered carefully over the sample (Figure 2). Electrophoresis was carried out at 250 V and 20 mA until the brown pigment penetrated the acrylamide gel. The current was turned off, the Sephadex layer transferred to a Buchner funnel where it was washed with buffer (ca. 120 mL) for removal of the protein. The filtrate was chromatographed on a small Sephadex G-50 column; elution with distilled water separated buffer salts from protein. The protein (high molecular weight) fraction was collected and lyophilized. The dry material was dissolved in 0.25 mL of 0.05 M phosphate buffer, pH 7.0, and subjected to electrophoresis by a standard disc acrylamide gel procedure (Davis, 1964). The gel was treated with amido black for detection of protein bands.

RESULTS AND DISCUSSION

Staining of the final gel with amido black revealed the presence of five protein bands (Figure 3). These bands were presumed to be protein based on the fact that (a) they were contained in a high molecular weight fraction from which the pigment had been removed, and (b) they reacted with amido black to give typical protein color. This procedure thus provided the first direct detection of protein in highly pigmented, normally cured tobacco.

Sephadex has been used previously as a support medium in zone electrophoresis, giving sharply defined zones with negligible protein adsorption (Gelotte et al., 1962; Hanson

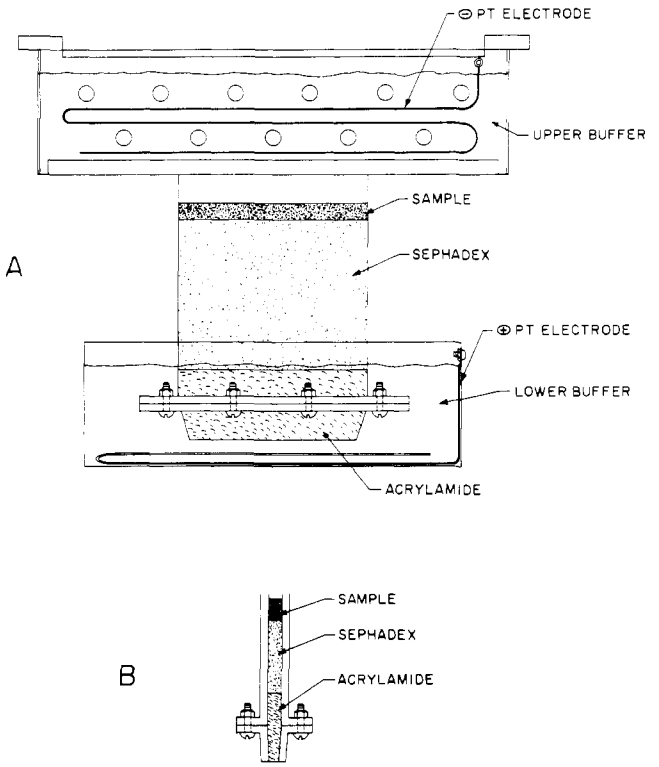

Figure 2. Assembled electrophoresis unit showing solutions and gels: (A) front view; (B) side view.

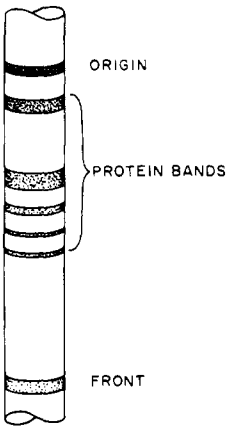

Figure 3. Protein bands on final disc gel after staining with amido black.

et al., 1966). It has also been used by Koppikar et al. (1970) in combination with acrylamide to increase resolution of serum proteins. In the present technique Sephadex G-10 was used not for molecular filtration, but as a support medium for the proteins. Once a protein sample penetrates an acrylamide gel, it is difficult to remove it without denaturation of the protein. However, the use of Sephadex as the gel support rather than acrylamide greatly facilitates the recovery of protein constituents for further processing.

Although we developed this technique mainly for cigar tobacco proteins, we believe it to be applicable to the detection of proteins in other plant materials that are highly pigmented. With the recent introduction of improved accessories for scanning gels in spectrophotometric instruments, quantitation of these proteins appears highly feasible.

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