Table 2. Nicotinic acid and nicotinamide content of smoke from various tobaccos as determined by microbiological assay. The totals were determined by assay of the raw smoke without use of chromatographic separations.

Tobacco type	Micrograms per cigarette		
	Nicotinic acid	Nicotinamide	Total
Bright	13	6	14
Burley	15	7	21
Turkish	9	6	13

determined by the pilot run were made on the paper chromatogram of tobacco smoke. The areas that measured 2 cm above and 2 cm below the lines and running the width of the chromatogram were cut from the paper. These strips were eluted with 80-percent ethanol by the use of a Soxhlet extractor, and the eluted solution was concentrated to dryness. The solids were taken up in water and assayed by the standard microbiological method (*6*) using *Lactobacillus arabinosus* 8014 organism. Table 2 shows the results when this procedure is applied to smoke from cigarettes made from three different types of tobacco.

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Solubility and Color Characteristics of Leaf Proteins Prepared in Air or Nitrogen

A characteristic property of extracts made from plant leaves is the intense brown color that develops almost immediately upon disruption of the living leaf cells. The browning reaction, presumably arising from the action of polyphenoloxidase, is often so severe that it interferes greatly with the analysis of the leaf proteins by such methods as electrophoresis and analytic ultracentrifugation, which depend upon an optical system for the location of protein components. Aside from this difficulty, browning also indicates that an undesirable change has occurred from the natural state of living protoplasm.

Browning of leaf extracts has been partially controlled by the use of chemical inhibitors (*1*). However, in our experience, inhibitors such as cyanide and dithiocarbamate have the undesirable property of precipitating proteins. Also, the addition of chemicals to plant extracts further complicates an already complicated system. The purpose of this communication is to show that browning can be entirely prevented when all operations during the homogenization and fractionation of leaf extracts are conducted in an inert atmosphere of high-purity nitrogen. Under such conditions, there is a striking difference in the solubility and color properties of two proteins that we have investigated, fraction I protein (*2*) and tobacco mosaic virus (TMV). We are presenting a general outline of the method because of its routine adoption in this laboratory for our work on plant proteins and viruses (*3*).

Leaves are first infiltrated repeatedly in a vacuum desiccator with 99.99 percent nitrogen which has been further purified by passage through alkaline pyrogallol and water. The sealed desiccator is then placed in a specially constructed plastic box (*4*). The box (Fig. 1) is designed to accommodate apparatus such as an Omnimixer, basket centrifuge, Servall centrifuge, electrodes for *p*H measurements, and glassware. Access to the apparatus within the box is provided by the neoprene gloves attached to the arm holes in the front panel.

In order to prevent browning of leaf extracts, we have found that a box atmosphere of more than 99.5 percent nitrogen is necessary. To obtain this, the box is sealed and then flushed with purified N_2 for 1 hour at a flow rate of 15 lit/min. Air and excess N_2 escape through small outlets at the top of the box. Nitrogen is played over all surfaces of the interior and apparatus and is bubbled for a few minutes through all liquids by means of a sintered glass gas disperser. After the flushing operation, the nitrogen flow is decreased to 8 lit/min, and the outlet tubes at the top are clamped off almost completely in order to maintain a slight positive pressure in the box.

We shall present here only one of the anaerobic protocols that we have followed. The problem was to prepare an anaerobic tobacco leaf extract free of cell walls, nuclei, chloroplasts, mitochondria, and sphaerosomes and to compare the remaining soluble proteins with a similar aerobic preparation. For this purpose, half-leaves of tobacco were used, one set for the anaerobic preparation, the other for the aerobic preparation.

The desiccator was opened in the flushed work box, and the leaves were

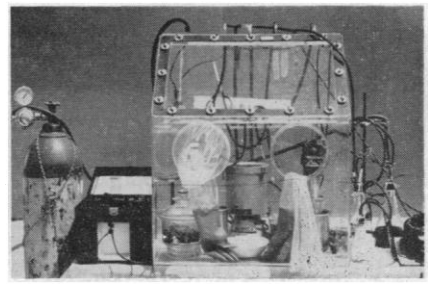

Fig. 1. Plastic box for maintaining an inert atmosphere during the preparation of leaf proteins.

cut into small pieces about 1 cm² in size. The leaf pieces were homogenized at top speed for 5 minutes in 0.5*M* *p*H 7.5 maleate buffer (1 part by volume to 2 parts of leaves by weight) in the Omnimixer, the Omnimixer cup and sleeve to which it was attached being immersed in an ice bath. The homogenate was passed through sharkskin filter paper with the aid of a basket centrifuge. The filtrate had a brighter green color than similar aerobic preparations.

The filtrate was placed in Spinco centrifuge tubes, the gaskets for the tubes were lightly greased, and the caps were tightened more than is customary in order to prevent entrance of air. The tubes were then removed from the box and centrifuged in the Model L Spinco ultracentrifuge, No. 30 head, at 25,000 rev/min for 90 minutes. The centrifuge tubes were returned to the nitrogen atmosphere of the work box before being opened. The clear, light yellow supernatants, designated whole cytoplasm, were set to dialyze under nitrogen against 0.1 μ maleate buffer at *p*H 7. They were then anaerobically transferred to a synthetic boundary cell for analytic centrifugation. The aerobic preparation of the opposite half-leaves resulted in a clear supernatant which was dark brown in color.

Analytic centrifuge patterns of both the air and the nitrogen samples appear in Fig. 2. The greater transmission of light through the anaerobic preparation permits the use of a narrower slit width, thus defining the baseline more sharply. The lesser difference in light transmission between the buffer and the solution makes for sharper definition of the forward limb of the slow moving peak. All in all, area measurements made with anaerobic preparations are more precise. The sedimentation constant of the faster component, representing fraction I protein, measured 17.8*s* for the aerobic whole cytoplasm and 18.1*s* for the anaerobic, a difference that is not considered significant.

By centrifuging the anaerobic whole cytoplasm, under anaerobic conditions as before, at 40,000 rev/min for 3 hours in the No. 40 Spinco head, clear, light yellow pellets, mostly consisting of frac-

tion I protein, are obtained. The aerobic counterparts of these pellets are clear but dark brown in color.

In addition to the striking difference in coloration of fraction I protein, a remarkable difference in its solubility characteristics is apparent. Fraction I protein pellets prepared in N_2 dissolve completely with great ease and rapidity merely by gentle shaking. Indeed, one difficulty in the anaerobic method is that the protein dissolves appreciably before the N_2 atmosphere can be reestablished and the supernatant fluid decanted. This difficulty can be partially overcome by not filling the tubes quite full and then immediately inverting the tubes after centrifugation, so that the pellets are separated from the supernatant liquid by a N_2 bubble during the interval necessary to reestablish an external N_2 atmosphere. When it is completely dissolved, a solution of fraction I protein is water clear, even when it contains as much as 1 percent protein. In contrast, fraction I protein prepared in air dissolves slowly and incompletely with an undissolved residue of protein remaining in spite of vigorous stirring. The protein solution is dark brown in color.

When the fraction I protein pellets are resuspended in weak neutral buffers or water, the anaerobically prepared pellets still require less time to resuspend completely than do the aerobic pellets. Thus, the same solubility relationships exist regardless of the resuspending medium. The following are some other observations comparing the behavior of the same leaf extracts prepared in air and under N_2.

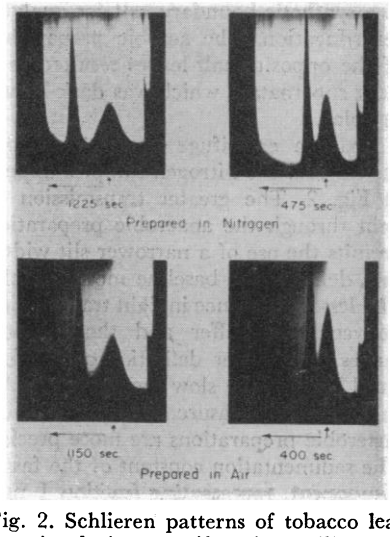

Fig. 2. Schlieren patterns of tobacco leaf proteins during centrifugation to illustrate clarity of pattern when browning is prevented. Arrows indicate direction of migration and position of starting boundary in synthetic boundary cell. Time is indicated after rotor attained speed of 51,100 rev/min; 0.1 μ K maleate buffer *p*H 7.0, protein concentration 0.8 percent. Fastest moving component is fraction I protein.

1) The ability to brown when exposed to air is retained for at least 3 weeks by anaerobic preparations of whole cytoplasm of tobacco leaves when the preparations are kept anaerobic at 4°C. With a similar preparation of spinach leaves, the ability of whole cytoplasm to brown is lost in 1 day.

2) Sixty hours of anaerobic dialysis (bubbling N_2) of anaerobic whole-leaf cytoplasm of Turkish tobacco, involving 10 anaerobic changes of a 1/50 dilution of the 0.5*M* maleate buffer, results in the loss of ability to brown in air. Presumably, a substrate or cofactor for the browning reaction has been lost during dialysis. On the other hand, *Xanthium* leaf whole cytoplasm will brown on exposure to air after a similar 60-hour anaerobic dialysis. Evidently, a less readily dialyzable component is contained in *Xanthium* extracts which is involved in oxidative browning.

3) Anaerobiosis permits the detection of pigments that are masked by browning. For example, tobacco leaf cytoplasmic proteins, dialyzed against phosphate buffer before analysis in a Cary recording spectrophotometer, display a minimum at 250 mμ, a maximum at 267 mμ, and shoulders at 290 and 325 mμ. The 325 mμ wavelength no doubt contributes to the yellow color of the protein solution. In contrast, a brown cytoplasmic protein solution fails to provide as much spectral detail in the ultraviolet and displays nothing more than a continuous absorption in the visible region of the spectrum.

4) During the summer and autumn months, a brownish purple pigment has been observed to occur in two species of tobacco. The pigment is partly dialyzable and is insoluble in all of the common organic solvents but is soluble in water. The cultural conditions of the plants under which the pigment appears are still undefined but may have some connection with nitrogen deficiency.

5) A red layer, lying between the starch and green chloroplast residue layers of the pellet formed by centrifuging a tobacco leaf homogenate at 30,000 rev/min (No. 30 Spinco head) for 1 hour, although observed to occur under aerobic conditions, is very much more pronounced under anaerobiosis.

6) Anaerobiosis permits the preparation of the common strain of tobacco mosaic virus in an uncolored condition, whereas TMV prepared in air under the same conditions is brown in color. As was shown previously (*5*), a brown host nucleoprotein associated with the virus can be removed by chelating agents to yield a colorless virus. Anaerobically prepared, uncolored virus also responds to the presence of a chelating agent by releasing, in this case, presumably an uncolored nucleoprotein. Anaerobically prepared virus also resuspends more rapidly than its aerobic counterpart.

As a result of our experience with the anaerobic method applied to a variety of plant physiological problems, we have come to believe that it represents a significant step forward in the direction of preparing protoplasmic constituents from plants which correspond more closely to their native state in the living cell. While the method requires some extra effort in the form of inconvenience, nevertheless the protoplasmic products obtained are of sufficient interest to warrant the inconvenience.

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* Fellow of the American Cancer Society, 1953–54, as recommended by the Committee on Growth, National Research Council.

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Reserpine and the Learning of Fear

It has been reported (*1*) that reserpine will attenuate a conditioned "emotional" or "fear" response in rats and monkeys. This finding was confirmed by other investigators (*2*) who, in addition, reported that monkeys that had been drugged prior to conditioning were "functionally impervious" to acquiring the emotional response. In contrast, the present paper presents the findings of two experiments that indicate that reserpine, even in large dosages, has a surprisingly minor effect on the learning of a fear response in rats (*3*).

The method used to establish and measure emotional behavior has been described in detail elsewhere (*4*). Briefly, thirsty rats are trained to press a lever for a water reward on a variable-interval schedule—that is, a lever press produces the reward only occasionally (average, once in 60 seconds). When the response rates are stabilized, the conditioned emotional response is superimposed on the lever-pressing behavior by repeated presentations of an originally neutral stimulus, a 3-minute clicking noise, terminated