muscle which acts against the elasticity of the spiracle lips; hence, it remains continuously under tension. An experimental analysis of the innervation of these spiracles has demonstrated that they receive motor innervation from the median nerves (*2*). In more than 200 instances, median nerve section has prevented the spiracle from cooperating in the ventilative rhythm. Nevertheless, the spiracle muscle can remain contracted after complete denervation and will open in response to relatively high concentrations of CO_2. Spiracle movements for the first 3 to 5 days following denervation, although no longer coordinated with the ventilative pattern, appear otherwise normal; muscle twitches are rapid and involve the whole muscle. During subsequent days these contractions become slower and are produced by asynchronous activity of parts of the muscle. This behavior, which perhaps should be called fasciculation, continues unabated for at least 90 days or until the muscle is reinnervated by the regenerating median nerve.

At any time during this period the muscle may be removed to a dish of saline where it will continue to fasciculate. Surprisingly, during this entire period the muscle remains responsive to electric stimulation. Although the rheobase varies from 1.8 to 4.0 volts there appears to be no consistent variation with time since denervation. Electric stimuli do not interfere with the spontaneous fasciculative rhythm at low stimulus frequencies, but tetanic contractions are induced by liminal stimuli at about 20 per second.

A study of methylene blue preparations indicates that the onset of fasciculation occurs at about the same time that the peripheral branches of the sectioned median nerve disappear.

The differences between the behavior of denervated thoracic spiracle muscles and that described for other insect muscles are not yet understood and currently are under investigation. One possible source of the difference is that the spiracle muscle is continuously under tension. Consequently its mechanical arrangements are rather similar to those of some insect flight muscles, in which one set of muscles contracts against the tension of another, or to the dipteran haltere muscle, which contracts in opposition to the elasticity of the haltere articulation. In both the dipteran flight and the dipteran haltere muscles contraction is not necessarily the direct consequence of the motor impulse (*3*). Rather, the motor impulse serves to increase sensitivity of the muscle to stretch to such a degree that a twitch occurs. By analogy, it seems possible that fasciculation of the denervated spiracle muscle is due to increased sensitivity to stretch. This is supported by the observation that a denervated muscle which has become momentarily quiescent may be readily started fasciculating again by mechanically opening the spiracle. A corollary hypothesis that the presumed stretch sensitivity of denervation involves increased muscle sensitivity to the neuromuscular mediator substance is currently under investigation.

JAMES F. CASE

Physiology Division,
Chemical Warfare Laboratories,
Army Chemical Center, Maryland

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Nicotinic and Glutamic Acids, Nicotinamide, and Glutamine in Cigarette Tobacco Smoke

During the course of studies of the constituents in tobacco smoke (*1*) it became of interest to investigate the presence of some naturally occurring, biologically active nitrogen compounds that had not previously been reported in the literature. The presence of the amino acids, glutamine and glutamic acid, and of nicotinic acid and nicotinamide are reported in this paper.

Cigarettes, 70 mm in length, were smoked according to a standard procedure reported elsewhere (*2*). An amphoteric fraction of the collected smoke containing nitrogen and giving a positive reaction to ninhydrin was chromatographed on Dowex-50 (8 percent cross-linked, 200 to 400 mesh, H^+ form), and six separate ninhydrin-positive fractions were eluted with 2*N* HCl. The fractions were compared directly with authentic samples of amino acids in different paper chromatographic solvent systems. Table 1 shows the close agreement of fracions 1 and 2 with glutamic acid and glutamine, respectively. After heating fraction 2 in 1*N* HCl for 1 hour at 100°C and rechromatographing, it was found that the spot which initially migrated exactly like authentic glutamine no longer existed. Instead, a new spot appeared which migrated parallel with known glutamic acid. The conversion after acid hydrolysis of fraction 1 into glutamic acid is additional evidence that fraction 1 was originally glutamine.

A quantitative determination that utilized the color developed by the reaction of the amino acids with ninhydrin showed that the smoke from a 50/50 blend of bright and burley tobaccos contained, respectively, 10 and 7 μg of glutamic acid and of glutamine per cigarette. The four remaining ninhydrin-positive amphoteric substances have not been identified.

The migration of the pyridine alkaloids and of nicotinic acid and nicotinamide in several solvents on buffered paper has been shown to be a function of the *p*H of the buffer (*3–5*). The pattern of movement of a given pyridine-containing compound in the system has been useful in its characterization (*4*). Nicotinic acid has been tentatively identified in tobacco smoke by use of such a procedure (*5*).

For a verification of the presence of nicotinic acid and a quantitative determination of the acid and of nicotinamide, the following procedure was followed. Smoke collected in Dry Ice-acetone traps was dissolved in ether and extracted three times with equal volumes of 2-percent HCl solution. The acid layer was adjusted to *p*H 10.0 with sodium hyroxide and extracted twice with an equal volume of ether to remove most of the nicotine-type alkaloids present. A check run with knowns showed that, after this treatment, all the nicotinic acid and 90 percent of the nicotinamide remained in the basic aqueous layer. This solution was then neutralized and concentrated to a small volume. An amount of solution corresponding to one cigarette was then applied in a 0.5-cm wide streak across the entire width of a dry, 20- by 40-cm Whatman No. 1 paper that had previously been soaked in *p*H 6.3 sodium citrate buffer.

On a separate 5- by 40-cm paper also buffered at *p*H 6.3, 20 μg of a known mixture of equal parts of acid and amide were applied and run simultaneously with the paper chromatogram containing the cigarette smoke. The R_f values of the acid and of the amide on the pilot paper were 0.30 and 0.80, respectively. The values were determined by developing a color reaction with *p*-aminobenzoic acid in an atmosphere of cyanogen bromide.

Two pencil lines that corresponded to the R_f of the acid and of the amide as

Table 1. Comparison of R_f values of glutamic acid and glutamine with elution fractions from Dowex-50 (H^+) ion-exchange resin.

Solvent system	Glutamic acid	Fraction 1	Glutamine	Fraction 2
Phenol/water (7/3)	0.27	0.27	0.56	0.56
n-Butanol/acetic acid/water (40/10/50)	0.22	0.22	0.12	0.13
Methyl ethyl ketone/propionic acid/water (75/25/30)	0.30	0.30	0.23	0.23

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.

DONALD A. BUYSKE
JOHN M. FLOWERS, JR.
PELHAM WILDER, JR.
MARCUS E. HOBBS
Department of Chemistry,
Duke University,
Durham, North Carolina

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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

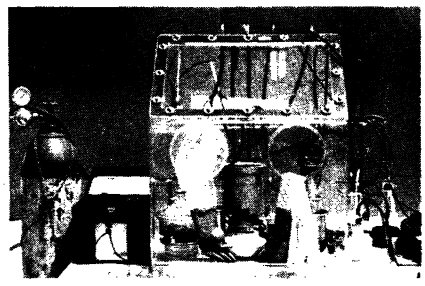

Fig. 1. Plastic box for maintaining an inert atmosphere during the preparation of leaf proteins.

cut into small pieces about 1 cm^2 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-