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.

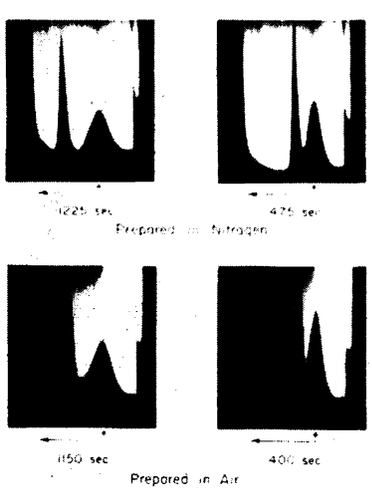

Fig. 2. Schlieren patterns of tobacco leaf proteins during centrifugation to illustrate clarity of pattern when browning is prevented. Arrows indicate direction of migration and position of starting boundary in synthetic boundary cell. Time is indicated after rotor attained speed of 51,100 rev/min; 0.1 μ K maleate buffer pH 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 0.5M 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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16 August 1956

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

Table 1. Emotional conditioning measured as suppression of lever responding in the presence of the conditioned stimulus during an extinction test period. Values presented express the rate of lever pressing during the presentation of the 3-minute auditory stimulus as a proportion of the rate in the immediately preceding 3-minute period.

Experiment I			Experiment II		
Rat no.	First test trial	Over-all extinction (15 trials)	Rat no.	First test trial	Over-all extinction (15 trials)
		Reserpine during conditioning			
E1	0.00	0.56	E4	0.03	0.80
E2	0.02	0.59	E5	0.01	0.15
E3	0.00	0.48	E6	0.00	0.64
Mean	0.01	0.54	Mean	0.01	0.53
		Saline (or no drug) during conditioning			
C1	0.00	0.52	C4	0.00	0.54
C2	0.00	0.26	C5	0.00	0.46
C3	0.00	0.55	C6	0.00	0.14
Mean	0.00	0.44	Mean	0.00	0.38

contiguously with a brief shock to the feet.

The conditioned fear response consists of suppression of lever responding, crouching, immobility, and frequently urination and defecation on presentation of the auditory stimulus. A quantitative index of the degree of behavioral suppression is obtained by calculating the ratio of the number of lever-pressing responses emitted in the 3-minute period of clicker presentation to that emitted in the 3-minute period immediately preceding the onset of the stimulus.

The present experiments were similarly designed, and both involve two main phases. In the first phase, two groups of rats were given emotional conditioning; an experimental group was trained under the influence of reserpine, and a control group was given identical training without the drug. In the second phase, both groups were tested for emotional conditioning after the effects of the drug had worn off. This kind of experiment requires two controls: the experimental group must be effectively drugged during conditioning, and the effects of the drug must have been dissipated prior to testing.

The first experiment investigated the chronic action (that is, conditioning sessions occurred 23 hours after drug administration) of reserpine on the learning of the fear response. Following stabilization of lever-pressing rates in daily 1-hour sessions, three experimental animals were given intraperitoneal injections of 0.5 mg/kg of reserpine daily after the lever-pressing run, and three control rats received saline injections of equal volume. Injections were continued for 6 additional days, at the end of which time a sharp drop in lever-pressing rates (on the average, to approximately 60 percent of the original level) indicated that the drug had "taken." On each of the four days immediately following, three pairings of clicker and shock (1.0 ma) were administered at 20-minute intervals during the 1-hour lever-pressing session. Every animal thus received a total of 12 emotional conditioning trials. Drug injection was continued during this period.

Prior to testing for emotional conditioning, a control phase was instituted to take account of the long-term residual effects of reserpine. For 10 days immediately following the conditioning phase, the drugs were switched between groups; the control group received a series of reserpine injections identical with that administered to the experimental animals prior to and during conditioning, and the experimental animals were given saline injections corresponding to those previously received by the controls. During this period there were no emotional conditioning trials. Following this control phase, all injections were discontinued, and the daily lever-pressing sessions were continued until the original response rates had been recovered by all animals (24 days). Both groups then received five daily extinction sessions under a procedure in which the clicker stimuli were presented as in the conditioning phase, but no shocks were administered.

The second experiment, which was concerned with the acute (3 hours between injection and conditioning) action of reserpine, essentially replicated the procedure of the first experiment with the following changes (*5*). On the three days prior to emotional conditioning, experimental animals received "preparatory" injections of 0.5, 0.5, and 1.0 mg/kg of reserpine, in that order. Twelve emotional conditioning trials were then administered to both experimental and control (noninjected) animals over two daily sessions, the experimental animals having been injected with 1.0 mg/kg of reserpine 3 hours prior to the first conditioning trial of each session. Injections were then discontinued, and daily lever-pressing runs were administered until the original response rates had been recovered by the experimental subjects (10 days). All animals were then given five daily extinction test sessions identical in procedure with that of the first experiment.

An index of lever-pressing suppression was calculated as described for every animal in both experiments for the first test trial and for the over-all extinction period. These data are presented in Table 1. A high degree of emotional conditioning for all animals, experimental and control, is indicated by the first extinction trial scores; the values in all cases equal or approximate 0.00, reflecting virtually complete suppression of lever responding during the presentation of the clicking noise. Further, examination of the over-all test period scores reveals only a slightly greater tendency on the part of the controls to resist extinction of the conditioned suppression.

In view of the fact that the experimental animals were drugged practically to the point of inactivity by the end of the conditioning phase (*6*), these data lend little support to the earlier report (*2*) that reserpine substantially impedes the learning of an emotional response. While this discrepancy may be a species phenomenon (rats versus monkeys), the similarity of reserpine effects on previously established emotional behavior in these animals (*1*) suggests that procedural differences are more likely factors. The use of a relatively objective criterion of emotional response in the present studies, as opposed to the observational criteria employed in the earlier investigation, is one difference in method that has, in other situations, not infrequently yielded conflicting findings.

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5. More accurately, this experiment investigated both cumulative and acute effects of reserpine.
6. For this reason, reliable estimates of suppression were not obtainable in the conditioning phase. However, bolli counts taken during conditioning in experiment I suggest that the experimental subjects were less fearful. The total counts for the three drugged anmials were 3, 11, and 14, while those for the controls were 32, 25, and 35.

20 September 1956.