greater final yield of water-soluble extract than does centrifuging at low speed a coarser suspension after standing overnight. By filtering after completion of the nutrient we are filtering a 1:20 instead of a 1:1 dilution of the substances extracted from the embryo tissue. This filtration can be carried out without hyaluronidase, but addition of the latter speeds up the process enough to justify its use.

Bryant *et al.* have made a notable contribution in elucidating many details of embryo-extract behavior. The simpler methods we have described produce a medium perfectly adequate for the maintenance of stock cultures of the cells of "strain L."

Received October 8, 1953.

Hiptagenic Acid, a Toxic Component of *Indigofera endecaphylla*

M. P. Morris, C. Pagán, and H. E. Warmke

***Federal Experiment Station, Mayaguez, Puerto Rico*[1]**

Various species of *Indigofera* have been used for many years in the Orient as forage and cover crops. One species, *Indigofera endecaphylla,* Jacq. (trailing indigo or creeping indigo) showed great promise when established in Hawaii and Latin America. In Puerto Rico a pasture cafeteria experiment using cattle as test animals, showed *I. endecaphylla* to be outstanding on the basis of yield, palatability, protein content, and recovery after heavy grazing (*1*).

Several years after *Indigofera endecaphylla* was introduced into Hawaii, it was observed that this legume produced severe toxic symptoms in herds of dairy cattle (*2*). These symptoms included loss of appetite, dizziness, abortion, and even death, if the cattle were not removed from the trailing indigo pasture. Extended feeding tests with guinea pigs were then carried out in Puerto Rico (*3*). These tests showed that guinea pigs were able to survive indefinitely (2 yr) on a diet in which *I. endecaphylla* constituted the only green forage. The test animals maintained normal weight and appeared normal in every way except for the fact that the pregnant females invariably aborted during early or middle pregnancy. Feeding tests with chicks showed that the lethal dose for a 1-wk-old chick was about 5 g of dry plant material.

In view of the outstanding agronomic characteristics of trailing indigo, a breeding program aimed at producing a nontoxic variety of trailing indigo has been initiated. If such a variety can be developed, it will constitute an important contribution to tropical agriculture.

The breeding program for trailing indigo required a method of analysis that would use no more than a single plant for each assay. Because individual plants may yield as little as 1 g of dry plant material, the possibility of using animal feeding tests as a method of assay was eliminated. In seeking a satisfactory chemical method of assay an investigation of the chemical nature of the toxic principle was made.

Extraction tests with various solvents showed that no toxic material could be extracted with nonpolar solvents such as chloroform, benzene, carbon tetrachloride, and ether. It was found that a substance which was highly toxic to chicks was readily extracted with hot water. Further, it was found that this toxic material could then be extracted from the aqueous solution by repeated extractions with ether. The toxic mixture obtained after the evaporation of the ether crystallized when taken up in hot benzene and cooled. The crude crystalline material melted at 63°–65°. A highly purified sample obtained from a silicic acid column melted at 67.5°–68°. Qualitative tests showed the presence of carbon, hydrogen, and nitrogen. Quantitative analysis for carbon, hydrogen, and nitrogen indicated an empirical formula of $C_3H_5NO_4$. Aqueous solutions were strongly acidic; and points of inflection on the titrametric curve indicated equivalent weights of 59 and 118. Molecular weight determinations in benzene gave figures of 235 to 245, thus indicating dimerization in nonpolar solvents. As the acid was dibasic, it seemed probable that the 4 oxygen atoms in the molecule could be accounted for by 2 carboxyl groups. The fact that this compound smoothly evolved carbon dioxide when heated to 130°–140° added weight to this idea. However, qualitative tests showed that the nitrogen atom was in an oxidized state, and therefore the toxic compound could not be a dicarboxylic acid.

A literature search based on the above information showed that this toxic, dibasic acid was identical with hiptagenic acid, the aglycone of hiptagin, a toxic glucoside first isolated by Gorter (*4*) from *Hiptage benghalensis* (metablota) a plant native to India. This conclusion was not obvious from a comparison of the available physical and chemical properties of the two substances. Fortunately however, 3 plants of *Hiptage benghalensis* had been introduced from the Orient by the Bureau of Plant Industry in 1939, and were being maintained here at this station (P. I. 116513). This permitted a direct comparison of the two compounds. Both gave the same crystalline forms, melted at 67.5°–68°, and had the same composition and identical titrametric curves. The mixed-melting-point was 67°–68°; the ultraviolet absorption curves were identical and showed a maximum at 276 mμ.

The unusual and often complex chemical reactions of hiptagenic acid have made the elucidation of its molecular structure difficult. Several different formulae were proposed before Carter (*5*) presented what appears to be conclusive proof that hiptagenic acid is identical with β-nitropropionic acid. Another group of investigators (*6*) has reported that β-nitropropionic acid is a metabolic product of *Aspergillus flavus*. If hiptagenic acid and β-nitropropionic acid are identical, then this compound is the first and only aliphatic nitro compound ever isolated from natural sources. This simple, three carbon, nitrogen-containing acid has now been isolated from several different spe-

[1] Administered by the Office of Experiment Stations, Agricultural Research Service, USDA.

cies of plants; and the possibility exists that it may play an important role in nitrogen metabolism.

References

1. WARMKE, H. E., FREYRE, R. H., and MORRIS, M. P. *Agron. J.* **44**, 517 (1952).
2. NORDFELDT, S., *et al. Hawaii Exptl. Tech. Bull.* No. 15, 1952.
3. FREYRE, R. H., and WARMKE, H. E. Legume Toxicity. Puerto Rico Fed. Expt. Station Ann. Report, 1952.
4. GORTER, K. *Bull. Jard. Bot. Buitenzorg* **2**, 187 (1920).
5. CARTER, C. L., and MCCHESNEY, W. J. *Nature* **164**, 575 (1949).
6. BUSH, M. T., *et al. J. Biol. Chem.* **188**, 685 (1951).

Manuscript received December 18, 1953.

The Determination of Chloride by Use of the Silver-Silver Chloride Electrode[1]

Martin Chanin[2]

Laboratory of Thoracic Surgical Research, City of Hope Medical Center, Duarte, California

Accepted methods for the determination of chloride may be grouped mainly under four headings: (a) gravimetric; (b) titrimetric, with color indicators; (c) potentiometric, with titration; and (d) optical. Attempts have been made to adapt these methods so that they may be used in dilute solutions and with biological materials, but such adaptions are usually time consuming and not too accurate. This paper describes an improved potentiometric method for chloride ion determination. The time required for a complete determination should be no more than one minute.

A consideration of the Nernst equation shows that, with the proper electrode pair, it should be possible to set up a concentration cell for the determination of almost any ion. The voltage will not be affected by the presence of biological material, and there should be minimal interference from other ions. Tremblay (*1*), Neuhausen and Marshall (*2*), Duxbury (*3*), and Katsu (*4*) decided that the method has only very limited application. One of the greatest difficulties has been drifting. Drifts have been eliminated in the present investigation by making all dilutions with a buffer solution of pH 7. These drifts are due to the changing pH, which is caused by the absorption of atmospheric CO_2.

There is a daily change in the voltage developed by known chloride concentrations, so it has not been possible to compute values from theoretical considerations (*1, 2*). It is necessary to plot a reference curve each time the electrodes are used, but because the voltage is a logarithmic function of concentration, only two points are needed. These are plotted on semi-log paper, and the straight line that connects them is the desired curve. Dilutions should be made so that the final unknown chloride concentration is in the range of 0.1 to 1.0 milliequivalents per liter. Also, the concentrations of at least two different dilutions of the same unknown should be determined. There should be insignificant variation when the original concentration is computed.

TABLE 1. Millivolt readings for various concentrations of chloride with the silver-silver chloride electrode.

Concentration (Milliequivalents per 1000 ml)	Reading (mv)
2.0	245
1.5	239
1.0	230
0.9	227
0.8	225
0.7	222
0.6	219
0.5	215
0.4	209
0.3	203
0.2	194
0.1	178

The electrodes used in the present investigation are a mercury-mercurous sulfate reference electrode (which has provision for a saturated potassium sulfate bridge built into it),[3] and a silver-silver chloride electrode.[4] The latter electrode is a rod of solid silver, and is shipped with a silver chloride coating already on it. This coating must be renewed completely before it can be used, as unexplained drifts occur with an insufficiently pure silver chloride coating.

The following method for renewing the silver chloride has worked best. Scour the silver electrode with cleansing powder until no purple brown color can be seen. Rinse with distilled water and then connect to the negative pole of a 1½-v dry battery. Connect a platinum electrode to the positive pole, immerse the electrodes in dilute ammonium hydroxide, and allow current to flow for about 1 min. Remove the electrode, wipe off any discolored material, and rinse with distilled water.

Coating with silver chloride is now effected by electrolyzing in a saturated solution of reagent-grade potassium chloride. Connect the clean silver electrode to the positive pole of a 1½-v dry battery, a platinum electrode to the negative pole, and allow current to flow for 20 sec. Without rinsing, place the freshly prepared silver-silver chloride electrode in distilled

TABLE 2. Millivolt readings and chloride concentrations for different dilutions of blood serum.

Dilution	Concentration (Milliequivalents per 1000 ml)	Reading (mv)
	1.0	233
	0.1	180
1–100	1.14	236
1–1000	0.114	183

[1] Supported by a grant from the National Tuberculosis Association and the California Tuberculosis Association.

[2] The author acknowledges the consultative assistance of Wendell H. Griffith, Chairman of the Department of Physiological Chemistry at the University of California Medical School at Los Angeles.

[3] Beckman 1170-9.

[4] Beckman 1264.