would escape detection today than a few decades ago. No activity during the course of this season or recent seasons has been of the order of magnitude of the explosive eruption of Mount Katmai in June, 1912. That eruption partly destroyed one of the highest peaks in this portion of the range, created a crater 1½ mi in maximum dimension, and ejected 25 to 50 times the volume of material involved in Trident's February eruption (7). The greater frequency of mention of volcanism in this area during recent months probably reflects a more general awareness of the activity but not necessarily greater frequency or intensity of eruptions.

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Filtration of Embryo Extract for Tissue Cultures[1]

Charity Waymouth[2] and Philip R. White

Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

The discipline commonly called "tissue culture" has long suffered from the apparent complexities of its procedures. Bryant, Earle, and Peppers have recently published on "The effect of ultracentrifugation and hyaluronidase on the filtrability of chick-embryo extract for tissue cultures" [*J. Natl. Cancer Inst.* **14**, 177 (1953)], which we wish to commend as a contribution to mass technics. We should like to note, however, that many of the complicated procedures and the correspondingly expensive equipment described may be omitted.

We have been using hyaluronidase-treated chick-embryo extract in the maintenance of Earle's "strain L" cells for about 8 mo.[3] Our cultures show a uniform cellular increase of sufficient rapidity to require subculturing (dividing into 3–10 aliquots, usually 6) at about 10-day intervals. This is not the maximum proliferation rate, which is not required for maintaining stock cultures.

Our procedure is as follows: We open a dozen "fertile" eggs, incubated 10 days. These will usually provide 8–10 embryos which are collected in a small Petri dish. They are then dropped into the barrel of a 20-ml Luer syringe. We do not use a screen insert. The plunger is inserted and the tissue pulp forced into 15-ml conical Pyrex centrifuge tubes, allowing about 5 ml of pulp per tube. No complex grinders are needed. We then add an equal quantity of Earle's balanced salt-dextrose solution, stir thoroughly with a spatula or glass rod, and place in the refrigerator at +5° C overnight. No freezing with CO_2 is required. The following day the extract is separated by centrifuging for 10 min at about 3000 rpm. No ultracentrifugation is needed. The clear supernatant is decanted, and can either be used immediately in the preparation of nutrient (which is our usual practice), or can be stored in the freezing part of the refrigerator at −15° C.

The complete nutrient comprises 1 volume of extract, 2 volumes of horse serum, and 7 volumes of balanced salt solution. About 10 mg of hyaluronidase are added to each 100 ml of nutrient, and the whole is filtered through a Selas candle of No. 03 porosity. We do not use a pressure filter. A water pump or mechanical vacuum pump is satisfactory, but the vacuum should be kept below 250 mm of mercury by use of a bleeder valve. One hundred and fifty milliliters of nutrient will filter without clogging a 2-in. filter and with almost 100 percent yield in about 10 min. The clear sterile filtrate is drawn off into tubes and either used immediately or stored at +5° C. A dozen eggs will usually provide about 15 ml of embryo extract (1:1) or 150 ml of complete nutrient. The solution has a final composition of 10 percent embryo extract, 20 percent horse serum, and 70 percent balanced salt solution. This is only half as concentrated for both embryo material and serum as recommended by Bryant *et al.*, yet we find it entirely satisfactory for routine maintenance of "strain L" cells in 3.5-cm Carrel flasks without substratum (cells growing directly on glass, no cellophane or clot). It is also to be noted that we do not "gas" the nutrient or the flasks with a special gas mixture. Air is quite satisfactory.

In this procedure we have utilized three of the steps recommended by the above authors: a Luer syringe as a grinder, hyaluronidase as a depolymerizer, and filtration through the No. 03 Selas candle. We have eliminated the use of a wire screen in the syringe, preliminary freezing with CO_2 ice, grinding in a Potter-Elvehjem grinder, ultracentrifugation, use of a pressure filter, and gassing of the culture flasks. The equipment needed is to be found in most laboratories. The procedures can be carried out by a student after a little instruction. We believe that the results are not inferior to those obtained by more complex methods.

It is our belief that many of the difficulties encountered by Bryant *et al.* have arisen from the series of procedures used. If one presses tissue through a 24-mesh wire screen, then freezes in CO_2 ice, and grinds in a Potter grinder, one obtains a suspension containing much fine particulate matter which clogs the filter. It is doubtful if this excessive trituration provides any

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[2] A.C.S.-B.E.C.C. Fellow 1952–53 from the Chester Beatty Research Institute, London, England.

[3] We are indebted to Dr. Earle for communicating to us information on the use of hyaluronidase for this purpose prior to publication.

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."

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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.