Production of Cardiac Glycosides by Plant Tissue Cultures I

Nutritional Requirements in Tissue Cultures of *Digitalis lanata* and *Digitalis purpurea*

By E. JOHN STABA

The techniques employed and the agar medium suggested for the establishment of *Digitalis lanata* Ehrh. and *Digitalis purpurea* L. seed and root callus tissue are reported. Particular attention is devoted to the concentration effects of the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), 2-benzothiazoleoxyacetic acid (BTOA), and 1-naphthaleneacetic acid (NAA) contained in a growth medium suggested for the two *Digitalis* species.

THE GENERAL procedures employed to establish *in vitro* plant tissue cultures have been well documented and reported (1–3). However, the establishment of a defined medium for a specific plant to be grown as either normal or callus tissue is still determined empirically.

Nickell (4), Steward (5), and Bergmann (6) have shown that callus plant tissue cultures could be transferred to liquid submerged cultures and grown as single plant cells or small aggregates of plant cells. Many investigators have since speculated on the possible application of plant tissue cultures for producing economically useful plant constituents (4, 7).

The experimental evidence pertaining to the capability of an *in vitro* plant tissue culture to biosynthesize a desired plant constituent is inconclusive. *Datura* crown-gall tumors are known to contain high amounts of hyoscine (7); *Datura tatula* L. root tissue cultures are known to biosynthesize the alkaloids hyoscyamine and hyoscine (8); and, *Atropa belladonna* L. root and callus tissue cultures are known to biosynthesize the alkaloid atropine (9). However, callus tissue cultures of *Agave toumeyana* Trel. leaves do not contain the sapogenin hecogenin (10).

This publication represents the first one of a series that will attempt to determine whether or not tissue cultures of cardiac glycoside-containing plants can biosynthesize cardiac glycosides.

EXPERIMENTAL

The digitalis seeds used on medium A (see Table I) were obtained from the Saier Seed Co., Dimondale, Mich., and those seeds used on medium B were obtained from the medicinal plant laboratory and garden, University of Minnesota.[1] Roots were obtained from digitalis plants approximately 6 months old and growing in the drug plant greenhouse, University of Nebraska.

Sterilization of Seeds and Roots.—Digitalis seeds were sterilized in a 5.25% sodium hypochlorite solution (Purex Corp., Ltd., South Gate, Calif.) diluted 1:4 with sterile distilled water for approximately 10 minutes in vacuum. The seeds were then washed with sterile distilled water and transferred to sterile Petri dishes containing approximately 10 ml. of sterile distilled water. Two seeds were transferred aseptically to a nutrient medium after approximately 80% of the seeds had begun to germinate. This occurred on the fifth day for *D. lanata* Ehrh. seeds and on the third day for *D. purpurea* L. seeds.

Root sections were cut from digitalis plants grown in soil, washed in tap water, and sterilized in the above solution in vacuum for approximately 15 minutes. By aseptic procedures, the sterilized root sections were washed with distilled water, placed in Petri dishes containing a small amount of distilled water, and chopped with a scalpel into small root sections of about 2 cm. The root sections were then transferred aseptically to a nutrient medium.

Auxin Stock Solutions and Coconut Milk.—All auxins were prepared as 30% ethanol stock solutions. The auxins and coconut milk were autoclaved with the constituents of medium A or medium B. With the exception of 2-benzothiazoleoxyacetic Acid (BTOA),[2] all auxin stock solutions were used within 48 hours of their preparation.

Experiment One.—Medium A, with 0.7% agar, was prepared in the following four variations: (*a*) medium A; (*b*) medium A with 2,4-dichlorophenoxyacetic acid (2,4-D), 2 parts per million (p.p.m.); (*c*) medium A without enzyme-hydrolyzed casein hydrolysate (EHCH) but with 2,4-D, 2 p.p.m.; and (*d*) medium A without EHCH, coconut milk, or 2,4-D. The pH after autoclaving of media (*a*) and (*b*) was 4.1, and of media (*c*) and (*d*), 5.4. The low pH of media (*a*) and (*b*) was due to the presence of EHCH and caused the media to liquefy.

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[1] Courtesy of the late Dr. Earl B. Fischer, Professor of Pharmacognosy, University of Minnesota.
[2] Supplied through the courtesy of the Organic Chemical Research Section, Lederle Laboratories, Pearl River, N. Y.

TABLE I.—DIGITALIS TISSUE CULTURE MEDIA

Medium A[a]		Medium B[a]	
Coconut milk, %	15.0	Coconut milk, %	15.0
Sucrose,[b] %	2.0	Sucrose,[b] %	2.0
meso-Inositol, %	0.5	Modified White's inorganic salts, mg./L.	
Vitamin-free, enzyme-hydrolyzed casein, %	0.2	$MgSO_4$	360.0
Murashige's inorganic salts,[c] mg./L.		$Ca(NO_3)_2$	200.0
KNO_3	1,900.0	Na_2SO_4	200.0
NH_4NO_3	1,440.0	KNO_3	80.0
$MgSO_4 \cdot 7\ H_2O$	370.0	KCl	65.0
$CaCl_2$	332.0	NaH_2PO_4	16.5
KH_2PO_4	136.0	$MnSO_4$	4.5
$MnSO_4 \cdot 1\ H_2O$	14.0	$ZnSO_4$	1.5
$ZnSO_4 \cdot 7\ H_2O$	8.1	H_3BO_3	1.5
H_3BO_3	4.8	KI	0.75
KI	0.8	Na_2MoO_4	0.021
$(NH_4)_6MoO_2 \cdot 4\ H_2O$	0.18	$CuSO_4$	0.002
$CuSO_4 \cdot 5\ H_2O$	0.02	Iron-ethylenediamine tetraacetic acid[d]	50.0
Iron-ethylenediamine tetraacetic acid[d]	79.5	Vitamins solution[e] (same as medium A)	
Vitamins solution[e]		Simple supplement	
Biotin	1.0	*meso*-Inositol	200.0
Choline	1.0	Glycine, anhydrous	2.0
Folic acid	1.0	Niacin	0.4
Nicotinamide	1.0		
Pantothenic acid	1.0		
Pyridoxal	1.0	pH Adjustments made with ammonium hydroxide solution	
Thiamine	1.0		
Riboflavin	0.1		
Seitz-Filtered Complex Supplement for Medium B			
	mg./L.		mg./L.
Glucose-1-phosphate	100.0	Chlorogenic acid	10.0
L-Glutamine	100.0	Methyl-β-L-arabinopyranoside	5.0
L-Glutamic acid	50.0	Adenosine triphosphate disodium salt	5.0
L-Aspartic acid	50.0	Adenine (hemi) sulfate	5.0
Urea	50.0	Desoxyribonucleic acid	3.0
L-Arabinose	50.0		
L-Asparagine	25.0		

[a] Media were prepared with inorganic chemicals of reagent grade, organic chemicals from either Nutritional Biochemical Corp., Cleveland, Ohio, or Calbiochem, Los Angeles, Calif., and distilled water, redistilled from glass. [b] Pfanstiehl Laboratories, Waukegan, Ill. [c] Dr. Toshio Murashige, University of Hawaii, formerly in the laboratory of Dr. Folke Skoog, University of Wisconsin. [d] Perma Green Iron 135, Refined Products Corp., Lyndhurst, N. J. [e] Eagle's vitamins solution, Hyland Laboratories, Los Angeles, Calif.

Murashige's mineral solution; Murashige's mineral solution, half-strength; and modified White's mineral solution were prepared with each of the above four variations.

Two sterile, germinated digitalis seeds were placed aseptically on 5 ml. of each medium contained in 17 mm. × 100 mm. sterile plastic test tubes.[3]

Experiment Two.—Medium B, with 0.7% agar, was prepared in the following three variations: (*a*) medium B without coconut milk; (*b*) medium B with coconut milk and EHCH, 0.2%; and (*c*) medium B with coconut milk and complex supplement.

To each of the above three media, auxins were added to yield the following p.p.m. concentrations: indoleacetic acid (IAA) 0.1 and 1; BTOA 2 and 6; 2,4-D 4 and 8; naphthaleneacetic acid (NAA) 6; BTOA 2 + IAA 0.1; BTOA 2 + 2,4-D 4; and IAA 0.1 + 2,4-D 4 + NAA 2. The following auxin concentrations also were used in medium (*a*): BTOA 2 + kinetin 0.5; and IAA 0.1 + kinetin 0.5.

The complex supplement was Seitz-filtered and, at all times, added aseptically to autoclaved and cooled medium B.

The above media were adjusted to pH 5.7 with ammonium hydroxide solution before Seitz-filtration or autoclaving.

Experiment Three.—Medium B was prepared in the following two variations: (*a*) medium B, and (*b*) medium B with complex supplement.

In each of the above two media, auxins were used in the p.p.m. concentrations shown in Table II and in the following auxin combinations: BTOA 1 + 2,4-D 5; BTOA 5 + 2,4-D 1; BTOA 5 + 2,4-D 5; BTOA 1 + NAA 1 + 2,4-D 5.

Approximately 15 ml. of each medium in experiment 2 and experiment 3 was added to autoclaved, 1-oz., plastic-capped, wide-mouth prescription bottles. Two sterile, germinated digitalis seeds were placed aseptically in each bottle. This procedure was repeated four times for each medium.

RESULTS AND DISCUSSION

Experiment One.—Germinated digitalis seeds were completely inhibited in growth if placed on medium A, or any of its variations containing

[3] Falcon Plastics, Los Angeles 45, Calif.

TABLE II.—RELATIVE GROWTH OF DIGITALIS SEED CALLUS ON MEDIUM B CONTAINING COMPLEX SUPPLEMENT[a]

Auxin Concn., p.p.m.	*Digitalis purpurea*, Days of Growth			*Digitalis lanata*, Days of Growth		
	25	65	130	35	80	115
BTOA						
0.5	0	0	0	0	0	+
1.0	0	0	+	0	+	+
5.0	0	+	++	0	+	+++
10.0	0	+	+++	0	+	+++
2,4-D						
1.0	+	++	+++	+	++	+++
2.0	+	++	++++	+	+	+++
5.0	+	++	++	+	++	+++
10.0	+	+	++	+	+	++
NAA						
1.0	0	0	0	0	0	+[b]
2.0	0	0	+	+	+	+
5.0	0	+	+	+	++	++
10.0	+	+	++	+	++	++

[a] Of 16 control Digitalis plants, one developed a small callus after 95 days of growth. [b] Of eight plants, one developed a callus on the 115th day.

EHCH. Although this substance is normally a good nutrient supplement for plant tissue cultures (11), it has affected tissue growth of Jerusalem artichoke tubers adversely in the absence of nitrate ions (12). Casein hydrolysate, in the absence of IAA and kinetin, also caused adverse growth effects in tobacco callus cells (13).

Germinated digitalis seeds grew normal-appearing roots and stems on medium A without EHCH, coconut milk, or 2,4-D. Root development was generally poor unless the medium contained IAA or NAA. One small callus appeared after approximately 45 days of growth on medium A without EHCH, but with 2,4-D, 2 p.p.m.

No significant influence on the growth of digitalis seeds by any of the three mineral solutions used was observed.

Experiment Two.—Medium B was prepared at a pH of 5.7 with EHCH in order to confirm that this substance was responsible for the inhibition of germinated digitalis seeds and not the high acidity of the medium. As previously observed in experiment 1, all germinated digitalis seeds in experiment 2 were also completely inhibited in growth on media containing EHCH.

Due to the adverse growth effects of EHCH, a chemically defined complex supplement (see Table I) was formulated to serve as the nitrogen and carbohydrate source. This supplement appeared to increase the vigor of digitalis seed growth, with the one exception of media containing IAA where it appeared to retard root development.

Small callus tissues were observed to form occasionally from sterile, germinated digitalis seeds or roots on media containing the auxins, 2,4-D, BTOA, or their combinations. Media containing NAA stimulated root production. Previous investigators have obtained questionable growth effects when digitalis was grown on a medium containing 2,4-D, 6 p.p.m., with NAA, 0.1 p.p.m. (1).

Experiment Three.—As the digitalis seeds consistently grew better on a medium with the complex supplement, the descriptions of growth given below and in Table II pertain only to media containing the complex supplement.

Digitalis seeds developed normal-appearing stems and leaves on media without auxins for about 65 days (Fig. 1, frame 1), with eventual drying and browning of the stem and leaves after about 115 days (Fig. 2, frame 2). Root systems in the control plants were poorly developed.

Effects of the Auxin, BTOA.—The stimulatory effects of BTOA on tissue growth of both carrot and Jerusalem artichoke tuber have been reported (11).

D. purpurea appeared slightly less sensitive (Fig. 1, frames 4, 5, 6) and more varied (Fig. 1, frame 2) in response to BTOA than *D. lanata* (Fig. 2, frames 4, 5, 6). The *D. purpurea* callus shown in Fig. 1, frame 2 is growing on medium B with BTOA, 5 p.p.m., and increased more than 6,000% when calculated as the wet weight over the original weight of the seed (0.1 mg.) in 95 days.

In both digitalis species callus appeared to form from the stem-root node, stem, or cotyledon tissue in contact with the medium.

Effects of the Auxin, 2,4-D.—All digitalis seeds on media containing 2,4-D formed very poor stems and roots (Fig. 2, frame 1) and eventually formed callus tissues.

Both digitalis species at the lower 2,4-D concentrations, developed excellent callus tissue within 25 to 35 days (Fig. 1, frames 7, 8, 9). The callus would generally develop from small nodules on either the roots, stems, or cotyledons (Fig. 2, frames 7, 8, 9). Root-like nodules were observed to form on some callus tissues of *D. lanata* (Fig. 2, center of frames 8, 9).

Effects of the Auxin, NAA.—*D. purpurea* seeds at the lower NAA concentrations generally produced good stem growth and a short, thick root system (Fig. 1, frame 3). At the higher NAA concentrations, the seeds developed practically no stems and were transformed into small callus tissues containing small rootlets (Fig. 1, frames 10, 11, 12).

D. lanata seeds at the lower NAA concentrations generally produced good stem growth, a short, thick root system, and occasionally callus tissue. At the higher NAA concentrations all the *D. lanata* seeds developed a rapidly growing callus containing an abundance of root nodules and root hairs (Fig. 2, frames 10, 11, 12). In contrast to the growth rate of *D. purpurea* tissue, the growth of *D. lanata* tissue on higher NAA concentrations was rapid.

Effects of Combinations of the Auxins, BTOA, 2,4-D, and NAA.—Digitalis seeds formed callus tissue on all auxin combinations containing 2,4-D. However, if the 2,4-D concentration was 5 p.p.m. or greater, the callus growth was generally less vigorous. Combinations of BTOA, 1 or 5 p.p.m., with 2,4-D, 1 p.p.m., resulted in very good callus growth formation for both digitalis species (Figs. 1 and 2, frames 13, 14, 15).

Digitalis Root Callus Formation.—The incidence of contamination in bottles containing digitalis roots of plant origin was about 50%. Callus tissue did occasionally form on medium B containing either BTOA, 2,4-D, NAA, or their combinations. *D. lanata* root callus formation is shown on medium B containing NAA, 10 p.p.m. (Fig. 2, frame 3).

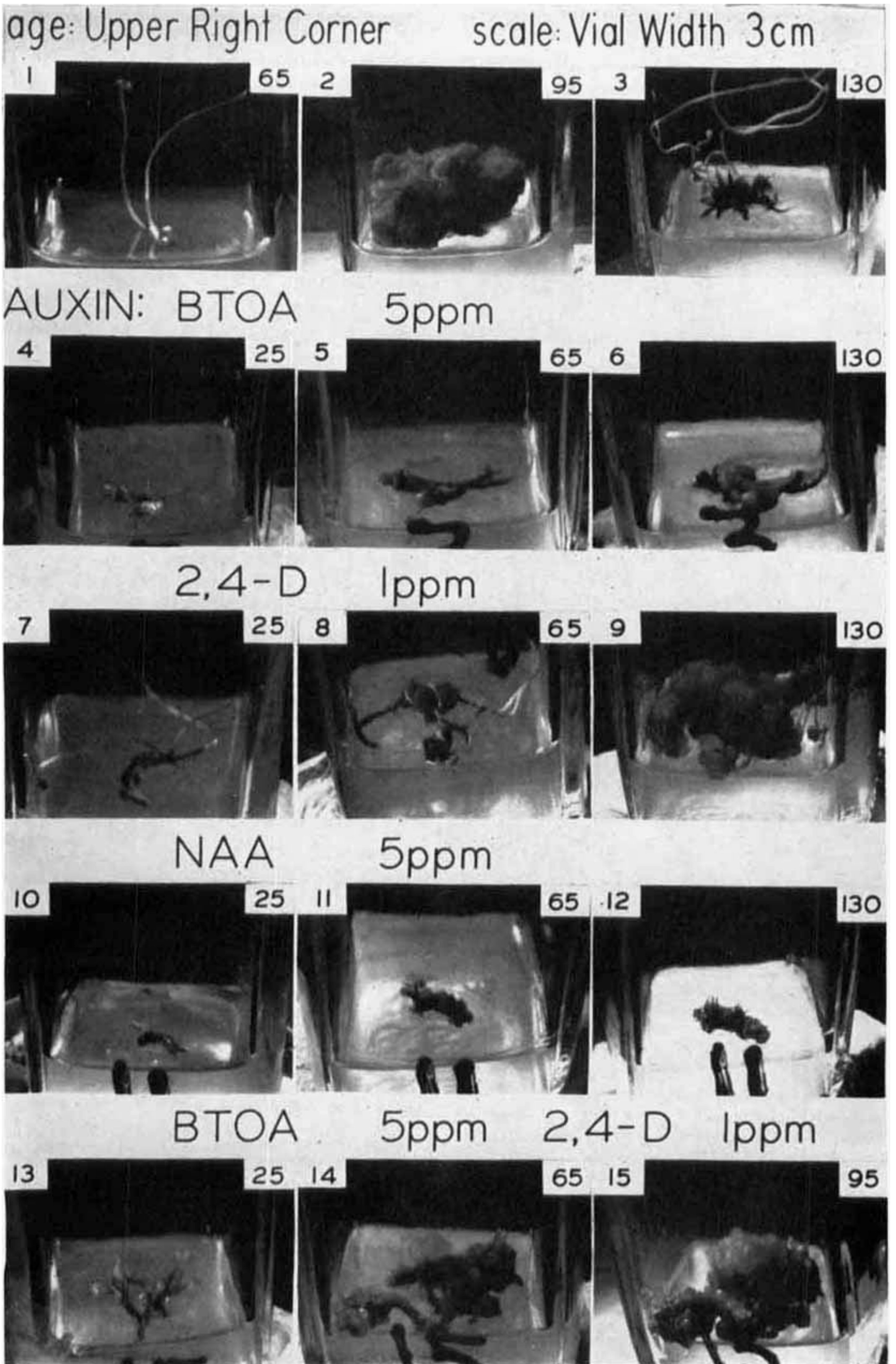

Fig. 1.—*Digitalis purpurea* L.

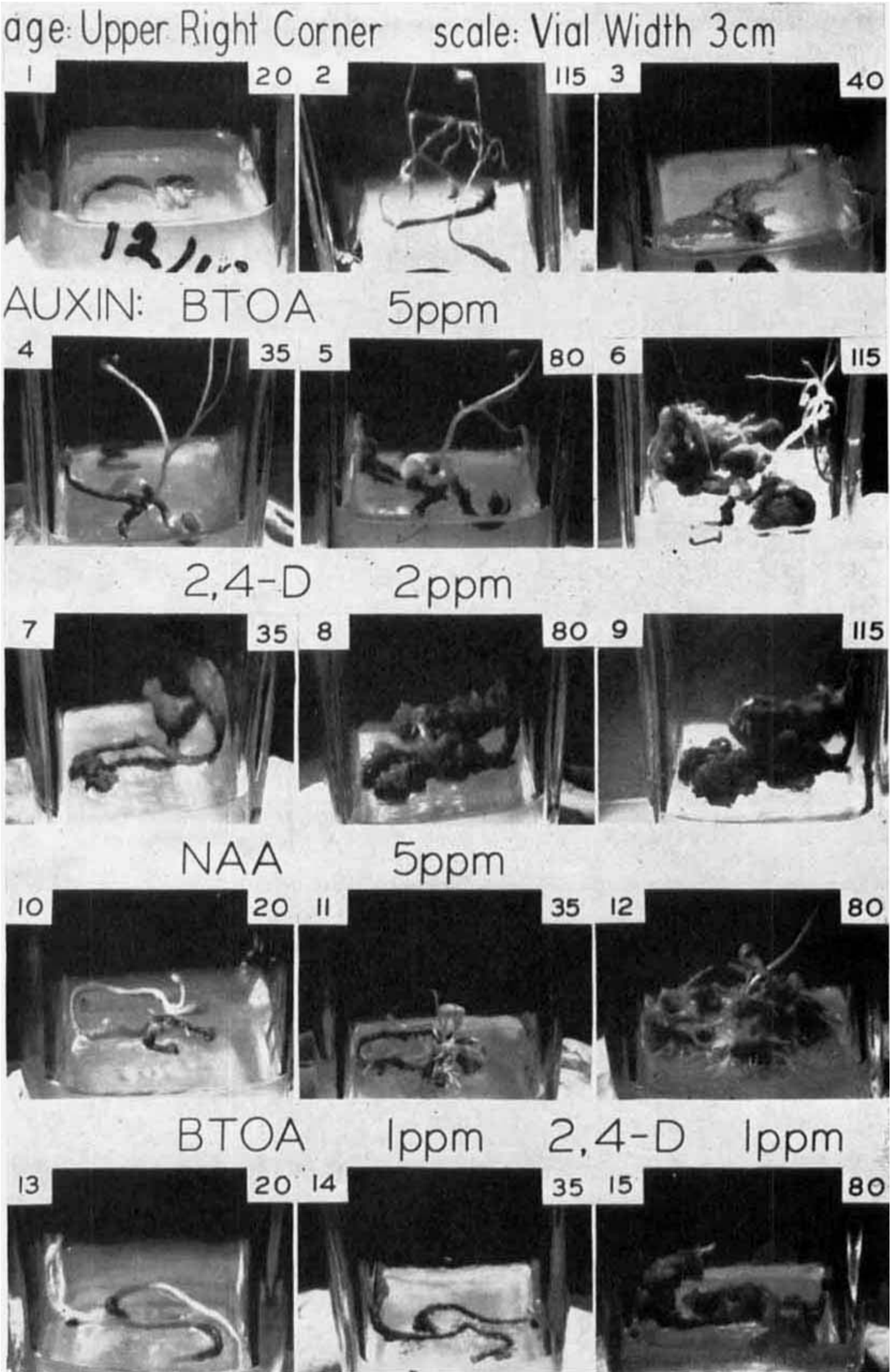

Fig. 2.—*Digitalis lanata* Ehrh.

SUMMARY AND CONCLUSIONS

1. On the media defined, enzyme-hydrolyzed casein will inhibit the growth of germinated digitalis seeds.

2. On medium B with complex supplement, the auxins BTOA, 5 p.p.m.; 2,4-D, 2 p.p.m.; NAA, 5 p.p.m.; or appropriate auxin combinations, BTOA, 1 to 5 p.p.m., with 2,4-D, 1 to 2 p.p.m., will cause digitalis seeds or roots to form callus tissues.

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Radioisotopic Method of Evaluating Dispersed Systems I

Emulsions

By JAMES B. APPINO†, JOHN E. CHRISTIAN, and GILBERT S. BANKER

Liquid petrolatum emulsions were prepared by a standard method and evaluated by a new radioisotopic method. The radioisotopic method required less time and was more sensitive than previously used methods. A radioisotopic creaming rate was determined for each emulsion and can be used to compare microscopically identical emulsions and/or production samples of an emulsion to a previously determined standard. The method is applicable to w/o or o/w emulsions.

THE PRECISE evaluation of emulsions has presented problems to pharmaceutical and other scientists for many years. In an attempt to appraise and control emulsion appearance, uniformity of dosage, and stability, the following emulsion properties have been evaluated: particle size, particle size distribution, viscosity, and creaming rate (1–3). The evaluation of these factors gives some insight into the question, "How long can the product remain pharmaceutically elegant on the pharmacist's shelf?" but may leave the question of uniformity either wholly or partially unanswered.

This study is concerned primarily with an evaluation of the creaming rate and/or phase uniformity of emulsions in less time than is now required by the conventional visual (4) or microscopic (5) methods, or the more tedious though perhaps more accurate chemical or analytical methods (6). Radioisotopes, because of their sensitivity of detection (7), were utilized as tools in evaluating these properties.

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EXPERIMENTAL

Preparation of Emulsion Samples.—The compositions of the emulsions evaluated in this study are given in Table I. The standard method of manufacture of all emulsion samples involved heating the liquid petrolatum and emulsifiers to 55° and the water and preservatives to 60°. The water phase was then added to the oils with constant agitation and the emulsion mixed until the temperature dropped to 38°. All emulsions were prepared in 1-L. quantities and allowed to deaerate for 24 hours before use.

Approximately 1 mc.[1] of sodium iodide (I^{131})[2] or iodobenzene (I^{131}) (8) per 100 ml. of emulsion was

[1] Gave 8,000–12,000 c.p.m. in end counting and 5,000–8,000 c.p.m. in side counting apparatus. Apparatus described later in text.
[2] Weight of sodium iodide is insignificant.