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The Isolation from Lemon Juice of a Growth Factor of Steroid Nature required for the Growth of a Strain of *Paramecium aurelia*

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SUMMARY: A steroid fraction obtained from lemon juice was found to be an essential metabolite for *Paramecium aurelia*, var. 4, stock 51.7 (s), in axenic culture. This steroid is closely related to, or is identical with, either β -sitosterol or γ -sitosterol.

Paramecium aurelia has been established in pure culture in a heat-sterilized medium, composed of equal parts of a 0.5% yeast autolysate (Basamin-Busch) and a 24 hr. culture of *Aerobacter aerogenes* in lettuce extract (van Wagtendonk & Hackett, 1949). Lettuce or other plant extracts were shown to be essential since no growth of *P. aurelia* could be obtained when these extracts were omitted. An axenic medium, consisting of a salt solution, dialysed yeast extract, a solution of water-soluble vitamins, proteose peptone, and guanylic and cytidilic acid has been developed (van Wagtendonk, Miller & Conner, 1952; van Wagtendonk, Conner, Miller & Rao, 1953). *P. aurelia* is unable to grow in this medium unless plant extracts are added. An essential metabolite for *P. aurelia* must therefore be present in plants. The isolation and identification of this metabolite is described.

EXPERIMENTAL

Method of assay

P. aurelia, variety 4, stock 51.7, sensitive (Sonneborn, 1949) is not able to grow in either axenic or bacterized media in the absence of plant extracts. Rapid screening assays for activity are desirable in an extraction of biologically active material. The test in axenic medium is slow, and for this reason fractions were screened using a bacterized medium. The basal medium for the bacterial assay consisted of: NaCl, 0.8 g.; KCl, 0.05 g.; Na_2HPO_4 , 0.1 g.; NaH_2PO_4 , 0.1 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, 0.125 g.; double distilled water, 1 l.; HCl to pH 6.5.

The extracts to be tested were prepared in serial dilutions, using the salt solution as a diluent. These dilutions were autoclaved at 120° for 15 min. They were then inoculated with *A. aerogenes* 24 hr. before use for the assay, as in the procedure routinely used for the culture of *P. aurelia* (Sonneborn, 1950).

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One-half ml. of each dilution of the fractions being tested was placed in each depression of a 3-depression slide. Single isolations of *P. aurelia* were placed in each depression, and the depressions incubated at 27°. After 24 hr. the number of animals present in each depression was counted and a single isolation from each depression was made into fresh culture fluid of the same composition. This was repeated three times. In each series a control, consisting of the basal medium with *A. aerogenes* alone, was included. No multiplication of *P. aurelia* took place in these controls, except during the first 24 hr.

In order to express quantitatively the activities of the different fractions as measured in the bacterized medium, it was necessary to adopt a unit of activity. One unit was arbitrarily defined as follows: a solution of an active fraction contains one unit when *P. aurelia*, var. 4, stock 51.7 (s), transferred to the medium, will undergo at least three fissions per day on three consecutive transfers of a single animal.

The highly purified fractions were tested qualitatively in an axenic medium prepared as follows: One pound of Fleischmann's bakers' yeast was finely crumbled and dried at 65°. A 1 : 4 (w/v) suspension of the dried yeast was prepared by continuously stirring the yeast in distilled water at 60° for 3 hr. The suspension was autoclaved for 20 min. at 120°, centrifuged, and filtered through a Seitz filter pad (no. 8) for sterilization and clarification. A 1% solution of proteose peptone (Difco) was made in distilled water and autoclaved for 20 min. at 120°. One volume of the yeast extract was added aseptically to 5 vol. of the proteose peptone solution. In this medium the factor from plant extracts is the limiting component. The fractions were made up in double distilled water in the optimal concentration as determined by the bacterial assay method. Such a fraction was considered to be active when *P. aurelia*, transferred to this medium, showed sustained growth for at least four consecutive transfers. This assay served to eliminate the possibility that the factor from lemon juice was altered by the bacteria and thereby made available to *P. aurelia*.

After the factor had been obtained in the crystalline state and identified as a steroid, direct comparisons between the population densities obtained with other steroids were possible. Serial dilutions of the factor and of the steroids were made in the following manner: a weighed amount was dissolved in 95% ethanol, so that the resulting solution contained 1 mg./ml. This solution was then added by means of a fine pipette to double distilled water heated to 70°; this resulted in the formation of a stable suspension. The final concentration of all compounds so prepared was 100 µg./ml. Further serial dilutions were made with distilled water. The tubes were then autoclaved. Desired amounts of these dilutions were added aseptically to the axenic medium described above. Approximately thirty animals were inoculated into each tube and incubated for 4 days at 27°. After four such transfers the number of animals/ml. in each tube was determined by counting the number of organisms present in suitable samples. (Nephelometric techniques could not be applied because of intense light scattering by the organisms.) Except for the first transfer,

no multiplication of *P. aurelia* took place in the absence of the factor from lemon juice. All concentrations were tested in duplicate and every assay was repeated at least three times.

Distribution of the growth factor

Various plant and animal sources were tested for growth-promoting activity. The results are given in Table 1. No preparations from animal sources were active. It is not known whether the activity displayed by the different plant extracts is due to the same factor. Lemon concentrate was the most active source and was selected for the extraction and purification of the growth factor.

Table 1. *Relative activity of various raw materials*

	Relative activity
Cerophyl extract*	1
Water extract of desiccated lettuce (Difco)	3
Water extract of baked lettuce	4
Orange juice (fresh)	10
Lemon juice (fresh)	15
Lemon concentrate†	60
Lemon peel infusion†	0
Wilson liver extract	0
Proteose peptone	0
Bacto tryptone	0
Casamino acids	0

* Obtained from the Cerophyl Laboratories through the courtesy of Dr G. A. Köhler.

† Obtained from the California Fruit Growers Exchange through the courtesy of Dr W. E. Baier.

Extraction and purification of the growth factor from lemon juice

The extraction procedure included the following steps: (1) precipitation of the active fraction with acetone; (2) extraction of the precipitate with hot ethanol; (3) saponification of the extract with methanolic KOH; (4) phase distribution; (5) chromatography. Steps 1–4 are given in Table 2, and step 5 in Table 3. In the first two steps, the precipitation and the extraction of the precipitate with hot ethanol, the total activity increased, presumably caused by the removal of inhibitory substances. The saponification of the ethanolic extract resulted in a large increase in potency/ $\mu\text{g.}$, as does the following phase distribution.

Three distinct bands appear in the chromatogram (Table 3). Most of the activity is present in the second band. After elution with hot ethanol a yellow solution was obtained from which white crystals separated after cooling to -10° . The inactive yellow material was removed by repeated recrystallization from 95% ethanol at 4° . A pure compound, representing 0.025% of the original solids and 20% of the original activity, was isolated. The chemical characteristics of this compound are given in Table 4.

The infra-red spectrum of the growth factor for *P. aurelia* is very similar to that of a sample of β -sitosterol (Fig. 1).

Table 2. *Precipitation, extraction, and phase distribution steps for the purification of the growth factor for Paramecium aurelia, var. 4, stock 51.7 (s)*

The yields and activities are based on assays with the bacterized medium.

Treatment	Fraction	Solids (%)	Activity (%)	Potency units/ μ g.
1 gal. of lemon concentrate, chill to 4° and add equal volume of cold acetone, stir well and centrifuge at 1000g at 4° (supernatant solution, inactive)	1	100	100	0.125
Precipitate	2	37	150	0.5
Suspend P_1 in hot 95 % ethanol (500 ml.), stir, filter while hot. Repeat 3 times, combine extract (residue inactive) and concentrate to solids	3	7	170	8
Dissolve E in 500 ml. of hot 80 % methanolic KOH (10 %). Allow to stand for 4 hr. with occasional shaking. Extract 3 times with 200 ml. petroleum ether (30–60°). Combine petroleum ether extracts and concentrate to solids	4	0.6	96	20
Methanol layer of E	5	6.4	3	0.5
Dissolve R in 100 ml. 80 % methanol and extract 3 times with CCl_4 . Dry with anhydrous MgSO_4 , filter and concentrate <i>in vacuo</i>	6	0.3	96	40
Methanol layer of R	7	0.3	2	10

Table 3. *Alumina column step in the purification for the growth factor for Paramecium aurelia, var. 4, stock 51.7 (s)*

4.5 g. of fraction 6 (Table 2) was dissolved in 25 ml. CCl_4 . 5 ml. portions were added to columns (1.5 × 10 cm.) of 12 g. cyanide-treated, acetic-acid-washed alumina (Eli Lilly and Co., Indianapolis, Indiana. Lot number 190-4B-292). The column was washed with 25 ml. CCl_4 and developed with CCl_4 -ethyl ether (9:1) till 3 bands separated. A total of 25 ml. of the developer was used. The column was cut in three portions corresponding to the bands, and each portion was eluted with hot ethanol. The combined fractions of five such chromatograms were combined and evaporated *in vacuo* to solids.

Band no.	Colour	Fraction	Solids (%)	Activity (%)	Potency units/ μ g.
1	Bright yellow	D_1	0.05	0	0
2	Deep orange	D_2	0.20	107	67
3	Light yellow	D_3	0.05	0.4	10

Table 4. *Chemical characteristics of the factor from lemon juice*

Melting point 136.5–137.5° (uncorrected).

Carbon and hydrogen analysis.

	C	H
Lemon factor	83.60	12.32
Calculated for: $\text{C}_{27}\text{H}_{46}\text{O}$	83.80	11.91
$\text{C}_{29}\text{H}_{48}\text{O}$	84.46	11.65
$\text{C}_{29}\text{H}_{50}\text{O}$	88.92	12.07

Colour tests

Liebermann-Burchard +. Salkowski +. Rosenheim –.

Fig. 1. Infra-red adsorption curves of the steroid from lemon juice and of β -sitosterol. The curves are tracings from original automatic recordings. Second curve: steroid from lemon juice (Paramecium factor), 11.0% in chloroform. Third curve: β -sitosterol, 11.8% in chloroform. Chloroform (first curve) was passed over SiO_2 and Al_2O_3 to remove traces of water and alcohol.

Fig. 2. Growth response of *Paramecium aurelia*, var. 4, stock 51.7 (s), in axenic culture to increasing concentrations of the steroid, isolated from lemon juice, β -sitosterol and clionasterol.

The growth-promoting activity of the factor from lemon juice was compared with that of β -sitosterol and clionasterol (inferred to be the 24a-epimer of β -sitosterol (Bergmann & Low, 1947; Fieser & Fieser, 1949)). It can be seen from Fig. 2, that the three compounds have very similar growth-promoting activities.

Because of (1) the close similarity of the infra-red spectra of the factor from lemon juice and β -sitosterol, and (2) the fact that the factor from lemon juice, β -sitosterol and clionasterol have similar growth-promoting activities, it is tentatively concluded that the growth factor for *P. aurelia*, var. 4, stock 51.7 (s), is closely related to, or identical with, either β - or γ -sitosterol. The possibility is not excluded that the fraction isolated from lemon juice is a mixture of these two isomers.

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